Stem Cell Biology and Regenerative Medicine

Barbara Zavan
Eriberto Bressan *Editors*

Dental Stem Cells: Regenerative Potential

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Barbara Zavan • Eriberto Bressan
Editors

Dental Stem Cells: Regenerative Potential

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Editors
Barbara Zavan
Department of Biomedical Sciences
University of Padova
Padova, Italy

Eriberto Bressan
Department of Neurosciences
University of Padova
Padova, Italy

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Contributors

Paul R. Cooper Oral Biology, School of Dentistry, University of Birmingham, Birmingham, UK

O.G. Davies Wolfson School of Mechanical and Manufacturing Engineering, Loughborough University, Loughborough, UK

Waruna Lakmal Dissanayaka Comprehensive Dental Care, Faculty of Dentistry, The University of Hong Kong, Hong Kong

Nileshkumar Dubey Discipline of Oral Sciences, Faculty of Dentistry, National University of Singapore, Singapore, Singapore

Henry F. Duncan Division of Restorative Dentistry and Periodontology, Dublin Dental University Hospital, Trinity College Dublin, Dublin, Ireland

Kitraki Efthymia Faculty of Dentistry, National and Kapodistrian University of Athens, Athens, Greece

Diamanti Evangelia Faculty of Dentistry, National and Kapodistrian University of Athens, Athens, Greece

Letizia Ferroni Department of Biomedical Sciences, University of Padova, Padova, Italy

Letizia Ferroni Department of Biomedical Sciences, University of Padova, Padova, Italy

Akhilesh K. Gaharwar Department of Biomedical Engineering, Texas A&M University, College Station, TX, USA

Department of Materials Science and Engineering, Texas A&M University, College Station, TX, USA

Chiara Gardin Department of Biomedical Sciences, University of Padova, Padova, Italy

Chiara Gardin Department of Biomedical Sciences, University of Padova, Padova, Italy

Paolo Ghensi Centre for Integrative Biology (CIBIO), University of Trento, Trento, Italy; Department of Neurosciences, University of Padova, Padova, Italy

Giovanna Iezzi Department of Medical, Oral, and Biotechnological Sciences, University of Chieti-Pescara, Chieti, Italy

Lucia Jimenez-Rojo Orofacial Development and Regeneration, Institute of Oral Biology, Centre for Dental Medicine, Medical Faculty, University of Zurich, Zurich, Switzerland

Amalia Kaparou Faculty of Dentistry, National and Kapodistrian University of Athens, Athens, Greece

Massimo Marrelli Department of Maxillofacial Surgery, Calabrodental, Crotone, Italy

Kyung-san Min Department of Conservative Dentistry, School of Dentistry, Chonbuk National University, Jeonju, South Korea

Thimios A. Mitsiadis Orofacial Development and Regeneration, Institute of Oral Biology, Centre for Dental Medicine, Medical Faculty, University of Zurich, Zurich, Switzerland,

Giovanna Orsini Department of Clinical Sciences and Stomatology, Polytechnic University of Marche, Ancona, Italy

Francesco Paduano Research and Development in Biomedicine – Stem Cells Unit, TECNOLOGICA Research Institute, Crotone (KR), Italy

Giorgio Pagni Private Practice, Florence, Italy

University of Milan, Milan, Italy

Adriano Piattelli Department of Medical, Oral, and Biotechnological Sciences, University of Chieti-Pescara, Chieti, Italy

Sara Ricci Department of Neurosciences, University of Padova, Padova, Italy

Vinicius Rosa Discipline of Oral Sciences, Faculty of Dentistry, National University of Singapore, Singapore, Singapore

B.A. Scheven School of Dentistry, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK

Jakub Suchánek Department of Dentistry, Faculty of Medicine in Hradec Králové, Charles University in Prague, Hradec Králové, Czech Republic

Fatima N. Syed-Picard Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA, USA

Marco Tatullo Research and Development in Biomedicine – Stem Cells Unit, TECNOLOGICA Research Institute, Crotone (KR), Italy

G.K. Thakral Department of Dentistry, SGRR Institute of Medical & Health Sciences, Dehradun, India

Petridis Xenos Faculty of Dentistry, National and Kapodistrian University of Athens, Athens, Greece

Chengfei Zhang Comprehensive Dental Care, Faculty of Dentistry, The University of Hong Kong, Hong Kong

Dental Stem Cells (DSCs): Classification and Properties

Chiara Gardin, Sara Ricci, and Letizia Ferroni

Embryonic Origin and Components of the Tooth

A tooth is a complex structure made up of two major parts: the crown and the root. The crown of the tooth is what is visible in the mouth, whereas the root is the portion normally not visible in the mouth because it is anchored within the bone. Embryologically, mammalian teeth develop from sequential and reciprocal interactions between oral epithelium and neural crest-derived mesenchyme [1]. Most of the dental tissues have a cranial neural crest origin, namely dentin, dental pulp, cementum, periodontal ligament, and alveolar bone. The outer mineralized layer of enamel covering the tooth crown is the only component of ectodermal origin (Fig. 1).

Dentin comprises the main portion of the tooth. It is a mineralized connective tissue secreted by the odontoblasts, which are specialized cells located at the periphery of the dental pulp. The thickness of dentinal layer increases with age due to the deposition of secondary and tertiary (reparative) dentin, reducing the volume of the pulp chamber and the root canals [2]. It has been shown that the presence of dentin is essential for the differentiation of inner dental epithelial cells into ameloblasts [3]. Ameloblasts start to produce and secrete specific enamel matrix proteins, and soon after tooth eruption in the oral cavity, they completely disappear. During that time, tooth root develops, accompanied by cementum deposition and periodontium formation [4]. The cementum is a mineralized tissue covering the root of the tooth and produced by the cementoblasts. The main role of cementum is to serve as a medium by which the periodontal ligament can attach to the tooth for stability. At

C. Gardin • L. Ferroni (✉)
Department of Biomedical Sciences, University of Padova, via Ugo Bassi 58/B 35131, Padova, Italy
e-mail: letizia.ferroni@gmail.com

S. Ricci
Department of Neurosciences, University of Padova, via Giustiniani 5 35128, Padova, Italy

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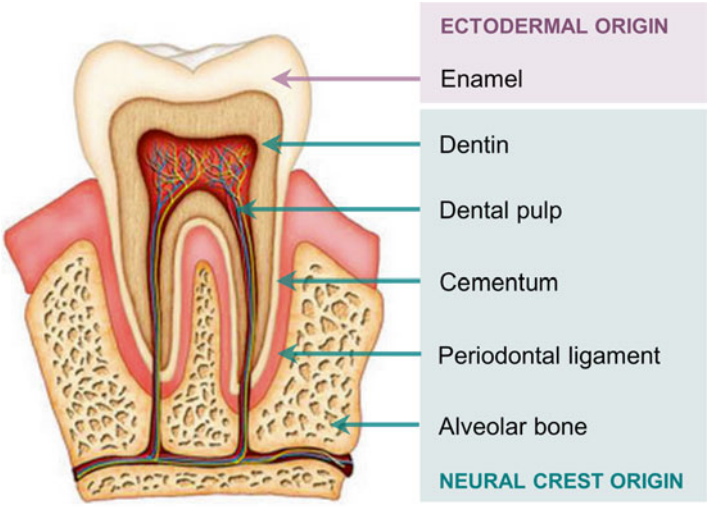

Fig. 1 Embryonic origin of the different tooth components. (Reproduced from www.bronxdental-spa.com/root-canal-bronx.htm)

the cementum-enamel junction, the cementum is acellular. This acellular type of cementum covers about two-third of the root; the apical portion of the root, that is more permeable, is covered by cellular cementum [5]. The periodontal ligament anchors the tooth to the alveolar bone, which is the bone in which teeth are encased. The outside wall of the alveolar bone is compact bone; the trabecular bone is inside and contains bone marrow. The number and the size of the trabeculae in this bone are determined by the function activity of the organ.

Dental pulp is the soft connective tissue of the tooth. It is located in its central cavity and is surrounded by the hard structures of enamel, dentin and cement. The dental pulp contains four layers. The external layer (*odontoblast layer*) is made up of odontoblasts producing dentin; the second layer (*cell-free zone*) is poor in cells and rich in collagen fibers; the third layer (*cell-rich zone*) contains fibroblasts and undifferentiated mesenchymal cells. From this layer, undifferentiated cells migrate to various districts where they can differentiate under different stimuli and make new differentiated cells and tissues. The innermost layer (*core of the pulp)* comprises blood vessels and nerves that enter the tooth mostly through the apical foramen. Other cells in the pulp include fibrocytes, macrophages, and lymphocytes [6].

Dental Stem Cells (DSCs)

Unlike other tissues such as bone, which have the ability to repair and remodel throughout life, human teeth have a very limited capacity to regenerate upon injury or disease [7]. Of all the dental components, the acellular enamel is incapable of

regenerating its original structure, whereas the remaining dental tissues possess that capacity in varying degrees, dependent on multiple factors [8]. Stem cells have been opening a promising future in regenerative medicine because of their two remarkable features known as self-renewal and multilineage differentiation. Stem cells reside in a dynamic and specialized microenvironment denoted to as niche, which is composed of heterogeneous cell types, extracellular matrix (ECM), and soluble factors [9]. The niche regulates stem cells behavior, by maintaining a balance between quiescence, self-renewal, and differentiation [10]. Based on their origin, stem cells can be generally classified in Embryonic Stem Cells (ESCs), which differentiate into all cell types found in the human body, and Adult Stem Cells (ASCs), whose differentiation potential is restricted to certain cell lineages. ASCs have been identified in almost every adult tissues from both epithelial and mesenchymal origin, including skin [11], bone marrow [12], adipose tissue [13], peripheral blood [14], cartilage [15], intestine [16], and periosteum [17]. For this reason, ASCs are also referred to as postnatal stem cells. ASCs are more applicable than ESCs in stem cell-mediated therapies and regenerative medicine because these cells lack ethical concerns. In addition, they have low immunogenicity and less tumorigenic potency than their embryonic counterparts, being promising candidates for regenerative therapies [18].

Several populations of ASCs have been identified also in various dental tissues, and they are collectively referred to as Dental Stem Cells (DSCs) [19]. DSCs are considered a promising source of ASCs since they are easily accessible by tooth extraction with a local anesthetic or when a primary tooth is replaced. More interestingly, DSCs can also be harvested from inflamed or diseased dental tissues, and their properties are similar to those of DSCs obtained from healthy tissues [20–22]. Therefore, it is believed that DSCs could retain, at least to some extent, the stem cell properties and tissue regeneration potential, making them an important tool for future developments in regenerative medicine. Another advantage of teeth as a source of stem cells is that, due to their ectomesenchymal origins, DSCs may display characteristics of both mesoderm and ectoderm [23]. This fact is very important because the association of mesenchymal (that will form odontoblasts, cementoblasts, osteoblasts, and fibroblasts) and epithelial (that will form ameloblasts) stem cells it is necessary for regenerating or building a new tooth.

Classification and Properties of DSCs

To date, seven different human dental stem/progenitor cells have been isolated and characterized: Dental Pulp Stem Cells (DPSCs) [7]; Stem cells from Human Exfoliated Deciduous teeth (SHED) [24]; Periodontal Ligament Stem Cells (PDLSCs) [25]; Dental Follicle Progenitor Cells (DFPCs) [26]; and Stem Cells from Apical Papilla (SCAP) [27]. DPSCs, SHED, and SCAP are generally referred to as dental pulp-related stem cells, PDLSCs and DFPCs as periodontium-related stem cells [28]. Other dental-related stem cells have been identified later. These are

Fig. 2 Timeline in the history of identification of the different DSCs

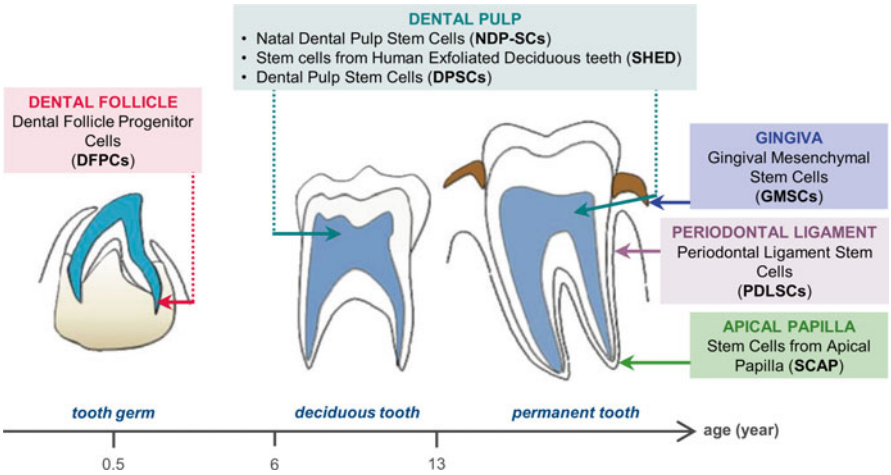

Fig. 3 Tooth developmental stages showing the anatomical localization of the different DSCs. (Reproduced from [31])

Gingival Mesenchymal Stem Cells (GMSCs) [29], and human Natal Dental Pulp Stem Cells (NDP-SCs) [30].

In this section, each type of DSCs will be described following the chronological order of their discovery (Fig. 2). Different biological aspects of DSCs will be discussed, starting from their first identification, the anatomical localization in the tooth (Fig. 3), the methods for their isolation, the peculiar expression of surface markers, and the differentiation potential in vitro and in vivo. The main biological properties of DSCs are summarized in Table 1.

Dental Pulp Stem Cells (DPSCs)

DPSCs Identification, Isolation, and Characterization

DPSCs from adult human dental pulp were first identified by Gronthos and colleagues in 2000 [7], even though the existence of stem cells in dental pulp has been reported by Yamamura in 1985 [58]. DPSCs were isolated on the basis of their high proliferation and frequency of colony formation that produced sporadic, but densely calcified nodules. The authors demonstrated that DPSCs could develop in vitro into odontoblasts, the cells that form the mineralized matrix of dentin. In addition, when

Table 1 Properties of DSCs

Type of DSCs	Location	Cell surface markers		Differentiation potential		References
		Positive	Negative	In vitro	In vivo	
DPSCs	Dental pulp of permanent tooth	CD9, CD10, CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD106, CD146, CD166, STRO-1, Nanog, Oct-4, Sox-2	CD14, CD34, CD45, HLA-DR	Odontogenic Osteogenic Neurogenic Adipogenic Myogenic Chondrogenic	Dentin/pulp-like complex	[7, 23, 32–39]
SHED	Dental pulp of deciduous tooth	CD13, CD29, CD44, CD73, CD90, CD105, CD106, CD146, CD166, STRO-1, Nanog, Oct-4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81	CD14, CD34, CD45, HLA-DR	Odontogenic Osteogenic Neurogenic Adipogenic Myogenic Chondrogenic	Dentin formation New bone formation by recruiting host murine cells	[24, 40–43]

(continued)

Table 1 (continued)

Type of DSCs	Location	Cell surface markers		Differentiation potential		References
		Positive	Negative	In vitro	In vivo	
PDLSCs	Periodontal ligament	CD9, CD10, CD13, CD26, CD29, CD44, CD59, CD73, CD90, CD105, CD106, CD146, CD166, STRO-1, Nanog, Oct-4, Sox-2, Rex-1, SSEA-1, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81	CD14, CD34, CD45, HLA-DR	Osteogenic Cementogenic Adipogenic Chondrogenic Insulin-producing cells	Cementum/periodontal ligament structure	[25, 33, 44–47]
DFPCs	Dental follicle of developing tooth	CD9, CD10, CD13, CD29, CD44, CD53, CD59, CD73, CD90, CD105, CD106, CD146, CD166, STRO-1, Nanog, Oct-4	CD34, CD45, CD117, HLA-DR	Osteogenic Adipogenic Neurogenic Chondrogenic	Mineralized tissue structure	[26, 28, 33, 48–51]

SCAP	Apical papilla of developing tooth	CD13, CD24, CD29, CD44, CD73, CD90, CD105, CD106, CD146, CD166, STRO-1, Nanog, Oct-4	CD18, CD34, CD45, CD150	Odontogenic Osteogenic Adipogenic Chondrogenic Neurogenic	Dentin/pulp-like complex	[27, 52, 53]
GMSCs	Gingiva	CD13, CD29, CD44, CD73, CD90, CD105, CD146, STRO-1, Oct-4, SSEA-4	CD14, CD34, CD45, HLA-DR	Osteogenic Adipogenic Chondrogenic Neurogenic Endothelial	Connective-like tissue	[29, 54–56]
NDP-SCs	Dental pulp of newborn	CD13, CD29, CD44, CD73, CD90, CD146, CD166, Nanog, Oct-4, Sox-2, Rex-1, FoxD3	CD14, CD34, CD45, HLA-DR	Osteogenic Adipogenic Chondrogenic Myogenic Neurogenic		[30, 57]

transplanted into immunocompromised mice, DPSCs mixed with hydroxyapatite/tricalcium phosphate (HA/TCP) were able to generate a dentin/pulp-like complex with a collagen matrix containing blood vessels and lined with odontoblasts [7].

The collection of stem cells from dental pulp is a common noninvasive surgical practice that can be performed in the adult during life. Dental pulp tissue from human third molars represents the most common source for DPSCs harvesting, since these teeth are often extracted and discarded. In order to isolate dental pulp, the tooth has to be sectioned at the cementum/enamel junction using dental instruments. Then, dental pulp tissue is gently separated from the crown and root, and minced into small fragments. From these, DPSCs can be isolated either by the enzymatic digestion (ED) method [7, 59] or by the outgrow (OG) method [60, 61]. Using the first approach, the dental pulp is digested in a solution of collagenase type I and dispase in order to obtain single cell suspensions. Conversely, the second technique is based on the outgrowth of stem cells from tissue fragments. Huang and colleagues compared the properties of DPSCs isolated with the two techniques [62], finding higher proliferation rate for the digested cells than for those isolated by the OG method. In addition, according to Karamzadeh and co-workers, DPSCs isolated by ED showed higher mineralization capacity than those obtained with the OG method [63]. In a later work of Hilkens and colleagues, it has been demonstrated that, when DPSCs were derived from the same donors and kept under the same culture conditions, there were no differences in cellular morphology, proliferation rate, stem cell marker expression and mesenchymal differentiation potential, regardless of the isolation method used [64].

Increasing evidence suggests that DPSCs can survive for long periods and can be passaged several times. Suchanek and colleagues showed that DPSCs achieved 60 population doublings in culture medium designed for bone marrow [65], whereas Laino and co-workers accomplished 80 passages by maintaining the DPSCs substrate interaction and cell-cell communication in the central region of the secreted ECM [66]. DPSCs can also be cryopreserved and stored for long periods without losing their multipotential differentiation ability [66].

In the dental pulp, stem cells are thought to reside in a perivascular niche within the tooth structure, as demonstrated by the expression of the endothelium-associated marker CD146 in blood vessel walls, but not in the surrounding fibrous tissue, odontoblast layer, and perineurium of the nerve [32]. DPSCs also express STRO-1, which is considered an early marker of different mesenchymal stem cells (MSCs). Positive staining for both STRO-1 and CD146 has been widely used to identify DSCs niches. In particular, STRO-1 and CD146 were found to co-localize on the outer walls of blood vessel in dental pulp, thus implying that the majority of DPSCs arise from the microvasculature [32]. Since DPSCs are considered a population of MSCs, markers that have been used for identifying MSCs are also used for DPSCs. Apart from CD146 and STRO-1, DPSCs result positive to other stromal-associated markers, such as CD9, CD10, CD13, CD29, CD44, CD73, CD90, CD105, CD106, and CD166, and negative to hematopoietic markers, such as CD14, CD34, and CD45, and HLA-DR [23, 33]. Nevertheless, different works have reported positive expression for CD34 in DPSCs [66, 67]. In humans, CD34 identifies a cell surface

antigen expressed by the most primitive stromal other than hematopoietic stem cells. The controversy concerning CD34 expression among studies can be attributed to the different experimental conditions, mainly the high serum concentration in culture medium, which has been found responsible for the rapid loss of the CD34 antigen during expansion [68]. Undifferentiated DPSCs also express Oct-4, Nanog, and Sox-2, and present the cytoskeletal proteins nestin and vimentin, all of which are characteristic of undifferentiated ESCs. Apart from stemness markers, DPSCs exhibit expression for a variety of markers associated with bone, such as alkaline phosphatase (ALP), collagen type I (COL1), osteonectin (ON), osteopontin (OPN), osteocalcin (OCN), and bone sialoprotein (BSP); smooth muscle, for example α-smooth muscle actin; and fibroblasts, including collagen type III (COL3), and fibroblast growth factor 2 (FGF2) [7]. The lack of expression of the odontoblast-specific dentin sialoprotein (DSP), which is restricted to the outer pulpal layer containing differentiated odontoblasts, implies that DPSCs represent an immature pre-odontogenic population [32]. It is important to remind that, like all MSCs, DPSCs are heterogeneous and the various markers listed above may be expressed by subpopulations of these stem cells [23].

Differentiation Potential of DPSCs

DPSCs are considered similar to MSCs not only because they share a fibroblastic morphology with selective adherence to solid surfaces and good proliferative potential, but also for their ability to differentiate into multiple cell lineages in vitro. After their original characterization, DPSCs have been differentiated into odontogenic, osteogenic, neurogenic, adipogenic, myogenic, and chondrogenic tissues.

Human DPSCs were initially identified on the basis of their differentiation into osteoblasts and odontoblasts, as previously reported [7]. It has been shown that the potentiality of DPSCs differentiation into odontoblasts in vivo is reduced after passage 9, when these cells can only differentiate along the osteoblast lineage [69]. A selected subpopulation of human DPSCs, called Stromal Bone Producing DPSCs (SBP-DPSCs), has been further isolated by Laino and co-workers [66]. The authors obtained these cells from dental pulps of subjects between 30 and 45 years of age. The aged cells seemed not to be different from those found in younger patients, thus concluding that 30 years should not be considered as a critical age limit for DPSCs isolation. The authors found that SBP-DPSCs, which represent 10 % of dental pulp cells, displayed a great ability to differentiate into osteoblasts, producing a living autologous fibrous bone (LAB) tissue in vitro. When transplanted into immunocompromised rats, SBP-DPSCs formed a lamellar bone containing osteocytes. In this setting, SBP-DPSCs produced bone but not dentin, as shown by mRNA expression of bone markers including OCN, Runt-related transcription factor 2 (RUNX2), COL1, ALP, but not dentin sialophosphoprotein (DSPP), which is specific for dentin. In a later work of the same group, it has been observed that about 30 % of SBP-DPSCs become endothelial cells during their differentiation. These cells were found lining the vessel walls of the newly formed woven bone. Interestingly, a complete

integration of vessels within bone chips took place after in vivo transplantation, leading to the formation of a vascularized bone tissue [34].

Although the majority of the studies have focused their attention on the ability of DPSCs to differentiate into odontoblasts or osteoblasts, further characterization revealed that DPSCs also possess adipogenic and neurogenic differentiation capacities [35, 36]. Remarkably, DPSCs are known to produce neurotrophic factors and even rescue motoneurons after spinal cord injury [37]. DPSCs were also found to undergo chondrogenic and myogenic differentiation in vitro. For example, Zhang and colleagues [38] demonstrated that human DPSCs were able to differentiate into neurogenic, osteogenic/odontogenic, adipogenic, myogenic, and chondrogenic lineages, when cultured under the appropriate inductive media. In a recent study of Paino and colleagues, it has been shown that DPSCs spontaneously differentiate in vitro toward the melanocytic lineage too [39]. The authors supposed that this spontaneous differentiation may be ascribed to the fact that both DPSCs and melanocytes arise from the neural crest cell population.

The ability of human DPSCs to differentiate into several cell lineages under defined conditions has been demonstrated also in a recent work [70]. DPSCs seeded onto a hyaluronan-based scaffold in the presence of a sequential cocktail of neuronal, glial, endothelial, and osteogenic factors proved to be a good strategy for the in vitro reconstruction of a dental pulp-like tissue. Once again, the plasticity and multipotential ability of DPSCs could be explained by the fact that dental pulp is made of both ectodermic and mesenchymal components, containing neural crest-derived cells.

Interestingly, Alongi and co-workers evaluated if DPSCs derived from Inflamed Pulps (DPSCs-IPs) could be used for dental tissue regeneration [20]. They found that DPSCs-IPs formed a dentin/pulp-like complex similar to healthy DPSCs when transplanted into immunocompromised mice. This is a very promising result since inflamed pulp, which is routinely discarded after pulpectomy, actually represents a possible source of stem cells.

Stem cells from Human Exfoliated Deciduous teeth (SHED)

SHED Identification, Isolation, and Characterization

Deciduous teeth is the formal name for what most people call *baby teeth* or, more colloquially, *milk teeth*. Children normally develop a set of 20 deciduous teeth, which appear after 6 months of life and generally are replaced, one tooth at a time, between age 6 and 13. The remaining crown of exfoliated deciduous teeth contains a living pulp remnant comprised of a normal dental pulp including connective tissue, blood vessels, and odontoblasts. Miura and colleagues were the first to identify in 2003 SHED from the remnant pulp in the crown of deciduous incisors of 7–8 years old children [24]. They isolated these clonogenic, proliferative cells using a technique very similar to the one Gronthos and co-workers used to isolate DPSCs

[7]. When cultured under a neuronal differentiation medium, SHED formed sphere-like clusters in which highly proliferative cells aggregated together in clusters that either adhered to the culture dish or floated freely in the culture medium [24]. The dissociation of these sphere-like clusters allowed cells to grow as individual fibroblastic cells. This phenomenon suggested a high proliferative capacity analogous to that of neural stem cells. In effect, SHED are characterized by a higher proliferation rate and increased cell population doublings, which are faster than those of Bone Marrow MSCs (BM-MSCs) [24].

SHED can be isolated using the same procedures described for DPSCs. These stem cells offer attractive advantages over other postnatal DSCs, as they are derived from a source which is non-invasive, readily accessible, naturally being disposed, and with very limited ethical or legal concerns [71]. Bakopoulou and colleagues compared the morphology, growth, immunophenotype, and in vitro osteo/odontogenic differentiation characteristics of SHED obtained using the two isolation methods of ED and OG [67]. They found that the enzymatically digested cells displayed a significant heterogeneity comprising different cell sizes and morphologies even within the same colonies; on the contrary, SHED isolated with the OG method showed a uniform fibroblast-like morphology. The two types of cultures showed differences also in the immunophenotypic profiles and in the mineralization rate, which was lower in SHED obtained with the OG method compared to the digested SHED. Despite these observations, no differences could be detected in the growth rates of SHED isolated using the two methods.

Similarly to DPSCs, SHED express the two early MSCs surface markers STRO-1 and CD146. Cells positive to STRO-1 and CD146 were found to be located around blood vessels of the remnant pulp, implying that SHED may originate from a perivascular microenvironment [24]. SHED have also been identified as Immature Dental Pulp Stem Cells (IDPSCs) by Kerkis and co-workers [40]. Besides confirming the findings reported above, the authors established that IDPSCs express the ESCs markers Oct-4, Nanog, stage specific embryonic antigens (SSEA-3, SSEA-4), and tumorigenic recognition antigens (TRA-1-60, TRA-1-81). Further characterization revealed that SHED were uniformly positive for CD13, CD29, CD44, CD73, CD90, CD105, and CD166; and negative for CD14, CD34, CD45, and HLA-DR [41]. SHED were also found positive to several neural and glial markers, such as nestin, βIII-tubulin, glutamic acid decarboxylase (GAD), neuronal nuclei (NeuN), glial fibrillary acidic protein (GFAP), neurofilament M (NFM), and 2′,3′-cyclic nucleotide-3′-phosphodiesterase (CNPase), possibly due to the neural-crest cell origin of dental pulp [72].

Differentiation Potential of SHED

Under appropriate culture conditions, SHED have demonstrated the ability to undergo differentiation not only into odontogenic, osteogenic, neurogenic, and adipogenic [24], but also myogenic and chondrogenic cell lineages [40, 42].

Miura and colleagues observed that, when SHED were transplanted with HA/TCP particles into immunocompromised mice, they were able to differentiate into odontoblasts and were immunoreactive to DSPP. Nevertheless, these cells failed to generate a complete dentin/pulp-like complex, as do DPSCs in vivo [24]. Additionally, the same authors observed that SHED could not be differentiated directly into osteoblasts; rather, they were able to induce new bone formation by recruiting host murine cells [24]. This is not a property attributed to DPSCs following transplantation in vivo. The osteoinductive potential of SHED was demonstrated in a further study, in which these cells were found able of repairing critical-size calvarial defects in immunocompromised mice [43]. Together, the potential for odontogenic differentiation and the high osteoinductive potential in vivo suggest that SHED may represent a population of multipotent stem cells that are more immature than DPSCs. This is not surprising since deciduous teeth are significantly different from permanent teeth with regards to their developmental processes, tissue structure, and function [42].

As reported above, SHED readily express a variety of neural cell markers under non-neuronal inductive conditions [24]. When stimulated with neurogenic medium, Miura and colleagues observed that expression of βIII-tubulin, GAD, and NeuN increased, whereas expression of the other neural markers remained unchanged. Under these conditions, SHED lost their typical fibroblastic morphology and developed multicytoplasmic processes. Neural developmental potential was further studied in vivo by injecting SHED into the dentate gyrus of the hippocampus of immunocompromised mice. These studies showed that SHED survived for more than 10 days inside the mouse brain microenvironment and continued to express both neuronal and glial cell markers [24]. Koyama and co-workers reported the differentiation of SHED into the adipogenic and chondrogenic lineages in vitro [42]. The adipogenic differentiation of SHED was detected by the in vitro accumulation of lipid droplets, and by the expression of adipogenic markers such as Peroxisome proliferator-activated receptor-gamma (PPARG), and Lipoprotein lipase (LPL). The comparison between SHED and DPSCs revealed a higher adipogenic differentiation capacity of SHED than DPSCs. SHED were also shown to express the chondrogenic markers Sox-9, collagen type II (COL2), and collagen type X (COL10) when cultured for 14 days with bone morphogenic protein 2 (BMP-2), a chondrogenic signaling protein in the transforming growth factor β (TGFβ) family [42].

The strong differentiation plasticity of SHED was demonstrated in engraftment studies in vivo. In particular, Kerkis and co-workers observed that, when IDPSCs were transplanted into immunocompromised mice via intraperitoneal injection, they engrafted into the lungs, liver, spleen, brain and kidney, producing a tissue that was indistinguishable from the host tissue for liver, spleen, brain and kidney [40].

All these observations suggest that deciduous teeth could represent an ideal source of stem cells for repairing damaged tooth structures, inducing bone regeneration, and possibly treating neurodegenerative diseases.

Periodontal Ligament Stem Cells (PDLSCs)

PDLSCs Identification, Isolation, and Characterization

The periodontal ligament, one of the highly specialized and complex connective tissues of the human body, is embedded between the cementum and the inner wall of the alveolar bone socket. Periodontal ligament not only has an important role in supporting teeth, but also contributes to tooth nutrition, homoeostasis, and repair of damaged tissue [73, 74]. Periodontal ligament contains heterogeneous cell populations that can differentiate into either cementoblasts or osteoblasts. The presence of multiple cell types within periodontal ligament has led to speculation that this tissue might contain progenitor cells responsible for maintenance of periodontal ligament homoeostasis and regeneration. In effect, PDLSCs were identified in the periodontal ligament of extracted teeth by Seo and co-workers in 2004 [25]. These cells have been reported to form adherent clonogenic clusters of fibroblast-like cells, and had a proliferation rate similar to DPSCs after 24 h in culture.

PDLSCs can be obtained from periodontal ligament tissue after its separation from the root surface of extracted third molars [25]. Tran and co-workers compared the biological properties of PDLSCs isolated using the OG or ED methods [75]. Cells showed a similar morphology, mainly polygonal with expansion of the cytoplasm, regardless of the isolation protocol used. Additionally, the two isolation methods did not influence the proliferation rates, as well as the immunophenotypic profiles of PDLSCs. Regarding the osteogenic differentiation potential, PDLSCs obtained with the OG method produced a minor total area of mineralized nodules compared to that generated by the enzymatically digested cells.

In the work of Wang and colleagues it has been found that periodontal ligament possesses asymmetrically distributed stem cells, and that PDLSCs properties vary depending on the harvest location [76]. Cells isolated from the alveolar bone surface exhibited strong proliferation capability, and they were shown able to better regenerate the alveolar bone when compared to cells obtained from the root surface.

PDLSCs are similar to other MSCs with respect to the expression of STRO-1 and CD146 markers, implying that these cells might also be derived from a population of perivascular cells. In effect, McCulloch and co-workers have previously identified progenitor cells in the perivascular region of the periodontal ligament of mice [77]. Later, Chen and colleagues localized small clusters of PDLSCs also in the extravascular areas and regions near the cementum [78]. Differently from DPSCs and SHED, PDLSCs were found to express high levels of scleraxis, a tendon-specific transcription factor associated with tendon cells [25]. This is not surprising as the dense collagen fiber structure of the periodontal ligament makes it similar to the tendon. Further characterization revealed that PDLSCs express the following cell surface markers: CD9, CD10, CD13, CD26, CD29, CD44, CD59, CD73, CD90, CD105, CD106, and CD166. Like DPSCs and SHEDs, PDLSCs do not express CD14, CD34, CD45, and HLA-DR [33]. Different works demonstrated that PDLSCs also express ESCs markers, including Oct-4, Nanog, Sox-2, Rex-1, SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 [44, 45], and a subset of neural crest markers, such as nestin, slug, p75, and Sox-10 [46].

Differentiation Potential of PDLSCs

PDLSCs were shown to be multipotent, having the ability to differentiate into osteoblasts, cementoblast-like cells, adipocytes, chondrocytes, and collagen-forming cells [25].

The osteogenic potential of PDLSCs was demonstrated through their ability to form a mineralized matrix containing calcium deposits after 14 days of exposure to osteogenic differentiation medium [47]. However, unlike BM-MSCs and DPSCs, these deposits were sparsely distributed in the culture. Under osteogenic induction medium, PDLSCs showed increased protein expression of the osteoblastic/cementoblastic markers ALP, BSP, OCN, and TGFβ receptor I. Despite the expression of these osteogenic/cementoblastic markers in vitro, PDLSCs were unable to form dentin or bone in vivo [25]. Nevertheless, PDLSCs loaded onto a HA/TCP-scaffold could give rise to a cementum/periodontal ligament structure when transplanted into immunocompromised mice and rats [25]. In particular, a thin layer of cementum-like tissue was formed along with condensed collagen fibers with sparse cells resembling periodontal ligament structure. In 2012, Song and co-workers compared stem cells derived from the periodontal ligament of permanent teeth (pPDLSCs) to those obtained from deciduous teeth (dPDLSCs) [79]. There were no significant differences in the proliferation rate, expression of stem cell markers, or in vitro differentiation between the two cell populations. Nevertheless, pPDLSCs generated more cementum/periodontal ligament-like structures in vivo than dPDLSCs, becoming better candidates for use in periodontium regeneration. In a recent work of Park and colleagues, PDLSCs from human healthy and inflamed periodontal ligament tissues were isolated and compared for their regenerative potential [22]. The proliferative potential did not differ between the two cell populations, and both of them were successfully able to differentiate under osteogenic/cementogenic and adipogenic culture conditions. Both cell types exhibited new cementum-like tissue and related periodontal ligament fiber regeneration after in vivo transplantation.

Although several cell types reside in the periodontal ligament tissue, adipocytes and chondrocytes have not been reported as native components. Nevertheless, PDLSCs were capable of differentiation into adipocytes after 25 days of culture in adipogenic inducing medium as demonstrated by the formation of lipid droplets, and the upregulation of adipocyte specific markers [25, 47]. Gay and colleagues also showed that PDLSCs could undergo chondrogenic differentiation after 21 days culture in vitro [47]. They not only observed cells with a chondrocyte cell morphology, but also the expression of chondrogenic markers, such as COL2 and proteoglycans. Another work demonstrated that PDLSCs differentiate into insulin producing cells, implying that these cells could also be able to generate cells of the endodermal lineage [46]. All these findings seem to suggest the pluripotency of PDLSCs and their ability to differentiate into cells from all three germ layers.

Dental Follicle Progenitor Cells (DFPCs)

DFPCs Identification, Isolation, and Characterization

The dental follicle is the ectomesodermal connective tissue surrounding the enamel organ and the dental papilla of the developing tooth germ prior to eruption [80]. Its main biological function is the coordination of tooth eruption through the regulation of the osteoclastogenesis and osteogenesis processes [81]. The dental follicle contains progenitor cells that form cementoblasts, periodontal ligament, and osteoblasts. It is thought that the dental follicle cells near the forming root differentiate into cementoblasts, whereas the cells towards the alveolar bone differentiate to osteoblasts. The dental follicle cells located between the cementoblast and osteoblast precursor cells develop into fibroblasts producing the ECM of the periodontal ligament [80].

Morsczeck and co-workers identified DFPCs in the dental follicle area of impacted wisdom teeth in 2005 using the same protocol that Gronthos and colleagues used five years earlier to discover DPSCs [26]. For the isolation of DFPCs, attached dental follicles are separated from the mineralized tooth, then minced and digested with an enzymatic solution [26]. It has to be reminded that DFPCs are only available from patients during wisdom tooth eruption, usually between 15 and 28 years of age [28]. DFPCs have been isolated by their ability to form clonogenic, fibroblastic-like colonies in culture that adhered to plastic [26]. DFPCs were found positive for nestin and notch-1 even after longer periods of propagation in culture. In addition, DFPCs expressed COL1, BSP, OCN, and FGF receptor 1-IIIC [26]. These cells also showed positive expression for STRO-1 and CD146, the two early MSCs markers also present on DPSCs, SHED, and PDLSCs. Furthermore, DFPCs have been reported to express CD9, CD10, CD13, CD29, CD44, CD53, CD59, CD73, CD90, CD105, CD106, and CD166 surface markers; these cells were negative for the hematopoietic antigens CD34 and CD45, CD117, and HLA-DR [28, 33, 48–50].

In order to establish if dental follicle contains heterogeneous cell populations, Luan and co-workers cloned 3 cell lines starting from murine DFPCs [48]. They found significant differences between the 3 dental follicle-derived cell lines in terms of proliferation rates, cell cycle distribution patterns, and cell surface markers. One cell line had high proliferation activity but it did not display any mineralization behavior, suggesting that it might be related to a periodontal ligament-type lineage. The second line was similar to the first one regarding proliferation potential but featured high ALP activity indicative of a highly undifferentiated state. The third cell line exhibited mineralization characteristics, indicating that it might be of cementoblastic or alveolar bone osteoblastic lineage.

Differentiation Potential of DFPCs

DFPCs have shown the potential to undergo osteogenic, adipogenic, chondrogenic, and neurogenic differentiation in vitro in both mice and rats [48, 51].

After 5 weeks of induction with osteogenic differentiation medium containing dexamethasone, human DFPCs at passage 6 were able to form a membrane-like structure. The structure of this membrane could be compared to that of the periodontal ligament, consisting of a fibrous tissue with parallel or net-like arranged collagen fibers and fibroblastic cells covered by a cell layer [26]. In contrast, when DFPCs at passage 17 were cultured under the same conditions for 4 weeks, they were able to produce compact calcified nodules. These globular or nodular elements were interconnected by connective tissue strands and surrounded by a connective tissue capsule. These results together would suggest that dental follicle derived from human wisdom teeth represents a source of precursor cells for periodontal ligament-fibroblasts, cementoblasts, and osteoblasts.

Morsczeck and co-workers also investigated osteogenic potential in vivo by transplanting DFPCs in conjunction with hydroxyapatite powder (HAP) into immunocompromised mice [26]. After 8 weeks from transplantation, the cells generated a structure lining the surfaces of the HAP particles, comprised of fibrous or rigid connective tissues. Nevertheless, transplanted DFPCs were unable to build a matrix for cementum or bone formation. More recently, the hard tissue-forming potential of human DFPCs has been evaluated by Yagyuu and co-workers after transplanting these cells with ceramics discs into immunocompromised rats [49]. After 8 weeks, a mineralized tissue structure containing cementocyte/osteocyte cells was formed in vivo, but the exact identity of the tissue type could not be determined as dentin, cementum or bone.

The multilineage potential of murine DFPCs has been assessed in the work of Luan and colleagues [48]. They obtained dental follicle tissues from mouse molar developing tooth organs of 8 days postnatal mice. The isolated DFPCs were induced to differentiate into adipogenic, chondrogenic, or osteogenic lineages using different induction media. Lipid droplets were observed in culture after induction of DFPCs with adipogenic medium for 6 days. A glycosaminoglycan (GAG)-rich fibrous cartilage-type ECM was generated following chondrogenic induction for 21 days; whereas the presence of a mineralized tissue was detected after placing DFPCs in osteogenic medium for 21 days.

In the study of Yao and colleagues, dental follicles were surgically removed from the first mandibular molars of rat pups at days 5–7 postnatally [51]. The isolated DFPCs were then subjected to osteogenic, adipogenic, or neurogenic differentiation in vitro. When placed in an osteogenic differentiation medium for 2 weeks, osteoblasts/cementoblasts formed and developed mineralization nodules, as revealed by Alizarin Red S and von Kossa staining. Adipogenic differentiation potential of DFPCs was revealed by the presence of Oil Red O stained cells. The incubation of DFPCs with a neuronal induction medium for 24 h resulted in the development of cells that resembled multipolar neurons. The neural differentiation in vitro has been demonstrated by Morsczeck and colleagues in a later work [50]. After stimulation with different serum-replacement media for 7 days, the authors observed that, like SHED, human DFPCs expressed neural stem cells-associated markers Sox-2, nestin, and ATP binding cassette, subfamily G, member 2 (ABCg2). Nevertheless, SHED and DFPCs did not have the same neural differentiation potential in vitro, as

demonstrated by the absence of neurosphere-like cell clusters in DFPCs differentiated cultures. In addition, the authors excluded a glial cell differentiation potential of DFPCs as revealed by the weak expression of the glial cell marker GFAP.

Stem Cells from Apical Papilla (SCAP)

SCAP Identification, Isolation, and Characterization

Sonoyama and co-workers were the first to isolate SCAP from the apical papilla of impacted wisdom teeth of 18-20 years old patients in 2006 [27]. The apical papilla is the soft tissue found at the apex of the developing permanent tooth that eventually becomes the pulp tissue in the mature tooth [82]. A further study conducted by Sonoyama and colleagues demonstrated that apical papilla appears to be histologically distinct from pulp and contains unique potent MSCs [52]. In particular, histological characterization of the apical papilla showed that there is an apical cell-rich zone lying between the apical papilla and the pulp. In comparison to the dental pulp, apical papilla seems to contain less blood vessels and cellular components.

The isolation of SCAP is achieved by gentle separation of root apical papilla from the surface of the root with immature apex during the extraction of third molars. Apical papilla is minced, then digested with an enzymatic solution to obtain single cell suspensions [27]. SCAP have been identified as adherent clonogenic cell clusters of fibroblast-like cells. These cells were shown to have a greater proliferation rate and population doubling than DPSCs isolated from the same tooth [27]. In particular, over 70 population doublings were measured for SCAP. Furthermore, it has been reported that SCAP display higher levels of survivin and telomerase, both of which are markers associated with cell proliferation, when compared to DPSCs from the same tooth [27].

SCAP show expression of the same surface markers as the other DSCs, that is STRO-1, CD13, CD24, CD29, CD73, CD90, CD105, CD106, CD146, and CD166. At the same time, these cells are negative for CD18, CD34, CD45, and CD150 antigens. Interestingly, SCAP express a unique marker, not detectable in other DSCs: CD24 [27, 53].

Differentiation Potential of SCAP

SCAP have shown the ability to undergo odontoblastic/osteoblastic, adipogenic, chondrogenic, and neural differentiation in vitro [27, 52, 53].

The potential of human SCAP to undergo odontoblastic/osteoblastic differentiation was evaluated after 4 weeks of induction with an osteogenic differentiation medium [27]. SCAP were able to form small round Alizarin Red-positive nodules, indicating calcium accumulation in vitro. Interestingly, the expression of the SCAP-specific marker CD24 was downregulated in response to osteogenic stimulation.

However, the biological significance of this finding is currently unclear. Similarly to DPSCs and SHED, SCAP have shown odontogenic potential in vitro. Nevertheless, SCAP express lower levels of DSP, matrix extracellular phosphoglycoprotein (MEPE), TGFβ receptor II, Flt-1 (VEGF receptor 1), Flg (FGFR1), FGFR3, and melanoma-associated glycoprotein (MUC18) compared to DPSCs [52]. When transplanted into immunocompromised mice, human SCAP mixed with HA/TCP particles underwent in vivo differentiation into odontoblasts, regenerating a dentin/pulp-like structure and connective tissue similarly to DPSCs [27]. Although SCAP are comparable to DPSCs, they represent a distinct source of dental stem/progenitor cells. It has been speculated that SCAP appear to be the source of primary odontoblasts responsible for the formation of root dentin, whereas DPSCs are likely the source of replacement odontoblasts that form reparative dentin [23]. In order to play a functional role in vivo, the root has to connect with the periodontal ligament. In a preliminary study using minipigs, co-transplantation of swine SCAP and PDLSCs onto HA/TCP particles produced the formation of dentine and periodontal ligament, respectively [27]. These findings suggest that SCAP, together with PDLSCs, could be used to create a biological root that could be used in a similar way as a metal implant, by capping with an artificial dental crown.

SCAP also demonstrated the ability to undergo adipogenic and chondrogenic differentiation following induction in vitro [27, 52, 53]. The adipogenic differentiation potential of SCAP was similar to that of DPSCs but much weaker than that of BM-MSCs. The chondrogenic differentiation was observed as the cells formed a pellet with a spherical appearance and expressed the cartilage extracellular protein COL2 [53].

An interesting feature of SCAP is that they express several neural markers even when not exposed to neurogenic stimulation, implying their possible origin from the neural crest [53]. However, when stimulated, more neural markers are expressed, such as βIII-tubulin, GFAP, GAD, nestin, NeuN, NFM, neuron-specific enolase (NSE), and CNPase [52].

Considering all these observations, SCAP seem to be more embryonic-like than other sources of DSCs [83]. Consequently, SCAP could have potential for applications not only in dentistry, but also in the treatment of neurodegenerative and ischemic diseases, diabetes research, and other applications in the field of regenerative medicine [84].

Gingival Mesenchymal Stem Cells (GMSCs)

GMSCs Identification, Isolation, and Characterization

Gingiva is a unique oral tissue attached to the alveolar bone of tooth sockets, recognized as a biological mucosal barrier and a distinct component of the oral mucosal immunity [29]. One of the most striking characteristics of gingiva is its remarkable regenerative and wound healing capacity, characterized by a rapid reconstitution of

tissue architecture and little evidence of scarring, in contrast to the common scar formation present in skin [85]. Histologically, gingiva is composed of three layers: an epithelial layer, a basal layer, and a lower spinous layer that is similar to the dermis of the skin. Zhang and co-workers were the first to report the isolation of GMSCs from the spinous layer of human gingiva in 2009 [29]. They used healthy gingival tissues obtained from remnant or discarded tissues following routine dental procedures. In this context, human gingiva represents an interesting alternative to the other dental tissues as source of DSCs. The major advantage of using gingiva is the possibility to achieve a large quantity of stem cells without the need to sacrifice a tooth irreversibly to obtain its pulp, periodontal ligament, or dental follicle. Furthermore, gingival tissues can be obtained from minimally invasive procedures at any time in life.

For the isolation of GMSCs, gingival tissues are generally incubated with dispase to separate the epithelial and lower spinous layer. The tissues are then minced and enzymatically digested with collagenase [29, 54]. Other methods described for the isolation of GMSCs involved the use of collagenase and dispase at the same time, followed by the removal of the first digested cell suspension for excluding epithelial cells, and by additional incubations with the same enzymatic solution [55, 86].

GMSCs were identified as plastic-adherent cells with a uniform fibroblast-like morphology [29]. These cells have been found to display stable phenotype, and to maintain normal karyotype and telomerase activity in long-term cultures [55].

Regarding the expression of surface markers, GMSCs result positive to CD13, CD29, CD44, CD73, CD90, CD105, CD146, STRO-1, Oct-4, and SSEA-4 antigens; at the same time, they lack expression of the hematopoietic markers CD14, CD34, and CD45, and HLA-DR [29, 54, 55].

Interestingly, GMSCs have been found to display immunomodulatory properties; in particular, they inhibit lymphocytes proliferation and express a wide range of immunosuppressive factors, such as Interleukin-10 (IL-10), IDO, inducible NO synthase (iNOS), and cyclooxygenase 2 (COX-2) in response to the inflammatory cytokine Interferon γ (IFNγ) [29].

Differentiation Potential of GMSCs

GMSCs demonstrated a multilineage differentiation potential into adipogenic, osteogenic, and chondrogenic cell lineages in vitro [29, 54, 56].

Under adipogenic stimulation, single colony-derived GMSCs could differentiate into adipocytes as demonstrated by Oil Red O staining, and increased expression of specific adipogenic markers, including PPARG and LPL [29]. The osteogenic differentiation was evaluated after incubation of GMSCs with an osteogenic medium. GMSCs were able to form mineralized nodules as determined by Alizarin Red S staining, and they showed increased expression of several osteogenic markers [29, 55]. When GMSCs were cultured as pelleted micromass under a chondrogenic differentiation medium, they were able to synthesize a multilayered sulphated

proteglycan-rich matrix, and express chondrocyte specific genes, such as aggrecan, Sox-9, and COL2 [55]. It has also been reported that GMSCs undergo endothelial and neural differentiation in vitro when stimulated with proper culture medium [29]. Endothelial differentiation was observed by the expression of the endothelial marker CD31; whereas neural differentiation was ascertained by the positivity for the neural markers GFAP, NFM, and βIII-tubulin.

The in vivo differentiation potential of human GMSCs has been evaluated by transplanting these cells with HA/TCP particles into immunocompromised mice [29]. GMSCs were able to regenerate a connective-like tissue containing collagen fibers; however, osteogenic differentiation of the transplanted GMSCs was not observed. The in vivo bone regeneration potential of GMSCs was demonstrated in a another study, in which GMSCs were seeded onto HA/TCP particles for 24 h, incubated in osteogenic medium for 10 days, then implanted subcutaneously in immunocompromised mice [55]. After 10 weeks, the retrieved implants showed the presence of a highly mineralized tissue.

Overall, these findings seem to indicate that human gingiva is an interesting source of DSCs, since GMSCs have shown ability of multilineage differentiation in vitro, and tissue regeneration in vivo.

Natal Dental Pulp Stem Cells (NDP-SCs)

NDP-SCs Identification, Isolation, and Characterization

Natal teeth are deciduous teeth that arise in newborn. These teeth are smaller than primary teeth and have little or no root development. Natal teeth are very rare since very few newborns are born with teeth, that is approximately one in every 2000–3000 births [87].

Human NDP-SCs are a unique type of dental pulp-related stem cells isolated only from the pulp of newborn teeth [30]. Karaöz and co-workers isolated NDP-SCs from 2 natal teeth obtained from a healthy newborn female in 2010 [30]. After fracturing of the dental crown, the dental pulp was uncovered and digested using collagenase to generate single cell suspensions. A small number of NDP-SCs adhered to plastic in culture, and displayed a fibroblast-like morphology that became flattened at later passages. When compared to SHED and DPSCs, NDP-SCs had higher proliferation rate [57]. NDP-SCs express CD13, CD29, CD44, CD73, CD90, CD146, and CD166 markers, but do not express CD14, CD34, CD45, and HLA-DR antigens, in a similar way to the other DSCs [30, 57]. Interestingly, NDP-SCs express detectable levels of the ESCs markers Nanog, Rex-1, and Oct-4, as well as the transcription factors Sox-2 and FoxD3, suggesting that these cells display some of the characteristics for pluripotency. Surprisingly, when growth in standard medium, NDP-SCs show a positive immune reaction for the osteogenic markers COL1, OCN, ON, OPN, BMP-2, and BMP-4. Furthermore, these cells express some myogenic markers, such as desmin and myogenin, and the chondrogenic

marker COL2 under the same culture conditions. NDP-SCs were also found positive to neural markers, including nestin, vimentin, GFAP, and βIII-tubulin [30]. In a recent study of Akpinar and co-workers the protein expression profile of human NDP-SCs was analyzed and compared to that of human DPSCs and SHED [57]. They found that NDP-SCs cell proteome was more similar to the SHED proteome than the DPSCs proteome, and they identified 61 proteins that were predominantly expressed by all the 3 DSCs. Further analysis on the molecular function of the identified proteins revealed that most of them played roles in cellular architecture. Proteins involved in the folding machinery were also described, as well as transcription, protein biosynthesis, and degradation related proteins, thus implying the presence of cellular self-renewal and proliferation.

Differentiation Potential of NDP-SCs

The multilineage differentiation potential of NDP-SCs has been demonstrated by their differentiation into osteoblasts, adipocytes, chondroblasts, myoblasts, and neuro-glial-like cells in vitro [30, 57].

Adipogenic differentiation of human NDP-SCs was confirmed by the formation of lipid droplets that enlarged and invaded the entire cytoplasm during cell differentiation. Following culture in osteogenic medium, it was possible to identify calcium deposits by Alizarin Red S staining. Chondrogenic differentiation was evidenced by the presence of round cells which resembled hyaline chondrocytes, and expressed GAG. When treated with 5-azacytidine, NDP-SCs acquired a myocyte phenotype in culture and, within 8 days following seeding, the myoblasts fused to form small myotubes. These immature myotubes had clustered nuclei that differentiated during the next 2–3 days into large multinucleate syncytia. The neuronal differentiation of NDP-SCs was demonstrated by the formation of neuron-like cells that displayed different morphologies, ranging from simply bipolar to branched multipolar cells, and expressed several neural-specific markers.

Conclusions

The observations reported in this chapter seem to indicate that DSCs are a viable alternative to other adult MSCs for regenerative medicine. The main advantage in using DSCs is that they are easily obtainable from several dental tissues which are often discarded. In addition, DSCs have been described as highly clonogenic and proliferative cells, possessing multilineage differentiation potential in vitro, and regenerative properties in vivo. A better comprehension of the biology of DSCs would result in significant benefits for the management of diseases affecting human dental tissues as well as other organs.

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Protocols for Dental-Related Stem Cells Isolation, Amplification and Differentiation

Jakub Suchánek, Klára Zoe Browne, Tereza Suchánková Kleplová, and Yvona Mazurová

If I have seen further, it is by standing on the shoulders of giants. Isaac Newton

Election of Research Material

To elect research material which would appropriately accommodate individual dental-related stem cell experiments it is essential to understand both the genesis of specific tissue-stem cell lineages and their consequent inherent properties such as population curve in time and behavior under various environmental conditions in vivo (in an intact organism) as well as in vivo modified (transplanted as modified autologous or heterologous material) and in vitro (outside of an organism) [1]. The below chapters provide comprehensive overview of the up-to-date scientific knowledge in this field of study concerning all the tissues of tooth and the tissues immediately adjoined.

Firstly, we outline dental-related tissues genesis and anatomical location, and thereafter the quality and quantity of their stem cell populations. We consider the complete range of postnatal dental-related tissues-stem cell populations, starting from the incident predeciduous dentition [2] to the primary and secondary dentition [3].

Specifically, the feasible dental-related postnatal sources of stem cells are all the mature tissues of all dentitions (natal/neonatal dental pulp [4], immature dental pulp [5], deciduous teeth dental pulp [6], adult dental pulp [7]; apical papilla [8], periodontal ligament [9] and gingiva [10] plus the immature tissues of the secondary dentition dental follicle [11] and dental papilla).

J. Suchánek, Ph.D. (✉) • T.S. Kleplová, M.D.
Department of Dentistry, Faculty of Medicine in Hradec Králové, Charles University in Prague, Sokolská 581, 500 05 Hradec Králové, Czech Republic
e-mail: SuchanekJ@lfhk.cuni.cz

K.Z. Browne, M.Sc. • Y. Mazurová, C.Sc., M.D.
Department of Histology and Embryology Faculty of Medicine in Hradec Kralove, Charles University in Prague, Sokolská 581, 500 05 Hradec Králové, Czech Republic

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Dental-Related Tissues' Genesis

Dental-related tissues—lifecycle spans from early stages on intrauterine life and depending on tissue type can last till the host's organism demise. These tissues histogenesis and odontogenesis start when the cells of the ectoderm and mesoderm germ layers interact, differentiate and form increasingly specialised tissues in the area of mouth cavity. The process does not stop until the tooth organ maturation and eruption by when all the dental-related tissues are mature. Seeing that different dentitions (predeciduous, primary, secondary) start forming at different times from tissues of different level of maturation, it is sensible that their dental-related tissues quality differs as well. The following overview of human teeth histogenesis and odontogenesis describes the developments in the dental-related tissues-quality over time.

Histogenesis

All the cells which form dental-related tissues originate in adjoined leaf-like layers of ectoderm and mesoderm. These germ layer-cells move around and create tissues which take on different morphological characteristics and purpose throughout the process of odontogenesis and regeneration.

While the tissues originating from mesoderm germ layer gives rise to oral mesenchyme (the source of all connective and supporting soft tissues and vessels composing the tooth and tooth socket), the ectoderm germ layer gives rise to two original types of tissues: the stomodeal ectoderm (oral epithelium) and the cranial neural-crest-derived ectomesenchyme (neural-crest cells which have undergone an epithelial to mesenchymal transition and manifest mesenchymal phenotype [12]); which together construct all the hard tissues of the tooth.

Organogenesis/Odontogenesis

The complex process of tooth organogenesis/odontogenesis starts at the 6th week of the human embryogenesis and, depending on the type of a tooth, lasts up to 18 years. Odontogenesis is divided into four stages: (1) *the initiation stage*, (2) *the bud stage*, (3) *the cap stage*, and (4) *the bell stage*.

The initiation stage starts with the migration of oral epithelium cells into the underlying mesenchyme and ectomesenchyme of the stomodeum where they form the primary (general) dental lamina, the first signaling centre of tooth development which gives rise to all the teeth buds of primary dentition (20 deciduous teeth) and 12 molars of secondary dentition. Subsequently, around 12th week, the secondary (successional) dental lamina evolves, as a lingual extension of the primary lamina. Finally, at 14th week of embryonic development, the secondary dental lamina gives rise to the first germs of permanent teeth with predecessors (20 teeth)/at the primary dentition bell stage.

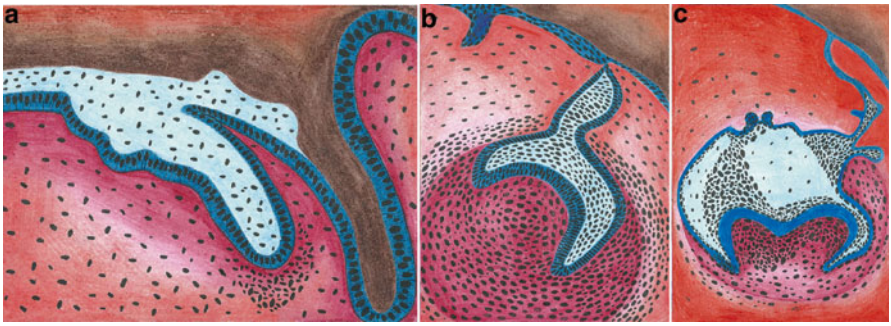

Fig.1 (**a**) developing tooth in a bud stage, (**b**) developing tooth in a cap stage, (**c**) developing tooth in a bell stage

The bud stage (Fig. 1a) is the first stage of the actual tooth germ development (at about 8th week for deciduous teeth). The ectodermal (epithelial) solid bud induces the proliferation and condensation of the underlying mesenchymal and ectomesenchymal cells to form the dental papilla which inductive capacity is essential for further development of ectodermal epithelial bud.

During *the cap stage* (Fig. 1b) (at about 9th–10th week), 3 basic structures develop, i.e. the enamel organ, dental papilla and dental follicle.

The bell stage (Fig. 1c) (approx. 11th–12th week) is primarily characterized by the activation of the enamel organ and the formation of the dentino-enamel junction. The enamel organ is composed of 4 epithelial layers—the outer enamel epithelium (OEE), reticular epithelium, intermediate stratum and inner enamel epithelium (IEE). The IEE starts to differentiate into preameloblasts which induce the proliferation of ectomesenchymal cells in the outer layer of dental papilla and their differentiation to (pre)odontoblasts. Odontoblasts start to secrete predentin which induces the process of preameloblasts maturation into ameloblasts, and so the secretion of the enamel commences. When the amelogenesis (i.e. the production and full mineralization of the enamel) is finished, the ameloblasts degenerate and disappear.

Later in the bell stage, the cervical loop (an area of the enamel organ located on the future crown-root border, composed of the IEE and the OEE) elongates apically through proliferation towards the epithelial diaphragm and eventually becomes Hertwig's epithelial root sheath (HERS).)The HERS then initiates the tooth root formation and activates the production of root dentin (in the same manner as in the crown region). After the activation of the local odontoblasts the root sheath disintegrates, this way the newly generated dentin becomes exposed to the ectomesenchymal cells in the inner layer of dental follicle, which eventually induces the differentiation of these follicular cells into cementoblasts and so the production of precementum. The exposed dentin further induces the differentiation of fibroblasts from the middle layer of dental follicle and so eventually the creation of periodontal ligament, and the differentiation of osteoblasts of alveolar socket leading to the creation of the outer layer of the dental follicle.

Eventually, the mature soft dental-related tissues are dental pulp, periodontal ligament, gingiva and apical papilla, and the mature hard dental-related tissues are enamel (oral epithelium), dentine and cementum (neural-crest-derived ectomesenchyme).

Dental-Related Tissues' Stem. Cell Lineages Differentiation Potential and Anatomic Location

Tissues' stem cell populations quality and quantity vary with their origin and stage of maturation. At maturation, the hard dental tissues posses no stem cell populations, while the soft dental-related tissues do and facilitate both self-regeneration as well as regeneration of them adjoined hard tissues [13, 14]. The only hard dental tissue which lacks the possibility to repair is enamel for the absence of communication with any soft tissue.

For the summary of up-to-date differentiation potential of dental-related stem cell lineages see Table 1.

Tooth Organ-Stem Cell Lineages

Immature Tooth Organ-Stem Cell Lineages

Dental Papilla Stem Cells: iTDPSC

Immature Tooth Dental Papilla is an engineering tissue of mesenchymal origin enclosed by the enamel organ on the top and by the dental follicle on the bottom, which eventually converts to pulp tissue. It plays central role in epithelial-mesenchymal interactions responsible for tooth morphogenesis. It is firstly responsible for the crown morphogenesis and later, when it becomes apical to dental pulp (a converted top part of dental papilla, after the onset of dentinogenesis/bell stage) it becomes secondly also responsible for the morphogenesis of the tooth roots (in the form of Apical Papilla) [54–56]. The most suitable teeth for obtaining dental papilla are third molars where the roots are not developed at all, this condition is fulfilled before the 14 years of age (Figs. 2 and 3a).

The up-to-date knowledge of the differentiation potential of the Dental Papilla Stem Cells is minimal and calls for further research. Most recently, in 2014, Vishwakarma et al. have stated that the dental papilla stem cells "can induce both enamel-dentine and dentine-cementum production", but only under certain specific conditions and combined with the appropriate cell populations.

Apical Papilla Stem Cells/Immature Root Papilla Stem Cells: SCAP/iRPSCs

The root formation of developing tooth starts when the cervical loop-epithelial cells proliferate apically and induce the differentiation of dental follicle mesenchymal cells into odontoblasts and cementoblasts [57] This apically extending epithelial

Table 1 Differentiation potential of dental-related tissues stem cells lineages

Differentiation capacity	DPSC	iRPSC (SCAP)	iTDPSC(germ)	NDP-SCs	SHED	DFSCs	PDLSCs	GMSCs
Adipogenic	Struys [15]	Sonoyama [16]	Yalvac [17]	Akpinar [18]	Miura et al. [6]	Kémoun [19]	Xu [20]	Gao et al. [21]
Chondrogenic	Koyama [22]	Wang [23]	Demerci [24]	Akpinar [18]	Kerkis [5]	Kémoun [19]	Xu [20]	El-Sayed [25]
Osteogenic	Mori [26]	Bakopoulou et al. [27]	Yalvac [17]	Akpinar [18]	Miura et al. [6]	Morsczeck [11]	Xu [20]	Gao et al. [21]
Neurogenic	Osathanon [28]	Sonoyama [29]	Yalvac [17]	Karaöz [30]	Miura et al. [6]	Morsczeck [11]	Shi [31]	Zhang et al. [32]
Myogenic	Zhang [33]	Abe et al. [34]	Tasli [35]	Karaöz [30]	Kerkis [5]	d'Aquino [36]	Shi [31]	Ansari [37]
Endothelial cells	Hilkens [38]	Bakopoulou [39]	Yalvac [17]		Cordeiro [40]	Dögan [41]		Zhang et al. [32]
Odontogenic	Huang [42]	Sonoyama [29]	Demerci [24]		Miura et al. [6]	Chen [43]	Shi [31]	Gao et al. [21]
Hepatogenic	Ishkitiev [44]	Patil [45]	Ikeda [46]		Ishkitiev [47]	Patil [45]	Kawanabe [48]	
Cementogenic	Kim [49]	Kim [49]				Kémoun [19]	Seo [9]	
Melanocytes	Paino [50]							
Cardiomyocytes	Armiñán [51]							
Epithelial cells			Dögan [41]					
Endodermal cells								Zhang et al. [10]
Periodontal ligaments						Morsczeck [11]		
Pancreatic islets	Ishkitiev [52]							
Cornea cells	Gomes [53]							

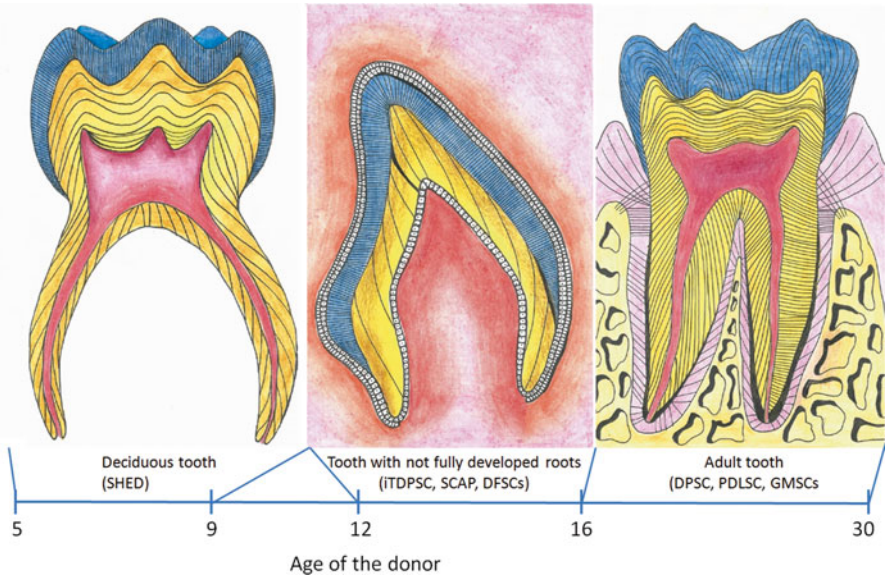

Fig. 2 Dental related stem cells lineages and the most common age of the donors of the different stem cells populations

wall forms Hertwig's epithelial root sheath (HERS) which guides the shape of the tooth roots. It is at this time dental papilla becomes apical to dental pulp tissue (hence apical papilla) and forms apical cell-rich zone as a divide between the two [55]. Overall, apical papilla is histologically different from dental pulp of immature tooth in multiple ways, according to Sonoyama et al., its stem cells proliferate 2 to 3 times quicker than those of the adjoined immature dental pulp, it contains less cellular and vascular components and it further contains unique potent MSCs. As apical papilla is a tissue derived from the neural crest, its stem cells express numerous neurogenic markers [58].

As SCAP are isolated from the apical papilla of a tooth with not yet fully developed roots, the most suitable teeth to extract are the first premolars of up to 12 years old patients and the third molars of 12–16 years old patients (Figs. 2 and 3b–d).

Mature Tooth Organ-Stem Cell Lineages

Dental Pulp

Dental pulp originates in dental papilla and is made up of connective tissue and four distinct cell-zones. The dental pulp connective tissue consist of an extracellular matrix (ECM) composed of ground substance with relatively high content of glycosaminoglycans, proteoglycans and other adhesion molecules (fibronectin, laminin, etc.) and the sparsely distributed type III collagen fibres which form a rich network only around vessels and nerves. The outmost cell-zone of the dental pulp is the odontoblast layer-zone (gives rise to the primary "predentine", secondary and tertiary

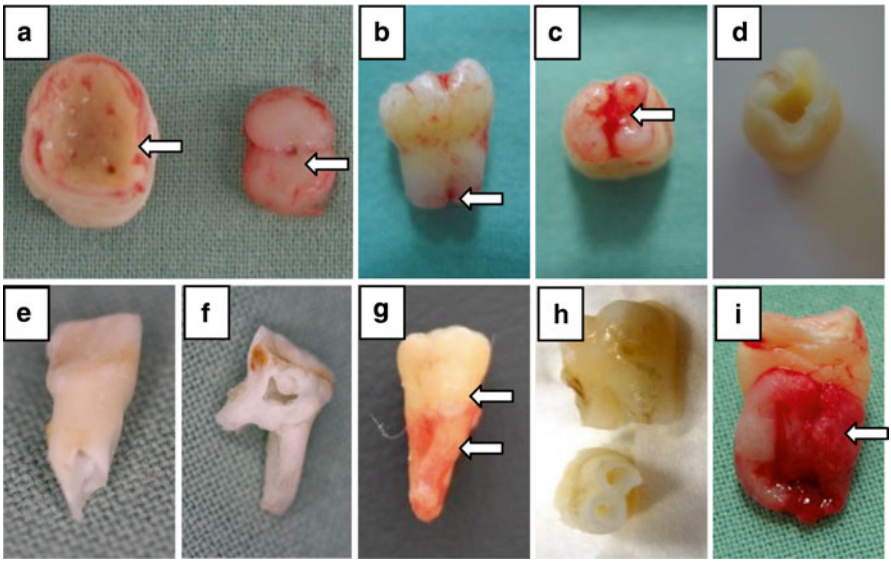

Fig. 3 (**a**) Extracted 3rd lower molar in a bell stage (germectomy) on the left side and its pulp on the right side. (**b**) Extracted 3rd lower molar with not fully developed roots. (**c**) Extracted 3rd lower molar with not fully developed roots with the apical papilla covering the root orificium. (**d**) Extracted 3rd lower molar with not fully developed roots from the (**c**) after extraction of the pulp tissue. (**e**) Extracted upper deciduous first incisor where the root apex is reabsorbed and the root canal widely opened. (**f**) Extracted second lower deciduous molar after splitting the crown using the bur to open the pulp chamber. (**g**) Extracted upper 3rd molar, the lower arrow indicates the area which is covered by the periodontal ligaments and presents the suitable source of the periodontal ligament stem cells, the *upper arrow* indicates the cement-enamel junction. (**h**) Two extracted upper first premolars after the isolation of the dental pulp. (**i**) Extracted lower 3rd molar with dental follicle tissue marked with the *arrow*

"reparative" dentine), followed inwards by a cell-free-zone called the Weil basal layer (contains dense capillary and nerve network), then cell-rich-zone, and finally arriving at the pupal core. The last two zones share similar structure as they are composed of larger vessels, nerves and great amount of cells, specifically: fibroblasts, cells belonging to the defense system, and undifferentiated mesenchymal stem cells.

Dental pulp stem cells lineages' quality and characteristics differ with dentition (predeciduous, primary, secondary) and level of their maturation.

Predeciduous Dentition Dental Pulp Stem Cells/Natal, Neo-Natal Dental Pulp Stem Cells NDP-SCs

The natal (erupted before birth) and neonatal (erupted within the days after birth) teeth are rarity found in some newborn babies within the first few days after birth. Natal teeth are more frequent than neonatal teeth, with the ratio being approximately 3:1 [59]. These teeth are usually indicated for extraction due to the danger connected to their inhalation, the risk that they could cause deformity and mutilation of tongue, dehydration, malnutrition, growth retardation of the following dentitions and due to

the health risks they can cause to the mother during breastfeeding. Histologically, the natal teeth have only thin or completely absent layer of both enamel and cementum. The predentin layer is often of various thickness compiled of irregular dentinal tubules. Furthermore, unlike dental pulp of teeth of the primary and secondary dentitions, the natal dental pulp has extra inflammatory areas and does not have the Weil basal layer and cell-rich-zone. These teeth tend to have a wide pulp chamber and underdeveloped roots which makes isolation of their dental pulp easier.

Primary Dentition Dental Pulp Stem Cells: SHED

The amount of stem cells from human exfoliated deciduous teeth decreases with the receding dental pulp (which is gradually replaced by gum). This implies that the deciduous teeth that fall out spontaneously are likely to have little to no pulp, therefore little to no SHED population. To ensure a sufficient amount of stem cells will be isolated, the rule of thumb is to focus on teeth which have at least 1/3 of the original root length (after the onset of primary dentition root resorption), and for the multi-rooted teeth it is best to isolate teeth with the furcation area still present (Fig. 3e, f).

The ideal time to extract the deciduous teeth for their dental pulp stem cells is between the ages of 5 and 9. Beware; dental pulp of teeth affected by dental carries is not fitting for use in research.

Secondary Dentition Dental Pulp Stem Cells: DPSCs

The teeth most commonly used for isolation of the dental pulp stem cells are the first premolars and the third molars (Fig. 3g, h), as they frequently attract preliminary extraction at early age of the dental tissue. The first premolars are usually extracted shortly after eruption, due to the orthodontic reasons, around the age of 12. The "youth" of their soft dental-tissues, not fully developed root, and very low probability of carries makes these teeth the ideal stem cell source. The third molars are often extracted to prevent health complication such as the surrounding tissue inflammation, as well as due to the orthodontic reasons. While the extraction for orthodontic reasons comes around the age of 16, the extraction due to tissue inflammation occurs later, usually between the ages of 16–30, hence involves at least partially-erupted and often fully developed tooth.

Tooth-Supportive Tissues-Stem Cell Lineages

Immature Tooth-Supportive Tissues-Stem Cell Lineages

Dental Follicle Stem/Progenitor Cells: DFSCs

The dental follicle is a loose connective tissue that surrounds the developing tooth and is separated from dentin by an epithelial layer (Hertwing's sheet). Once this epithelial layer disintegrates and dental follicle touches the dentin, it is prompt to differentiate into periodontium including the alveolar bone, cementum, and

periodontal ligament [60]. According to Vishwakarma et al. [61], the tissue contains at least three distinct stem cell populations (hDF1, hDF2, hDF3) with distinct morphologies, gene expressions and differentiation potentials.

Most commonly, the unerupted third molars are used for isolation of these stem cells, usually before 14 years of age (Fig. 3i).

Mature Tooth-Supportive Tissues-Stem Cell Lineages

Periodontal Ligament Stem Cells: PDLSCs

The periodontal ligament connects alveolar bone and cementum to support teeth in situ and preserve the hard tissues homoeostasis [9]. Periodontal ligament stem cells are commonly isolated from the soft tissue adjoined to the root below its enamel-cementum border (Fig. 3g); they form adherent clonogenic population of fibroblastic-like cells, forming flat and loose aggregates [62]. In 2010, Kawanabe et al. [62] have shown that PDLSCs express embryonic stem cell-associated antigen SSEA-4, implying these stem cells are great source of building material for regenerative medicine, being capable of differentiating into cells of all three germ layers.

The periodontal ligament tissue is usually isolated from around the first premolars and third molars from donors who have no history of periodontal disease and healthy periodontium.

Overall, PDLSCs were demonstrated to be able to differentiate into adipocytes, collagen-forming cells, cementoblast-like cells [9, 63], chondrocytes, neurons, hepatocytes [62], and osteoblasts [32] in vitro; and into cementum- and periodontal ligament-like tissues in vivo modified [9].

Gingiva-Derived Mesenchymal Stem Cells: GMSCs

The gingiva is a mucosal soft tissue which forms a shield-like physical protection of the adjoined dental-related tissues by sealing the gap between tooth's exposed enamel and periodontal ligament protected cementum; it further exhibits both immunomodulatory and anti-inflammatory capacities, which allow it to help the adjoined tissues to regenerate [10].

The GMSCs are usually derived from the spinous layer of human gingiva [64]. This highly homogenous population of stem cells carries not only the usual mesenchymal stem cell markers but also a positive expression of extracellular matrix proteins [10]. GMSCs have been successfully differentiated into osteoblasts, chondrocytes, adipocytes.

Donor

Theoretically any vital tooth and its enveloping tissues provide possible source of dental-related stem cells. However, it is important to narrow the pool of possible donors to those who are most likely to donate viable and research feasible stem cell

populations, will be the least impacted by the extraction procedure, populations, while undergo the least impactful extraction procedure. Eventually, electing the right research material is directly related to choosing a fitting donor.

The three basic recommendations in choosing the right donor:

1. *Dental-related tissues to be extracted*
   - Different stem cells lineages can only be isolated from donors of certain age/development status
2. *Health status of the donor*
   - General medical history—as certain illnesses and diseases (e.g. genetic predispositions) can cause researcher bias as they may influence the qualities of the research material
   - Possible impact of tissue extraction on the donor's health
3. *Clinical status of the tissue*
   - The general condition of the tissue and its immediate environment represent yet another research bias (e.g. caries lesions represent risk of bacterial transmission, inflammation of the soft tissue can adversely impact the research material, erupted tooth is contaminated by the oral micro microflora, fully developed roots complicate the process of the dental pulp isolation)

Election of Research Method

Bioethics, Legislation and Regulation of Human Stem Cell Research

The principal goal of human biomedical stem cell research is to relieve and avert suffering caused by both genetic and acquired impediments to health condition [65].

Due to the interdisciplinary character of the knowledge base and the worldwide network of research which aims to build upon each other, while oftentimes towards different goals, it is eminently important to unify the standards for information integrity, full disclosure, and respect for research participants—their health and freedoms [65]. While the research focused on the unipotent, multipotent and pluripotent stem cells avoids the major ethical and legal issues connected to the totipotent embryonic stem cells and oocytes, there are other concerns which remain standing.

The international consensus over the stem cell research guidelines is conveyed by the *ISSCR—International Society for Stem Cell Research*, established independent non-profit organisation founded in 2002, which releases yearly updated guidelines for stem cell research and clinical trials and provides templates of legal documents connected to biomaterial donation and transfer [65]. The ICSCN—*International Consortium of Stem Cell Networks* then provides the contacts and means to bridge varying national stem cell legislation and regulation to foster best practice international research collaborations. The consortium is managed by a

secretariat comprising of nationally funded representatives from Australia, Canada, Germany and Scotland and meets annually in conjunction with ISSCR.

Beyond the international organisations, the research ethics and regulations are further narrowed by supra-national consortia such as the European *EuroStemCell* and the American USFDA—*Food and Drug Administration of the United States of America*; national legislation and regulations issued by National Academies; and eventually by the institutional SCRO—*Stem Cell Oversight Committee* [66]. The SCRO must respectively consist of proxies of both scientific and ethical communities (also referred to as: IRE—Institutional Review Board/IEC—Independent Ethics Committee/ERB—Ethical Review Board or REB—Research Ethics Board), from which a part is non-affiliated. The ethical community further demands membership of representatives of ethical expertise and those representing the lay public [67]. Finally, the text summarising the institutional professional standards and self-regulations, issued by the institutional SCRO, deals with all the above issues in two separate parts: Part 1: *Respect for research participants* and Part 2: *Integrity of the research enterprise* [67]. The SCRO standards for non-totipotent stem cells research should, according to the ISSCR 2015 Draft Guidelines [65] and Hyun [68], address the following:

1. *Respect for Research Participants*

   The respect for research participants remains accompanied with the issue of donor/patient consent to share genetic information and the issue of donor/patient mental and physical state now and in the future; which prohibits scientists to treat the biomaterial freely and subject it to downstream research which has not been approved by the SRCO and the donor in advance [68]. The above issues require written and legally valid informed consent to donate (if the donor is not sui juris, their legal representative has to subscribe the informed consent instead), which according to the ISSCR Draft 2015, should address both the donor and research organisations' rights and obligations towards each other, specifically:

   (a) The provision of accurate information on risks to donor health connected to tissue retrieval.
   (b) The provision of accurate information on risks, limitations, possible benefits, and available alternatives to patient treatment.
   (c) The donated tissue treatment and storage—genetic modification, recipient profile, discard protocol, ad cetera.
   (d) The genetic and disease screening of the donated material.
   (e) The full disclosure of the derived information.
   (f) The donor/patient private information (identification, genomic information, ad cetera) treatment and discard protocol.
   (g) The future accessibility of the donor/patient to obtain additional consent, additional material or information.
   (h) The commercial potential of the donated material and intellectual property rights of the researchers/donor.
   (i) The treatment of incidental findings.
   (j) The treatment and legal liability of the researchers and medical staff in case of unforeseeable impacts on patient health [65].

2. Integrity of the Research Enterprise
   The treatment of the biological material founded upon valid, timely, reliable and reproducible protocol; executed following the GLP—Good Laboratory Practices; and reported in transparent, reliable manner and accessible format ensures integrity of research enterprise. All of the above is pertinent for independent peer review and downstream research.

The US FDA provides a step-by-step toolkit to institutional SCRO organisation, operation, administration and reporting which can be used as the building blocks of the institutional standards [65].

Dental-Related Tissue Extraction and Transport

Dental-Related Tissues Extraction

The extraction of tissues for stem cell research is performed under the same condition as an ordinary prescribed extraction, while we are interested in preserving the biological material as intact as possible, the health of the donor/patient comes first. Fundamentally, there are two types of extraction, the simple extraction and the surgical extraction; where both are performed under local anesthesia.

The simple extraction is adopted in the case of fully erupted tooth and is executed using various dental tools including elevator and forceps to break the tooth' bonds to periodontal ligament and to widen its alveolar bone socket, and so allow for smooth removal of intact tooth.

The surgical extraction is adopted in case of an impacted or not yet fully erupted tooth (the most common source of dental-related stem cell material) (Fig. 4).

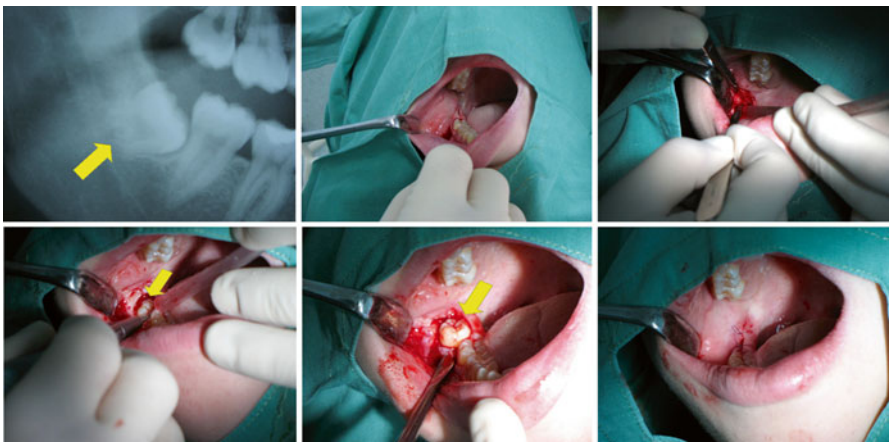

Fig. 4 The procedure of complicated extraction of lower 3rd molar. First picture represent the cut of the ortopantomogram. Second and third pictures show the moving of the mucoperiostal flap. On the fourth picture the bone covering the tooth is removed and on the fifth picture we can see the extraction using the levers

This procedure involves rising of mucoperiosteal flap/tissue flap on the intra-oral side of gums covering the tooth (for esthetic reasons) and trimming the exposed bone present around the tooth roots to allow for smooth root removal (here the prime consideration is to limit the amount of bone being removed). At this point, depending on situation, the surgeon either retains the tooth as a whole, which is preferable, or sections it to further ease its extraction. Once sectioned, however; the dental-related tissues are compromised together with their value in research.

Immediately after the extraction it is recommended to disinfect the tooth using any disinfectant solution commonly used in the dental cavity, to decrease the possible amount of bacterial presence. Then, the tooth needs to be fully immersed in the Hanks balanced salt solution enhanced by antibiotics and antifungals (composed of 1 ml of Hanks balanced salt solution, 9 ml water for inj., 200 μl/10 ml gentamycin, 200 μl/10 ml streptomycin, 200 μl/10 ml and 200 μl/10 ml penicillin) to fully eliminate any remaining bacterial presence.

Dental-Related Tissues Transport

The temperature of the solution during transport is highly recommended to be kept at 4 °C. Even though stem cells are theoretically resistant to hypoxia and survive longer than somatic cells, it is absolutely essential they are isolated from the extracted tissues as soon as possible (no later than 24 h after extraction). For this reason it is necessary to invest in proper preliminary planning to avoid any wastage.

Isolation of the Dental-Related Tissues

Tooth-Organ Tissues Extraction

Immature Tooth-Organ Tissues Extraction

Dental Papilla and Apical Papilla Extraction

Dental papilla tissue is extracted from tooth without developed roots, hence sourced from very young donors/patients. Such extraction is called germectomy (Fig. 3a) (an identical procedure is used to obtain dental follicle tissue (Fig. 3i). The most common source of the dental papilla tissue for stem cell research are third molars of donors/patients aged 10–14 years, as those teeth are commonly indicated for extraction. After the onset of root dentine formation and root development, the dental papilla splits into the soft tissues of dental pulp and apical papilla.

Apical papilla is localized at the area of root growth, below dental pulp (Fig. 3b–d). The dental pulp and apical papilla are clearly separated by apical papilla adjoined apical cell-rich zone which however does not stick to dental pulp and so allows smooth separation of the two tissues.

Whilst dental and apical papilla tissues can be obtained from immature teeth of both dentitions, it is rare to find immature deciduous teeth indicated for extraction which have no caries lesion; therefore the SCAP and iTDPSC are widely considered as stem cell lineages of secondary dentition.

Mature Tooth-Organ Tissues Extraction

Dental Pulp Extraction

Dental pulp consists of connective tissue and odontoblasts locked together inside the dental pulp chamber. During the process of tooth maturation, dental pulp tissue becomes increasingly enclosed in hard dental-related tissue, until only a tiny opening at the roots' apex (physiological apical foramen) is left (0.25–0.35 mm wide at maturation) to allow the nervous connections and blood vessels. To ensure the extracted dental pulp tissue is as intact as possible, we aim to use the least destructive method which varies with the tooth level of maturation (including all postnatal immature and mature dental pulps of all dentitions).

The three most commonly used methods of dental pulp extraction are: (1) Extraction of the dental pulp through the physiological apical foramen, (2) Splitting the tooth using forceps and (3) Splitting the tooth using burr (rotary file).

The first method is regarded as the easiest, fastest and the most qualitatively efficient of all; however, it can only be performed on teeth with wide apical foramen (approx. $x > 2$ mm), therefore reserved exclusively for teeth with roots which are not yet developed (isolating immature dental pulp tissue of primary and secondary dentition) or for teeth which roots are resorbing (isolating primary dentition dental pulp tissue). The first step of this extraction process is to gently detach the dental pulp from the adjoined dentine (commonly performed using a needle). The following step is to pull the pulp out downwards using tweezers (commonly performed using anatomical stainless steel hard tweezers, model ADSON) (Fig. 5a).

When the physiological foramen is too narrow to use as the extraction canal, we are left with other methods which involve mechanical disintegration of the hard dental tissue surrounding the pulp, opening the dental chamber from the top. To access the soft dental pulp, we tend to aim to split the tooth at the enamel-cementum junction (cervical area) using either forceps (crashing the junction) (Fig. 5b) or a burr (employing a diamond burr on a fast micromotor handpiece—red stripe, about 200,000 rpm; cooling the tooth with water spray; performing horizontal cut at the junction (Fig. 5c) (it is also possible to employing a disc shaped burr and performing vertical cut, though this approach is regarded as very risky as far as the pulp integrity and quality is concerned (Fig. 5d)). It is also important to mention that the burr method has potentially detrimental impact on the dental pulp due to the heat emitted during filing. If successful the dental pulp can be pulled out upwards using the same method as described above. While these mechanical methods are relatively complicated, slow and less efficient in the amount of tissue harvested (in comparison with the first method), they allow researchers access to much greater number of donors/donated tissues.

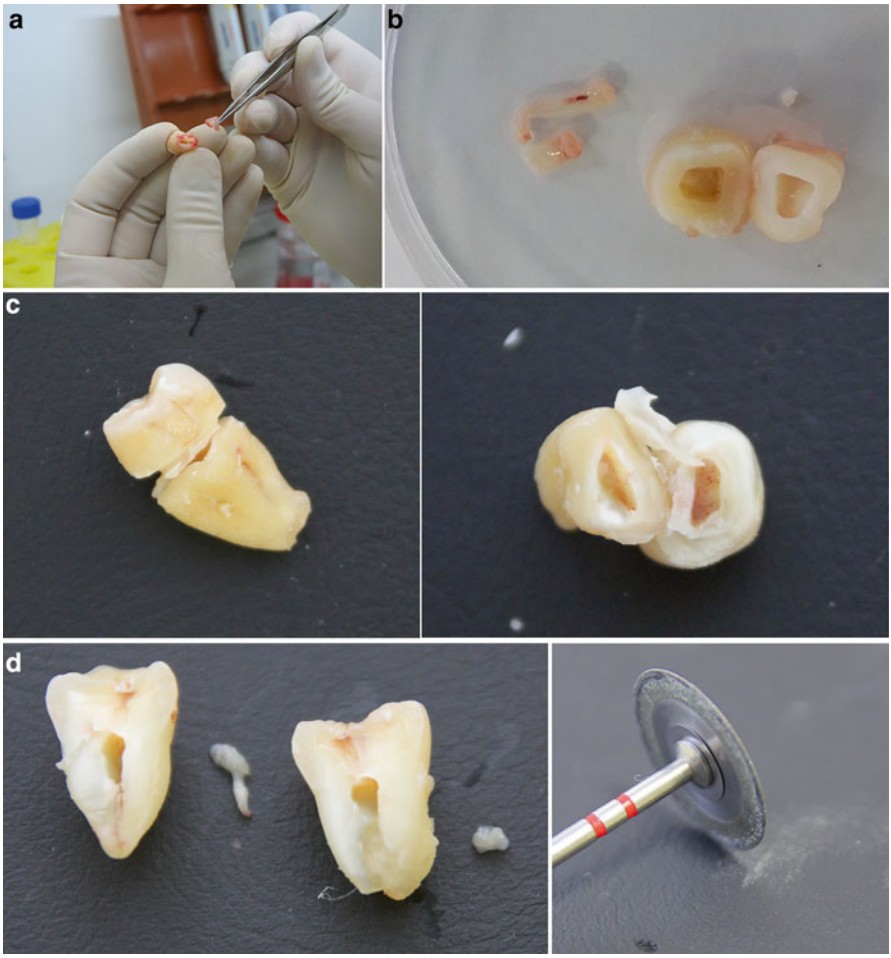

Fig. 5 (**a**) Extraction of the pulp tissue from the tooth with not fully developed roots, where the pulp chamber is widely opened. (**b**) Extracted lower 3rd molar where the crown was separated from the root in the using the forceps and the isolated dental pulp tissue. (**c**) Extracted upper 3rd molar where the crown was separated from the root in the using the diamond burr on the *left side* and the same tooth still containing the pulp tissue on the *right side*. (**d**) Extracted upper 3rd molar where the crown was separated from the root in the using the forceps soflex discs (the bur is on the *right picture*)

Tooth-Supportive Soft Tissues Extraction

Immature Tooth-Supportive Tissues Extraction

Dental Follicle Extraction

Dental follicle is loose connective tissue surrounding the developing tooth germ (Fig. 3i) which degenerates after the tooth erupts and finishes its development. It's

harvest after tooth organ extraction is relatively easy as it is not firmly adjunct to the tooth. Due to this timing issue, the most frequent source of this tissue are the third molars extracted from the young donors (around 10–14 years old). These teeth are frequently extracted for orthodontic reasons and are commonly completely embedded in the jaw bone.

Mature Tooth-Supportive Tissues Extraction

Periodontal Ligament and Gingiva Extraction

Periodontal ligament is specialized connective tissue localized between the alveolar bone and root surface, and is hidden under gingiva. While not all extracted teeth carry a research-sufficient amount of gingiva tissue, as the gingiva is commonly raised from the tooth surface preceding the tooth organ extraction, it commonly carries enough periodontal ligament tissue. Both the tissues are harvested off the tooth root surface by scraping.

Dental-Related Stem Cells Isolation

In general, dental-related stem cells are isolated from their maternal tissue using one of the two following approaches: (1) Enzymatic Digestion (ED) of tissues or (2) Spontaneous Outgrowth (OG) from the maternal tissue mince [64]. Recent scientific research suggests that isolation method most probably impacts the qualities of the isolated stem cell lineages [27, 69]. For example, while some researchers, comparing the impact of the above isolation methods on the resulting dental-related stem cell populations, concluded that OG isolated stem cell population had weaker stem cell-markers expression (SHED-ED × SHED-OG [70]) and lower proliferation rate (DPSC-ED × DPSC-OG [69], other studies concerning non-dental-related stem cells report contradicting results, mostly arriving to the decision to opt out from using the collagenase enzyme as it was believed to alter the stem cell phenotype [38, 71]. Ergo, it is important to keep an eye on the upcoming research in this field to help us narrate the most probably bias-free methodology.

Enzymatic Digestion/Dissociation: ED

During enzymatic digestion the extracted tissue is submerged in digestive enzymes solution (most commonly Hank's balanced Salt Solution (HBSS) carrying diluted collagenase I (3 mg/ml), dispase (4 mg/ml) and phosphate-buffered saline (PBS) (buffer solution) in a ratio of 1:1:1:1) for about 30–60 min, depending on the size of pieces of the tissue, at 37 °C. The result of ED is a single-cell suspension which contains many different cell types and remnants of vessels and extracellular matrix (Fig. 6). While it is not imperative to remove the remnants from the suspension, it

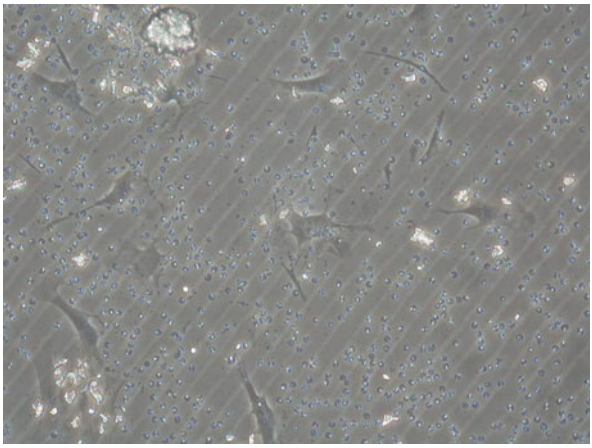

Fig. 6 Dental pulp stem cells in the prime culture 6 days after seeding with the remnants of the soft tissue and the erythrocytes

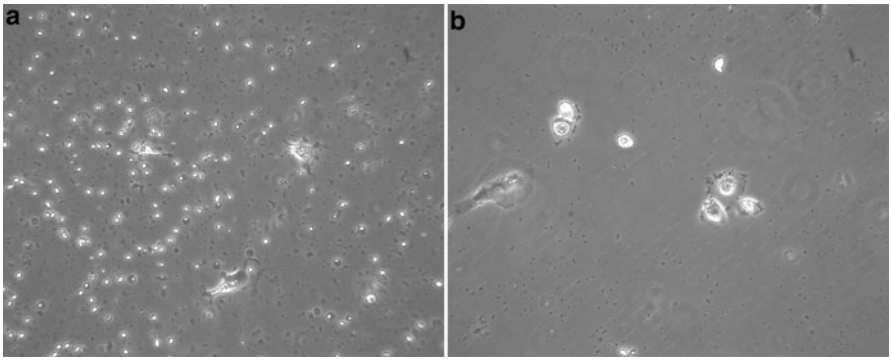

Fig. 7 (**a**, **b**) The same lineage of dental pulp stem cells before and after changing the media

can be done by either sieving through a strainer (3 μm followed by 20 μm), by magnetic-activated cell sorting (MACS) or by stem cell colony cultivation [64]. Whether researchers decide to work with suspension containing remnants or not, the single-cell suspension is centrifuged (600 g (g-force), 2000 rpm for 5 min) and the cell pellet is finally seeded into cultivation dishes where it is resuspended in amplification media. After 7 days of amplification the stem cells adhere to the surface of culture dish. At this point all the other material is washed away and new amplification media is added (Fig. 7a, b).

Spontaneous Outgrowth/Explant Culture: OG

The outgrowth method is technically easier and faster than ED. The soft tissue is minced to pieces smaller than 2 mm (explants) and planted on cultivation dish, fully immersed in amplification media. After 3 to 5 days of amplification we can observe stem cells adherent to the culture dish everywhere under and around the minced tissue (Fig. 8) [64].

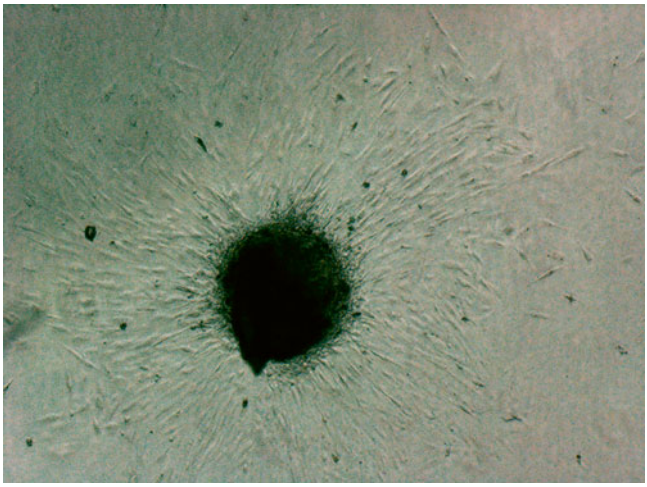

Fig. 8 Part of the dental pulp tissue with outgrowing stem cells. The photo was taken 12 days after seeding

Dental-Related Stem Cells Amplification and Differentiation Culture Systems

After we have successfully isolated our stem-cell lineages we aim to expand their population to research-relevant amounts (their amount at isolation varies by maternal tissue and method of extraction [72]. Depending on the future use of the amplified population it is important to choose the right amplification conditions and environment (physical characteristics of the culture system (adherent, non-adherent/sphere-forming), amplification media sera (xenogeneic, autologous serum, serum-free) and co-culture (epithelial/ectodermal-mesenchymal interaction) [64]) as they do affect the quality of the resulting stem cell population [73]. Especially, when human regenerative medicine is concerned, the researchers should avoid using xenogeneic sera as their use carries multiple legal, ethical and medical issues ranging from research results bias, to transfer of zoonoses (interspecies infectious agents); viral, bacterial and fungal contamination; and permanent alteration to the stem cell population genetic code [74].

Amplification and Differentiation Culture Systems

Generally, dental-related stem cells are amplified and differentiated at 37 °C at 5 % CO_2 atmosphere. To maintain high proliferation rate, cultivated stem cells should be passaged when the population reaches 70 % confluence (confluence >70 % leads to decrease in proliferation due to the physiological contact inhibition). The scientific

community recognises two basic physical cultivation environments for stem cell amplification and differentiation: the (1) *Adherent Culture Systems* and the (2) *Non-adherent (Suspension/Sphere-Forming) Culture Systems*; plus one extra type of differentiation environment, the (3) *Co-Culture Systems*. Overall, while the non-adherent culture systems are easier for passaging and the growth of cells is not limited by the surface area of the cultivation dishes, the adherent culture systems allow for easier visual inspection and provide less opportunities for cell heterogeneity [75]. The co-culture systems then provide "site-specific" lineages interaction which leads to differentiation of specific progenitor/adult cells [64].

Adherent Culture Systems

The adherent system consists of a dish which allows the cultured stem cells to adhere to it. At the end of cultivation, the dish is washed with phosphate-buffered saline, which safely removes all cultivation media. Once the stem cells are the only content of the dish, it's whole surface is covered by trypsin which detaches the stem cell off the surface of the dish. The trypsin is neutralised after 5 min by solution of α-MEM and serum (in concentration of 20%). The resulting suspension with cultivated cells is centrifuge (600 g, at 2000 rpm for 5 min). After centrifugation, the supernatant is removed and the resulting cell pellet is resuspended in new cultivation media and placed on new culture dish, in concentration of 2500 cells per cm^2. This culture system is the default system in stem cell research, except for cultivation and differentiation of non-adhesive cell lines (e.g. neural and hematopoietic cells).

Non-adherent/Suspension/Sphere-Forming Culture Systems

Spheres are free-floating cultures of stem cells. The non-adherent sphere-forming culture is generally serum-free and contains epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (LIF) and rests upon either super-hydrophilic (non-adherent) plates or in a culture flask [76, 77]. While this system has been widely applied for assaying neural stem cells up to neurogenesis [78] it has been also studied an effective system for generation of mineralized tissues (from SCAP) [34].

Jensen and Parmar's report on the applicability of this system raises a few important issues, they argue that the cultured cell types properties appeared to be sensitive to the culturing method used, variation in cell density in the system, as well as the concentrations of factors in the media, method and frequency of passaging and the number of passages after isolation [78]. Furthermore, multiple researchers report that the sphere-forming cells become increasingly heterogeneous with further passage as the growing spheres contain cells at various stages of differentiation, from stem cells to progenitor cells and specific cell types [75, 79].

Co-culture Systems

Co-culturing of various dental-related stem cells is not used for their amplification as much as it is used for their differentiation. These co-cultivation systems applied in the dental-related stem cell research are based on our knowledge of the dental-tissue genesis (epithelial-mesenchymal co-culture/ectodermal-mesenchymal co-culture). For example, Bai et al. [80] have demonstrated that co-cultivation of DFSCs and SCAP (rat) can lead to formation of bone-like particles and Xiao and Tsutsui [81] demonstrated that co-cultivation of oral epithelial cells (OECs) and DPSCs can build an epithelium invagination like model. These systems are very promising tool for future research as they replicate the original in vivo environment more accurately than the above single-cell systems.

Amplification Media

The cultivation media, as well as any component of extracellular environment affects the properties of cultivated cells. Due to the vast heterogeneity of media composition used through dental-related stem cell studies, it becomes challenging to make any generally applicable judgements about the media on the amplified or differentiated cell populations. Therefore it is imperative that when outlining a research method, one should critically and attentively evaluate the validity of preliminary research' methods and their results to eventually arrive at scientifically credible conclusion and results in their work, results which can be build upon. The three general groups of media are: (1) *Serum-Free Medium,* (2) *Allogeneic-Serum Medium,* and (3) *Xenogeneic-Serum Medium*. The serum-free media are currently the most preferable solution due to absence of risks associated with the xenogeneic and allogeneic media (e.g. transfer of viruses and zoonoses).

Serum-Free Media

The serum-free medium, most frequently used in dental-related stem cell research, is a combination of the following additives: Dulbecco's modified Eagle's medium, insulin, transferrin, sodium selenite supplement, Embryotrophic Factor [82]. While the serum-free media are most possibly the future of stem cell research, they are not yet universally effective when used for various types of stem cell lineages, and their efficiency in boosting stem cell proliferation and accessibility remains low [83]. Today, the serum-free media are used as a complementary media to amplification and differentiation processes powered primarily by either allogeneic or xenogeneic media.

According to a study, conducted by Tarle et al. [84], on use of serum-free media for amplification of PDLSCs and SHED; the stem cells cultivated partially under serum-free condition express higher positivity of stem cells markers then when amplified in xenogeneic medium alone. They also manifested similar differentiation capacity, while their proliferation rate was significantly slower [84].

Allogeneic Serum-Containing Amplification Media

The most commonly used allogeneic sera are human plasma (HP) or platelet rich plasma (PRP) [85–89]. While HP does deliver on its promise, it is proven that PRP, due to its boosted contents of platelets/platelet lysate, is more potent in maintaining the cultured dental-related stem cell properties and its use results in greater cells' proliferation rate, immunosuppressive activity and migration activity [87, 90, 91].

The disadvantages of these sera are the hazards involved in transferring heterologous material which can carry diseases (e.g. HIV, Hepatitis), drug metabolites, and depending on the donor also irregular concentration of growth factors and other components which vary within physiological borders. Nonetheless, the disease and drug metabolite hazards can be managed and the concentrations of the components can be successfully averaged by pooling material sources from multiple donors (>4) [92].

Xenogeneic Serum-Containing Amplification Media

The xenogeneic media contain around 2–20 % of fetal calf serum/bovine serum, where concentrations of >10 % are the most common. The advantages of this serum include its high content of growth factors and low concentration of inhibitory factors. The disadvantage is; however, that the concentration of those, together with the serum nutrients, enzymes and proteins, is not stable and varies sample to sample, within the species physiological borders. In general, the higher concentration of the bovine serum in media the better cell adherence, the more colony forming units, and the greater proliferation potential. Nevertheless, these research beneficial outcomes are offset by research detrimental impacts on the cultivated cells such as chromosomal instability, phenotype amalgamation, malignant transformations, and at concentrations >20 % even toxicity.

When attempting to minimise the above mentioned detrimental effects of high concentrations of xenogeneic serum on cultured cells while retaining the high proliferation rate and good adherence, it is recommended to use media with <10 % bovine serum and to make up for the higher concentrations by adding growth factors like platelet derived growth factor and epidermal growth factor to boost its performance. According to some studies the addition of insulin-transferrin-sodium selenite solution results in higher proliferation rate as well [92].

Nevertheless, there exists no solution for one of its major shortcomings, its xenogeneic origin, which brings a whole lot of possible hazards to the table. Eventually, its application can lead to internalization of bovine serum proteins by the cultured stem cells and finally stimulation of the recipient immune system and unwanted immunological reaction [93–95], or to transmission of the zoonoses [96, 97], and induction of metabolic and morphological changes in the cultured cells [98, 99]. Overall, while this serum is easily obtainable and relatively cheap, it is recommended it is phased out off the stem cell scientific research as it provides biased results which are not applicable for human medicine [100, 101].

Dental-Related Stem Cells Differentiation and Evaluation

According to their origin dental-related stem cells are pre-programmed to differentiate into certain mature cell lineages and it is harder, although not impossible, to induce them to differentiate into foreign lineages. To prove that the amplified dental-related stem cells are indeed dental-related stem cells we would test their capacity to differentiate into osteoblasts, chondroblasts and adipocytes, which capacity is characteristic for all mesenchymal stem cells. Yet again, the results of the differentiation process are highly dependent on the artificial extracellular environment and hence this step attracts good planning and thorough preliminary research. Besides choosing the basic culture system, as per chapter *i. Amplification and Differentiation Culture Systems*, we have to choose the right differentiation method (there are multiple approaches to the same end) and follow its evaluation process to produce valid results. (*Interesting new fact, in* 2015, Zhang et al. *have proven that LMHF mechanical vibration promotes PDLSCs osteogenic differentiation. This discovery implies that vibration may be one of the mechanisms of external environment which impacts the biological processes, hence should be considered as a variable when planning and reporting research results.*)

The below overview lists the most commonly used differentiation protocols in dental-related stem cell research:

Osteo-Differentiation Protocol and Evaluation

The dental-stem cells undergoing osteogenic differentiation are cultivated in adherent cultivation dish in a monolayer and are supposed to become almost 100 % confluent. The differentiation is induced by osteogenic differentiation media composed of α-MEM, 0.2 mM ascorbic acid 2-phosphate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM β-glycerophosphate, 0.1 μM dexamethasone and 10 % fetal calf serum and the process takes >4 weeks.

To prove a successful differentiation we use standard histological methods like Masson's blue trichrome and Von Kossa stain, to identify calcium deposits and collagen I fibres; or immunodetection of osteonectin, osteopontin, or procollagen I. Karbanová et al. [102] states that, by the week 3–4, we should be able to visually identify small extracellular aggregates of bone tissue.

Chondro-Differentiation Protocol and Evaluation

The dental-related stem cell chondro-differentiation can be successfully performed in either an adherent system (monolayer cultivation dish) and a non-adherent system (sphere-forming system). The chondrogenic differentiation medium is composed of high glucose DMEM, 0.2 mM ascorbic acid 2-phosphate, 0.1 μM dexamethasone, 1 % ITS + 1 supplement, 100 U/ml penicillin; 100 μg/ml

streptomycin, 40 μg/ml proline, 100 μg/ml sodium pyruvate, 10 ng/ml TGF-β3. Four weeks into the chondro-differentiation the acid mucins within the extracellular matrix can by visualized by alcian blue staining [102], proving the success of the process.

Adipo-Differentiation Protocol and Evaluation

Nowadays, adipogenic differentiation is most often induced using copyrighted adipogenic media. While the precise formula is not public, it is usually some mixture of dexamethasone, 3-isobutyl-1-methyl-xanthine, insulin, and indomethacin and standard cultivation media [103]. The results can be evaluated 4 weeks after the onset of the differentiation and the proof is provided by 0.18 % Oil red O staining of the intracellular lipid droplets in mature adipocytes with.

Neuro-Differentiation Protocol and Evaluation

The dental-related stem cells differentiation into neuro-like cells can be done, alike the chondro-differentiation, in both adherent and non-adherent systems using neurogenic differentiation medium composed of 50 ml DMEM, 50 ml F12 HAM, 2 ml B27 Supplement, 1 ml N2 Supplement, 10 ng/ml bFGF, 20 ng/ml EGF, 1 ml L-Glutamine, 1 ml Penicilin. The positive differentiation is proven using immunodetection methods to detect the markers typical for neural tissue—β-III tubulin, FORSE-1, GFAP, Nestin and PanNF.

Myogenic-Differentiation Protocol and Evaluation

Myogenic differentiation can be induced by treating the dental-related stem cell population with 3 μM 5-azacytidine in serum-free medium DMEM for the duration of 24 h. After this treatment, cells are washed with PBS and maintained in DMEM containing 10 % bovine serum [104]. To prove a successful myogenic-differentiation we should be able to visually identify myocyte markers using immunofluorescence-labeled antibodies against human cardiac actin, cardiac actinin, cardiac troponin C, cardiac troponin T, connexin 43, sarcomeric actin).

Hepatogenic-Differentiation Protocol and Evaluation

The dental-related stem cells undergo hepato-differentiation in media composed of 20 ng/ml recombinant human hepatocyte growth factor and 2 % bovine serum, for the first 5 days, and then a mixture of 10 ng/ml Oncostatin M, 10 nM Dexamethasone and 1 % Insulin-Transferrin-Selenium-X (ITS-X), for another 15 days [47].

The success of the differentiation is evaluated by either using imunohistochemistry detection of albumin, α-fetoprotein, hepatic nuclear factor 4α and insulin like growth factor, or periodic-acid-shift staining for visualising the stored glycogen.

Dental-Related Stem Cells Cryopreservation

SHED and third molar DPSCs represent the majority of dental-related stem cells material which is cryopreserved for either research or future personal-medical use. There is no clear winner amongst the cryopreservation methods available today as there are important questions pending to be answered: (1) What is the optimal cryoprotective agent and in which concentration should it be used to reduce or eliminate the side effect of cryopreservation?; (2) How many cells should be cryopreserved to obtain viable lineage after thawing?; and (3) What is the optimal process of cryopreservation, storage time and storage temperature to maintain maximum amount of viable cells?

Preceding cryopreservation the stem cell population should be harvested from the cultivation system. After centrifugation the cell pellet is resuspended using the chosen cultivation media and the amount of viable cells is measured. It is recommended to cryopreserve stem cell populations composed of at least one million cells. To avoid damage to the cells inflicted by the cryopreservation itself we expose the cells to cryoprotectants (e.g. glycerol or dimethyl sulfide—DMSO) in the concentration of maximum 10 % (as DMSO is cytotoxic)at room temperature for about 30 min, to allow the protectant to enter the cells but not damage them. The DMSO concentrate should be premixed before it touches the cells as it releases heat during the process of dilution. Finally, the treated cells should be fastly frozen using liquid nitrogen.

Good luck researchers!

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Isolation and Cryopreservation of Stem Cells from Dental Tissues

O.G. Davies and B.A. Scheven

Introduction

One of the principal aims of cell-based therapies is to deliver personalised medicine for the repair and regeneration of tissues lost to accidents or disease. To achieve this aim sub-zero temperatures (−196°) are applied that halt biological activity, thus preserving the cells for future clinical applications. The idea of banking stem cells as a means of '*biological insurance*' has seen a recent rise in popularity that is at least in part due to increased media attention and a greater public awareness of regenerative medicine. Consequently, several companies now exist offering individuals the opportunity to store their own multipotent cells, with the aim of future therapeutic application to restore or regenerate a multitude of tissues throughout the body.

In order for cellular therapies to be effective and widely available a number of biological and pragmatic considerations need to be taken into account. Firstly, the cryopreservation procedure employed must assure the quality and safety of the final biological product. This is of particular importance when the banked cells are intended for clinical use. In order to ensure that cryopreserved stem cells are suitable for clinical application standardised protocols and procedures are required that adhere to current good manufacturing practice (cGMP) [1, 2]. This means that cryoprofiles including type, concentration, equilibration, and dilution of cryoprotective agent (CPA) as well as freezing rate and thawing conditions must be optimised and

O.G. Davies (✉)
Wolfson School of Mechanical and Manufacturing Engineering,
Loughborough University, Loughborough, Leicestershire LE11 3TU, UK
e-mail: O.G.Davies@lboro.ac.uk

B.A. Scheven
School of Dentistry, College of Medical and Dental Sciences,
University of Birmingham, St Chad's Queensway, Birmingham B4 6NN, UK

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validated for particular cells/tissues [3]. Additional factors to be considered when banking stem cells for clinical applications are: potential cytotoxicity of the CPA and the immunogenicity of animal or human serum components often incorporated within the cryopreservation medium (CPM). Implementation of these standards will help ensure that cell viability, proliferation, genetic and epigenetic stability, and differentiation are not compromised, allowing banked stem cells to be as clinically effective as freshly isolated cells. One final point concerns the more pragmatic aspects of space, cost-effectiveness, and the establishment of GMP standards governing freezing protocols and the use of CPAs [4]. The scaling of stem cell therapies to meet clinical demand is by no means a simple task and the future success of cellular therapies is equally likely to depend on advances in the field of healthcare engineering as well as in basic science and medicine [5]. This concise review will focus on issues surrounding the cryopreservation of dental tissues, in particular stem cells isolated from dental pulp, which are an emerging and promising source for a wide range of regenerative therapies.

Principles of Cryopreservation

Cryostorage causes a physical insult to cells which could result in structural and molecular changes. The principal aim of any cryopreservation procedure is to provide adequate protection from damage caused by the formation of intracellular and extracellular ice crystals during both freezing and thawing stages [6]. In order to achieve this, CPAs are incorporated in the freezing medium. An ideal CPM is one in which cells maintain their stem cell characteristics over long periods of time (durability), with no loss in biological viability or vitality. CPAs work by penetrating the cell membrane where they bind to water molecules in solution. This acts to prevent an efflux of water from the cytoplasm during freezing, avoiding cellular dehydration and maintaining stable intracellular salt concentrations and pH [7]. To date, a number of different CPMs have been tested for the storage of mesenchymal stem cells (MSCs) derived from a variety of tissues, with the most common solution incorporating dimethyl sulfoxide (DMSO) as a CPA [8]. In addition to the choice and concentration of CPA used, the rate of freezing is also important for maintaining stem cell viability. Cells may incur damage caused by the process of rapid cooling (cold shock) or by the low temperatures used to enable long term preservation (chilling injury). The optimal cooling rate is likely to be specific for each tissue, and is largely determined by the ratio of cell volume to surface area, and by the permeability of the membrane to water and the CPA. The optimal cooling rate is also likely to depend on the concentration of CPA within the cryopreservation medium. Ideally, a CPA solution should be non-toxic for cells and patients, non-antigenic, chemically inert, ensure high cell viability after thawing and allow quick and simple transplantation without the need for additional washing steps [9]. However, producing a freezing environment that is optimal for both cells and patient will require further research, and it is likely that a compromise will have to be reached.

Dental Stem Cell Banking

This chapter focuses on the long-term storage of dental tissues, in particular stem cells derived from the vital central gelatinous tissue called the dental pulp. Dental pulp stem cells (DPSCs) were first identified at the turn of the twenty-first century and have since been shown to have the capacity to differentiate down multiple lineages [10]. DPSCs originate from the neural crest and are responsible for the formation of dentine producing cells called odontoblasts [11].

Barlett and Reade were the first scientists to cryopreserve dental tissue [12]. However, at that time dental cryopreservation was intended for the storage of whole teeth required for auto-transplantation rather than for stem cell isolation [13]. Recently the focus has shifted away from auto-transplantation towards the use of adult dental stem cells as a means of restoring or replacing lost or decayed dental tissue [10]. Indeed, adult stem cells are becoming increasingly popular as a cell source for regenerative medicine since they are not subject to the same ethical concerns as embryonic stem cells. Teeth in particular contain a significant proportion of adult mesenchymal stem cells (MSCs) in relation to their tissue mass [14]. Dental stem cells from extracted teeth have further appeal due to the fact that the process of isolating these multipotent cells is relatively simple and avoids invasive procedures associated with other popular sources of MSCs, such as bone marrow or fat. One of the most promising sources of dental stem cells resides within the primary dentition, which becomes lost during maturation. When human deciduous teeth are lost and replaced by permanent teeth, stem cells located within the pulp chamber can be harvested and stored as a convenient and pain-free source for regenerative medicine. The stem cells from human exfoliated deciduous teeth, or SHED cells, are a rapidly proliferating and multipotent source that are often favoured over other bankable dental stem cells located within the pulp of wisdom teeth or orthodontically extracted teeth [15]. The process of isolating and storing cells from teeth is relatively cost-effective when compared with MSCs derived from cord blood—a popular source for banking [16]. Additionally, the efficacy of these cryopreserved dental MSCs has been validated by a number of studies showing that post-thaw viability and differentiation following short and long term storage remains unaffected [17, 18]. These data highlight the promise of dental MSCs as a source of regenerative cells for long-term banking and clinical application. However, for the creation of cell banks the question of whether to bank intact whole teeth or the isolated stem cell population needs to be further considered.

Banking Intact Whole Teeth

Frequently stem cells are isolated, purified and expanded (to approximately 80–90 % confluence) prior to cryopreservation and the majority of this chapter will primarily focus on this method. However, one should be aware that this method requires

resources for stem cell culture and scaling to be available upfront, thereby increasing workload and subsequent cost, with the further caveat of introducing possible phenotypic changes in the cells during expansion. It has been argued that a more rational and cost effective approach would instead be to bank whole teeth, allowing for the isolation and expansion of stem cells only when required. At present research into whole tooth banking for stem cell isolation is limited, and several groups attempting to cryopreserve intact teeth have shown a 25 % incidence of longitudinal fractures after freezing, which may compromise cell viability [19]. Methods for the banking of whole teeth have also encountered difficulties in enabling the CPA to reach the central pulpal region where the MSCs are localised. This consequently leads to problems with pulp necrosis, and can significantly reduce the viability, and hence the availability and clinical application of the cells [20, 21]. Importantly, current evidence demonstrates that MSC immunophenotype and differentiation is unaffected following whole tooth banking [22]. However, there are mixed reports concerning the effects of whole tooth banking on cell proliferation rates and morphologies [19, 22–24]. In order to improve the efficacy of whole tooth banking modern methods such as laser-piercing have been used to introduce microchannels, allowing CPA to reach the central pulp [21]. However, the procedure of whole tooth banking for MSC extraction is still in its infancy, and further advancements will be required if such a method is to be applied on a global scale.

International Licensed Dental Stem Cell Banks

To deliver effective autologous regenerative therapies dental stem cells need to be stored in a state of stasis. To achieve this purpose tooth banks are being developed that provide members of the public the opportunity to bank these clinically useful cells for an annual fee. Currently, the number of international licensed dental stem cell and tooth banks is low. However, due to increased media and public awareness of regenerative medicine and the potentially revolutionary impact of these technologies on the healthcare sector the number is likely to increase. Currently, a number of international laboratories offer collection and storage of dental stem cells, and the ease of the process typically makes it simpler and cheaper than banking cord blood stem cells. Historically, the world's first tooth bank was developed in Japan at Hiroshima University in 2005 [25]. The country's national tooth bank was opened soon after in 2008—a collaboration between Hiroshima University and Taipei Medical University. A similar development has since taken place in Norway as collaboration between the Norwegian Institute of Public Health and the University of Bergen [26]. However, tooth banking is not restricted to academic institutions, with a number of commercially licensed tooth banks existing in developed countries such as the UK and USA, as well as in developing countries such as India. At present the USA dominates the commercial tooth banking market with several companies such as BioEden, Pharmacells, Store-A-Tooth and StemSave expanding throughout North America, Europe and the rest of the world.

Cryopreservation Procedure

If banked stem cells are intended for therapeutic use it is important that current standards of good manufacturing practice are implemented throughout the entire process, from cell collection to freezing, storage and post-thaw assessment. The development of a standardised cryopreservation protocol for dental stem cells will require specification of the following:

Tooth Collection

One of the most significant problems related to the cryopreservation of dental tissue is the time elapsed between tissue harvesting and banking [27]. As soon as the tooth is lost or removed the vital pulp will begin to degrade, causing irreparable damage to the cellular component. A previous study has shown that in order to maintain DPSC viability, cell isolation and cryopreservation must occur within 120 h of tooth extraction [23]. Currently, to minimise tissue degradation, teeth are placed in sterile hypotonic phosphate buffered saline solution, which prevents dehydration. This can be done by the individual, the individual's parents/guardians (in the case of SHED cells) or a dental health professional. The tooth is then transferred into a vial, which is carefully sealed and placed into a thermette (a temperature phase change carrier) and transported to the banking facility in an insulated metal transport vessel—these materials are often provided by the tooth bank [16]. The procedure is designed to maintain the sample in a hypothermic state, and the process is referred to as sustentation. It is of critical importance that at the time of extraction, the central pulp region appears red in colour, indicating sufficient blood flow at the time of isolation. Teeth should be excluded if the central pulp appears grey, as this indicates pulp necrosis. The most appropriate source of deciduous teeth for isolation and banking are the canines and incisors, since these teeth often contain a healthy source of pulp that is starting to loosen. Primary incisors and canines with no pathology and at least one third of the root in-tact can also be banked [28]. Primary molars are not recommended for SHED banking as they take a longer time to resorb, which can have a negative impact on the pulp chamber and consequently on the viability of the stem cells [29].

Cell Isolation and Expansion

The majority of tooth banks isolate and expand the cells prior to freezing. However, before the dental pulp can be isolated the tooth must undergo a rigorous disinfection procedure. Sterilization can be achieved by washing the cells with PBS and povidone iodine [16]. Washes with commercially available bactericidal solutions (e.g.

chlorhexidine) may also be used to reduce the number of microorganisms and help further decrease the risk of cross-contamination [23]. After the tooth has been washed and disinfected DPSCs are isolated using protocols originally developed by Gronthos et al. in the following way [30]:

1. Pulp tissue is removed from the tooth manually using sterile forceps or a dental excavator. The pulp may also be removed by flushing the pulp chamber with sterile salt water or saline solution.
2. The excavated pulp is placed in a sterile petri dish and washed several times with PBS to further reduce the risk of bacterial contamination.
3. Pulp tissue is digested with a Collagenase type-I (3 mg/mL)/Dispase II (4 mg/mL) solution or 0.5 % Trypsin/EDTA solution for a period of 1 h at 37 °C.
4. Digested pulp tissue is passed through a 70 μm cell strainer to obtain single cell suspensions.
5. Cell suspensions are cultured at 37 °C/5 % CO_2 in growth medium; Alpha-modified Eagle's medium (α-MEM) supplemented with 20 % foetal bovine serum (FBS), 2 mM L-glutamine, 100 μM L-ascorbate-2-phosphate, 50 U/mL penicillin, and 50 μg/mL streptomycin.
6. At this stage there is the option to use the expanded MSCs to obtain differentiated cell lines such as odontogenic [31], adipogenic [32] or neural [33] by making adjustments to the culture medium.

In order to characterise or improve the homogeneity of MSC cultures fluorescence activated- (FACS) or magnetic activated- cell sorting (MACS) can be applied to select for Stro-1^+ or $CD146^+$ cells. Previous studies have demonstrated that DPSCs can be accurately identified through the presence of these cell surface antigens [34]. However, it should be noted that purification of MSC cell populations may not always be beneficial as it can adversely affect their differentiation and therapeutic potential [32].

Introduction of a Cryopreservation Solution

Incorporation of a CPA within the freezing medium prevents the formation of intracellular ice crystals that damage the cytoplasmic membrane during freezing. Protocols utilising DMSO as a CPA are widely used both in research and the clinic, since this method consistently allows high post-thaw recovery and differentiation [35]. However, there are a number of documented issues concerning the use of DMSO. Firstly, DMSO is recognised as a cytotoxic chemical that can cause adverse side effects such as sickness if introduced to a patient during cell therapy [36]. Secondly, DMSO has been reported to cause epigenetic changes and cytotoxicity at levels routinely used for MSC cryopreservation [37, 38]. Surprisingly, given the known adverse effects of DMSO there are currently no regulations or guidelines governing the use of this chemical for cell banking. In fact, a review of the current literature has identified that 10 % DMSO is most commonly incorporated within the

freezing medium—currently used by approximately 80 % of cell therapy centres [39]. However, due to the documented negative effects of this CPA on both cells and patient there is a need to find effective alternative methods. Reducing the concentration of DMSO has been postulated as one way of reducing associated cellular toxicity [8]. However, reports have shown that the survival and number of colonies formed by MSCs was significantly decreased as a consequence, and that the magnitude of this decrease was inversely proportional to DMSO concentration [23]. Furthermore, concentrations below 7 % have been shown to cause a significant decline in DPSC viability [2, 23]. Therefore, it was proposed that an alternative approach that employs a simple post-thaw washing step may represent an effective way of reducing the patient-associated effects of this CPA without affecting cell recovery [40]. However, this approach fails to take into account any epigenetic changes associated with the incorporation of 10 % DMSO. Furthermore, if applying such an approach, one has to be careful not to trigger a damaging osmotic shock [23]. To avoid triggering an osmotic shock a similar result might achieved by avoiding post-thaw washes and instead simply fractionating the delivery of stem cells [41].

Alternatively, one could reduce the concentration of DMSO in the CPM by incorporating biocompatible CPAs that reduce the risk of cellular toxicity and accompanying side effects upon cell transplantation. It is important that these non-toxic alternatives are able to provide a level of efficacy similar to DMSO without the documented side-effects. Lowering the concentration of DMSO by incorporating sugar derivatives such as hydroxyethyl starch or trehalose has been shown to provide comparable levels of protection from freezing-induced cell damage to traditional 10 % DMSO-based solutions [9, 42]. The incorporation of CPAs such as methylcellulose or polyethylene glycol has also shown promise, reducing the concentration of DMSO required in the CPM. Importantly, these freezing mediums provide a level of protection that virtually eliminates the need to incorporate serum, thereby reducing potential immunological complications resulting from cell transplantation. Indeed, the formulation of an effective serum-free CPM is likely to represent an important step required for the clinical translation of cellular therapies [43–46].

Complete replacement of DMSO with non-toxic alternatives has proven a more difficult task. For instance CPAs such as glycerol and proline are effective when used in combination with DMSO but provide inadequate protection when used independently [47]. In fact only a small number of CPAs have been identified that provide adequate cryoprotection without the need to incorporate DMSO. Currently, only CPMs containing polyvinylpyrollidone (PVP) or ectoin have been shown to achieve levels of MSC post-thaw viability and differentiation comparable to DMSO-based solutions [45, 47]. However, at present these studies are not comprehensive enough to draw any significant conclusions, and the efficacy of these CPAs will need to be evaluated in long-term studies before their clinical applicability can be comprehensively assessed. In conclusion, significant progress is being made in the identification of serum-free freezing mediums that contain non-toxic CPAs for the cryoprotection of MSCs. However, at present no studies have analysed the effects of novel biocompatible CPAs on multipotent dental stem cells. Therefore, considerable further investment is required into research that comprehensively examines the efficacy of non-toxic CPAs for dental stem cell banking.

Freezing Protocol

Controlled-Rate Freezing

Damage caused by cell dehydration and ice crystal formation can be minimised by controlling the freezing rate. A slow and controlled freezing rate of 1–2 °C per minute is generally considered optimal for maintaining MSC viability during cryopreservation [48, 49]. Controlled rate freezers (CRFs) are frequently used to accurately control the freezing rate, and can be either manual or programmable [50, 51]. Although controlled-rate freezing has the advantage of reducing the likelihood of cellular damage occurring through ice crystal formation, one of the fundamental issues with CRFs is that they cannot be scaled to provide uniformity of temperature to all vials during large scale banking [52]. This represents a significant problem, and if cell therapies are to be applied on a national or international scale advances in CRF technology will be required. Furthermore, CRF development, along with vapour phase storage in liquid nitrogen is costly, and could impact the clinical translation of regenerative medicine. Ultra-slow programmable freezing systems (USPF) represent an alternative method that apply a highly controlled freezing rate of −0.3 to −0.6 °C per minute, allowing comparatively high recovery rates and increased levels of efficiency that are in compliance with cGMP requirements [4, 53]. However, a significant caveat is that USPFs are more complex and costly than traditional slow rate freezing (SRF) [54]. Much like CRFs, scaling is also a significant pragmatic consideration when applying USPFs for the high-volume banking of dental tissue. Ultimately, the choice between programmable or manual freezing will be dependent on the resources available and the experience of the scientists or technicians on site.

Uncontrolled-Rate Freezing

An alternative approach where samples are first cooled at −4 °C and then directly deposited into a freezer at −80 °C or liquid phase nitrogen can also be applied [55]. Uncontrolled-rate freezing represents a comparatively cheap and simple way of cryopreserving cells that requires no specialist training. This method has been most frequently applied for the preservation of peripheral blood stem cells with consistent results [56, 57]. Initial studies have also shown that uncontrolled freezing can be successfully used for the long-term cryopreservation of dental pulp stem cells [58]. Based on the parameters of the study the authors found that this method was as effective at maintaining cell viability, proliferation, and differentiation as CRFs with subsequent liquid nitrogen storage. However, to date uncontrolled-rate freezing has only been tested in the presence of 10 % DMSO, and studies will be required to test the efficacy of this method for dental stem cell preservation in combination with low DMSO concentrations or non-toxic DMSO replacements.

Rapid Freezing (Vitrification)

Vitrification represents a viable alternative to controlled-rate freezing for the preservation of tissue and stem cells [59, 60]. The process of vitrification and its application for the cryopreservation of biological material was first described by Luyet during the early part of the twentieth century [61]. This approach applies a high concentration of cryoprotectant and rapid cooling rate to produce an arrested liquid state and a glass-like solidification [53]. Faster cooling is considered advantageous because it reduces the toxic effects of the CPA and minimises the formation of ice crystals during freezing and thawing [6]. The process is simple and achieved by placing small volume samples held on vitrification carriers, such as cryoleaf, cryoloop, or ministraw directly into liquid nitrogen [62–64]. The method has become increasingly popular as a means of preserving cells that are particularly sensitive to cryoinjury such as embryonic cells [53]. However, current evidence suggests that the rapid freezing of whole teeth may be problematic due to progressive root resorption, and conditions will need to be further optimised if this method of cryopreservation is to be used for whole tooth banking [48]. A second potential caveat associated with the vitrification method is that rapid freezing may also generate an increased immunological response within the pulp [65]. Pragmatically, this method is also limited due to the large volumes of liquid nitrogen that would be required to scale up the technology to meet clinical demand.

Magnetic Freezing

Magnetic freezing applies a weak magnetic field to water or tissue that lowers the freezing point by approximately 6–7 °C. The first programmable device was developed by Hiroshima University and marketed as the cells alive system (CAS) [66]. It operates by producing an alternating magnetic field that prohibits water molecules from clustering during the freezing process, thereby preventing the formation of ice crystals [67]. This method is ostensibly cheaper than cryogenics and has been shown to provide comparatively high post-thaw viability for intact whole teeth and DPSCs alike [68, 69]. Furthermore, magnetic cryopreservation has been shown to reduce the concentration of DMSO required (5 %) to maintain cell viability and vitality [68, 69]. Magnetic cryopreservation has been successfully utilised for tooth banking with satisfactory implantation outcomes, indicating that viability of the dental pulp and periodontal ligament is adequately maintained [70]. Importantly, this method of cryopreservation may also eliminate the need for the addition of serum to the freezing medium, putting it in compliance with GMP.

Thawing Conditions

The standard method for thawing cryovials is to place them in a water bath at 37 °C. This facilitates a relatively quick thawing process that acts to remove and prevent the formation of ice crystals [71, 72]. However, studies examining the

influence of temperature, time and method of thawing are limited. Of the few studies examining the effects of thawing on cell viability and vitality, some groups have examined the use of dry heat applied using 37 °C gel pads and compared this method with cells thawed using a water bath. It has been hypothesised that the application of dry heat provides a cleaner way of thawing cells that are to be used for clinical applications. These studies identified that samples thawed using dry heat had similar viabilities and clonogenic potential to those thawed using the standard procedure [73, 74]. Of the limited number of studies comparing the effects of thawing temperature, a report by Yang et al. identified no significant differences between cells that were thawed at 0, 20 and 37 °C for 20 min [75]. However, a comprehensive examination of the effects of thawing temperature and duration are required before any valid conclusions can be drawn. Lastly, upon thawing, removal of the cryoprotectant causes each cell first to shrink and then swell. Swelling can lead to damage as the cells expand. Such damage can be prevented by step-wise removal of the CPA using graded isotonic solutions [76]. An automated method designed to remove the CPA from haematopoietic grafts after banking has been shown to be feasible in preclinical models [77, 78] and the post-thaw removal of CPA is now standard for cells of bone marrow and peripheral blood. However, at present few studies on dental stem cell cryopreservation incorporate a step-wise removal of the CPA, instead opting to wash the thawed cells with a single isotonic solution to remove the CPA or often incorporating no washing step at all.

Post-Thaw Assessment

There are a number of possible negative cellular effects associated with cryopreservation, and at present it remains unclear whether post-thaw MSCs retain the same potential for therapeutic applications as their non-cryopreserved counterparts. Detrimental cellular effects associated with cryopreservation can include a reduction in cell viability due to cold-shock and/or the toxic effects of DMSO, changes in the expression of stem cell-related markers, cytoskeletal disassembly, delayed apoptosis, and osmotic and oxidative stresses [5, 79]. Consequently, these factors may have a downstream influence on the functionality of MSCs, thereby reducing their applicability for regenerative therapies. Additionally, ultrastructural changes in stem cell morphology remain largely unexamined during post-thaw assessment. However, such changes have been documented in cryopreserved adipose-derived stem cells (ADSCs) [80]. The subsequent downstream effects of these ultrastructural changes remain unknown. However, it is important that the consequences of these changes are fully determined before cell banking is applied on a national scale. At the most fundamental level it is crucial that stem cell viability, differentiation and other important characteristics required for effective tissue regeneration are extensively studied for each cell type, since these have been shown to vary for stem cells isolated from different tissue sources [55]. Here we highlight the importance of establishing protocols that are based on human studies and are tissue/cell

specific. This is necessary since previous studies have shown that the viability and vitality of stem cells isolated from different species differs, with tissue-specific differences observed in post-thaw viability and gene expression [81].

Conclusions

Dental stem cell banking is becoming increasingly popular, with stem cells isolated from the dentition providing individuals with means of '*biological insurance*' to facilitate the regeneration of tissues lost to trauma or disease. In order to achieve this goal research and development is required to define optimum methods for long-term cryopreservation. The next phase of research will focus on the formulation of cryopreservation mediums that facilitate the safe and efficient transfer of cellular therapies within the clinic. This will mean eliminating the use of serum and toxic CPAs without compromising cell viability and vitality. Standardised protocols and procedures will also need to be developed that adhere to current good manufacturing practice (cGMP). Furthermore, pragmatic considerations relating to cost and scalability of cell banking procedures will need to be taken into consideration if cell therapies are to be applied on a national and international scale. Finally, perhaps one of the fundamental caveats concerning stem cell banking is that no studies have examined the effects of long-term cryopreservation (>10 years) on their viability and phenotype. This is a fundamental issue, and long-term studies will be required to demonstrate the efficacy of current cryopreservation procedures for future clinical applications.

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Epigenetics of Dental Stem Cells

Henry F. Duncan and Paul R. Cooper

Introduction

Stem cells (SCs) are critical to tissue regeneration [1] and they are either embryonic (embryonic stem cells [ESCs]), or post-natal/adult in origin (SCs) [2, 3]. ESCs are in an epigenetic-state which enables them to self-renew or differentiate into any bodily cell type (pluripotent). ASCs, however, have more restricted lineage potential (multipotent) [4, 5]. Current research is aimed at understanding cellular regulators that control this lineage commitment to enable novel stem cell-based regenerative strategies to be developed in medicine and dentistry.

Epigenetic modification of histone proteins by acetylation or via DNA methylation, are key regulator processes involved in SC self-renewal and pluripotency [6, 7] and occur in many tissues including dental ones [8]. Epigenetic modifications occur due to environmental factors and result in gene expression modulation, independent of DNA sequence [9] (Fig. 1). In healthy tissues, synchronous genetic patterns and epigenetic modifications regulate transcription, however, these processes are often disrupted in disease [11]. Whilst epigenetic changes are heritable, and accumulate throughout life, they are however reversible and therefore provide exciting opportunities for clinical application via pharmacological inhibition and modulation [12]. Key epigenetic modifications include direct DNA methylation [13], the post-translational modifications of acetylation, methylation and phosphorylation of histone proteins [14] and nucleosome positioning [15]. Therapeutically, a range of inhibitors targeting these molecular events have been developed with selected

H.F. Duncan (✉)
Division of Restorative Dentistry and Periodontology, Dublin Dental University Hospital, Trinity College Dublin, Lincoln Place, Dublin 2, Ireland
e-mail: Hal.Duncan@dental.tcd.ie

P.R. Cooper
Oral Biology, School of Dentistry, University of Birmingham, Pebble Mill Road, Birmingham B5 7SA, UK

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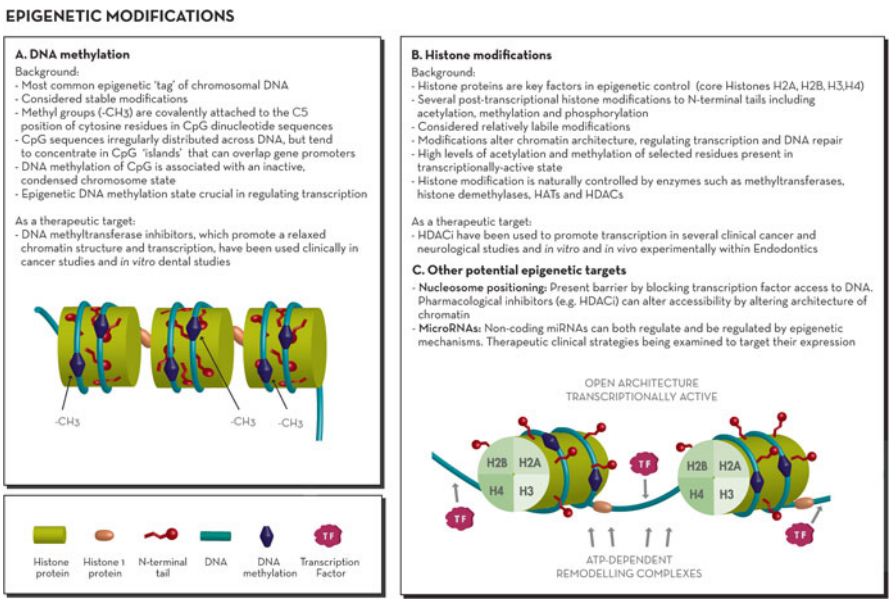

Fig. 1 Didactic classification of epigenetic modifications (reproduced with permission from [10]). (**a**) DNA methylation schematic illustrating the repeating nucleosome, which a single strand of DNA wound round the histone core. Methyl groups are covalently attached in CpG islands. (**b**) Histone modifications: the nucleosome comprises eight core histones. Linker DNA, connects the nucleosome structures and an additional histone protein (H1), enables further DNA compaction. The nucleosome core has a relatively long N-terminal amino acid extension, which is subject to several covalent epigenetic modifications (methylation, acetylation, phosphorylation, ubiquitination and SUMOylation). Histone modifications control both the conformation of the chromatin structure leading to an open or closed chromatin state and also nucleosome positioning. (**c**) The varying architecture of chromatin and position of the nucleosome regulates transcription, with an open conformation being transcriptionally active and a closed structure being transcriptionally repressive

therapeutics including Suberoylanilide hydroxamic acid (SAHA), Romidepsin and 5-azacytidine, already having United States Food and Drug Administration (FDA) clinical approval [16, 17].

Pharmacological agents, which epigenetically modify histone acetylation, include histone deacetylase inhibitors (HDACis). These molecules can reversibly change gene expression resulting in altered SC fate [18, 19]. Several HDACis, including Trichostatin A (TSA), Valproic acid (VPA) and Butyric acid, can induce SC proliferation, differentiation and also have anti-inflammatory activity [18, 20–22]. From a dental perspective, HDACis have been shown to epigenetically-modulate postnatal dental pulp SC (DPSC) differentiation and self-renewal potential due to their ability to alter the balance between histone deacetylases (HDACs) and histone acetyl transferases (HATs) [23]. Application of HDACis prevents deacetylation of the N-terminal histone tail within the nucleosome, leading to changes in chromatin

architecture and transcription activity [24]. Eighteen human HDAC enzymes have been identified which differ in their expression profiles and cellular localisations [25]. While the entire functional role of HDACs is still to be fully elucidated [26], pan-HDACis and isoform specific inhibitors have been developed. Notably application of pan-HDACi have been shown to accelerate differentiation and mineralisation events in dental pulp cell populations [18, 27], although de-differentiation induction has also been shown in some immature SC populations [28].

In endodontics, tissue-resident and local SC populations including DPSCs and Stem Cells from the Apical Papilla (SCAP) may be key to regenerative responses [4]. These SCs are relatively easily accessible and have therapeutic application at other oral and bodily locations. For repair processes SC migration, proliferation and differentiation are necessary [29] and reportedly these cellular regenerative-associated events are epigenetically regulated with the role of HDACis in modifying these processes being recently highlighted [30, 31]. 'Regenerative endodontics' utilising SC technology continues to gather significant momentum and involves concerted academic and clinical effort to develop novel tissue engineering approaches which aim to utilise cell-free homing, platelet–derived plasma and triple antibiotic paste [32–35]. Targeted restorative dental materials, which harness innate biological reparative processes utilising epigenetic therapeutics may also have clinical utility in the more immediate future [36].

Furthermore for clinical application of dental SC technologies a detailed working knowledge of their cell isolation, expansion, and biobanking combined with knowledge of genotoxicity will be essential for clinical application. Training of the clinical team in these areas will be important to realising patient benefit with this technology. The aim of this chapter therefore is to review current knowledge of dental SCs and how epigenetics may play a role in their responses during health, disease and dental clinical application.

Review

Dental Stem Cells

Postnatal SCs, in particular ones derived from dental sites, provide a readily available source for tissue engineering and repair strategies throughout the body [2]. DPSCs and stem cells from exfoliated deciduous teeth (SHED), reportedly account for 1–5 % of total permanent and 2–9 % deciduous pulp cell populations, respectively [4, 37, 38]. Other dentally relevant SC sources have also been identified [2] and include stem cells of the apical papilla (SCAP), dental follicle progenitor cells (DFPCs) and periodontal ligament stem cells (PDLSCs) [38–40].

DPSCs are one of the most readily available SCs as they can be isolated by the dental practitioner from extracted third molar teeth which is regarded clinically as 'waste tissue'. Notably harvesting from this site avoids the donor site morbidity

typically observed at other surgical locations. While the therapeutic application of DPSCs, such as dentine-pulp regeneration, appears quite promising, their overall utility may be limited due to the availability of autologous molar teeth for extraction along with the need for their in vitro expansion to obtain sufficient numbers for use [41]. Indeed during culture expansion, phenotypic change [42] may occur which also limits their clinical application. An understanding of the mediators, which are often epigenetic in nature, controlling self-renewal and lineage commitment, is therefore important. For regenerative dentistry other dental SC sources, such as DFPC and PDLSC may either be limited in their application due to diminished differentiation potential or in their availability [2, 8, 38]. SHED, however, are considered a rich source of dental SCs which can be harvested from waste tissue from extracted or exfoliated deciduous teeth. These dental SCs can be commercially biobanked as intact pulp tissue and subsequently SHED are isolated and expanded in vitro following enzymatic digestion of pulp tissue combined with fluorescence-activated cell sorting [43]. Although SHED are relatively easily obtained [43], currently their routine harvesting and clinical application is reportedly hampered by infection, lack of viable pulp tissue as well as lack of a standardised medicinal good manufacturing practice [37, 38].

Epigenetic Modifications and Dental Stem Cells

Classically ESCs exhibit an open chromatin structure characterised by relatively low levels of DNA methylation and high levels of histone acetylation [44]. After cellular lineage commitment and differentiation, the chromatin changes to a more tightly-bound conformation with reducing levels of acetylation and increasing levels of DNA methylation [45]. It is now evident that ESCs are maintained in a self-renewing state by a complex network of transcription factors [46], while differentiation and developmental potential are under epigenetic control [47]. During differentiation the pluripotency genes, Oct4 and Nanog, are repressed [48], an effect which is mediated initially by histone modifications, with the down-regulation maintained and cell reprogramming prevented by DNA methylation processes [49].

DNA methylation is cell type dependent and was traditionally thought to occur exclusively at CpG islands (Fig. 1), however, recently it was noted that up to 25 % of all ESC methylation was in a non-CG context [50]. Indeed the observed pattern of non-CG methylation disappeared when differentiation processes were induced in ESCs supporting a crucial role for methylation in determining cell fate [50]. Further characterization of epigenetic modifications in ESCs identified hypermethylation at 3 % of promoter regions in CgG islands, and notably the regions included developmental genes such as Rhox2 [51]. It has been suggested that although global methylation levels of CpG may be similar between cell types the distributions of methylation marks in ESCs are unlike any other cell type [52]. Other epigenetic modifications are also important regulators of SC function and self-renewal with

HDACs being vital to maintaining the self-renewal capabilities of mesenchymal SCs [7, 53] by maintaining the expression of key pluripotent transcription factors [6]. Amongst the best characterised cellular mediators of acetylation and methylation processes in SCs are the polycomb and trithorax protein complexes, which occupy ESC gene promoter sites and assist in catalysing specific histone modifications [54, 55]. Over the last 10 years knowledge of non-coding (nc) RNA, such as microRNA, has developed in relation to their epigenetic control due to their dysregulation in diseases such as cancer [56], their regulation of epigenetic mechanisms such as DNA methylation [57] and conversely their expression is regulated by epigenetic modifications [58]. Other ncRNAs, such as long-ncRNAs, also epigenetically modulate chromatin architecture by recruiting and binding chromatin-modifying proteins to specific genomic sites [59], while also guiding proteins, such as histone methyltransferases to those sites [60, 61].

Recently, interest in the epigenetic control of DPSC behaviour has led to suggestions that dental developmental anomalies, such as dentine dysplasia and dentinogenesis imperfecta, may be related to epigenetic modifications during odontoblast differentiation [62]. However, current research in this area is preliminary and has not, to date, analysed pure SC populations [63]. In a recent SC study, epigenetic states and related differentiation profiles were analysed in DPSCs and DFPCs via their expression of odontogenic genes, such as dentine sialophosphoprotein (DSPP) and dentine matrix protein-1 (DMP-1). Transcript levels were epigenetically-suppressed in DPFCs, while osteogenic stimulation in vitro demonstrated significant mineralisation increases only in DPSCs [8]. Interestingly, a highly dynamic histone modification response was demonstrated in mineralising DPFCs, but not in DPSCs, and the latter cells also expressed relatively high levels of the pluripotency-associated transcripts, Oct4 and Nanog. It was concluded that these two neural crest-derived SC populations were distinguished by epigenetic repression of dentinogenic genes with dynamic histone enrichment in DPFCs during mineralisation. These data demonstrated the essential role of epigenetic mechanisms in DPSC terminal differentiation and in controlling cell phenotype and lineage commitment [8].

Histone Acetylation Related Stem Cell Effects

Histone acetylation controls cellular transcription by altering the conformation of chromatin, a process balanced homeostatically by two cellular enzymes histone deacetylases (HDACs) and histone acetyl transferases (HATs). HDACs in particular have been a focus of attention as potential therapeutic targets [21], with 18 human HDAC family members characterised and divided into four classes [64]. Class I members (HDAC-1, -2, -3, -8) are ubiquitously expressed predominately in the cell nucleus [25], while the expression of class II enzymes (HDAC-4, -5, -6, -7, -9, -10) are tissue-restricted and dynamically shuttle from nucleus to cytoplasm [21, 65]. A recent analysis of selected HDAC expression in extracted third molar teeth demonstrated that HDACs -2 and -9 were expressed in selected pulp cell

populations, while HDACs -1, -3 and -4 appeared only relatively weakly expressed [66]. The reported variations in tissue expression suggest different cellular roles for HDACs and indications are that class I HDACs regulate cell cycle progression and maintain ESC pluripotency, while class II HDACs are central to stem cell differentiation and mineralisation processes [6, 67, 68]. However, it is likely that their roles inter-link, with both Class I and II HDAC enzymes playing crucial roles in maintaining the pluripotency of stem cells and in governing cell fate [6, 53]. The labile effects of histone acetylation can be therapeutically targeted by HDACis, which alter the homeostatic HAT/HDAC balance, leading to changes in transcription and subsequently, stimulating pleiotropic cellular effects involving growth, differentiation and regeneration [31, 69, 70].

HDACi-Induced Stem Cell Effects

Several HDACi including TSA, VPA and SAHA, have been used already in clinical cancer trials, and their promise in treating neurodegenerative. conditions, as well as in bone engineering and regenerative medicine has also been highlighted [16, 71, 72]. Indeed, several HDACi, at relatively low concentrations, have been demonstrated to induce differentiation and increase mineralisation dose-dependently in primary dental pulp cell (DPC) populations containing stem cells [18, 23, 27]. Furthermore, HDACis and VPA in particular has also been shown to improve both the efficiency of somatic cell reprogramming to iPSCs, as well as increasing the expansion capacity of selected ASC populations [19, 73].

Certain HDAC members, -1 and -2, have been identified as playing crucial roles in controlling cellular proliferation and stem cell self-renewal by maintaining the expression of key pluripotent transcription factors such as Oct4, Nanog, Esrrb, and Rex1 [6]. In vitro HDACi stimulate the expansion of hematopoietic SCs, while decreasing the relative number of differentiated cells [19, 74], whereas in vivo HDACi-induced an increase in SC markers following digit amputation in mice [75] and during kidney organogenesis in zebrafish [30]. Conversely, several studies using various cell types have demonstrated that HDACi can reduce stem cell proliferation and promote terminal differentiation [76, 77, 78, 53]. These apparently divergent effects of HDACi were attributed to the level of baseline cell differentiation with stem cells tending to de-differentiate, while precursor blast cells differentiated [74]. Other researchers have further speculated that in addition to cell status, HDACi-dose may all be critical to the cell response, with undifferentiated cells and high doses of HDACi tending to promote differentiation, while low doses applied to cells undergoing self-renewal are more likely to stimulate a de-differentiation effect [28, 79].

Within dentistry several recent in vitro studies using mixed primary cell cultures [27], and pure DPSC populations [18, 23] have demonstrated an HDACi-induced stimulation of odontoblast-like cell differentiation with an associated increase in

matrix mineral deposition. The effect of epigenetic modulation on regenerative processes highlighted differences between normal and immortalised cells with lower concentrations of HDACi and shorter durations of exposure being required to promote reparative effects in primary cells [27]. The effect of HDACis on human DPSC populations in vitro demonstrated increases in mineralisation, associated mineralisation-marker gene expression and down-regulation of HDAC-3 [18] and HDAC-2 [23], respectively. In vivo the response to a pre-natal systemic injection of TSA in dentine-pulp development was studied by histological analysis of tooth development at 7-days post-natal [23]. The volume of dentine deposited was thicker (1.64-fold) and the number of odontoblasts in the measured area was higher (1.74-fold) in the TSA group compared with the control [18]; this was attributed to an accelerated HDACi-induced differentiation effect. This reported ability of HDACi to manipulate stem cell fate renewal, although complex, is interesting from a regenerative medicine perspective as it potentially enables the scientist and clinician to promote differentiation, for example in tissue engineering, or electively maintain pluripotency if expanding SCs for cell-based therapies.

Induced Pluripotent Stem Cells (iPSCs)

The translational potential of pluripotent ESCs use has been somewhat limited due to expense, storage issues, ethics, as well as issues relating to immunological rejection [80]. In an attempt to address these issues, patient's own somatic cells have been reprogrammed into induced pluripotent stem cells (iPSCs) using a combination of four transcription factors (Oct4, Sox2, Klf4, c-Myc) and a viral induction protocol [81, 82]. However, as safety concerns were raised over the use of the proto-oncogenes, Klf4 and c-Myc, and low reprogramming rates (<0.001 %), the epigenetic modifiers, HDAC and DNA methyltransferase inhibitors, were used with and without these transcription factors [73]. The use of HDACi, in particular VPA, combined with all four transcription factors improved reprogramming efficiency up to 100-fold [73]. The presence of VPA, but absence of c-Myc, also increased reprogramming efficiency levels; however, when klf4 was eliminated the efficiency was greatly reduced indicating the importance of this molecule in the process [83]. It subsequently appears that VPA acts by inducing Oct4 predominately through the PI3K/Akt/mTOR pathway [84]. Further optimisation of iPSC techniques have been developed focusing on viral-free methods and avoidance of genomic modification approaches by using synthetic modified mRNAs for Oct4, Sox2, Klf4, and c-Myc, [85]. Although, VPA supplementation has also been combined with the synthetic mRNA methodology, only minor increases in reprogramming efficiency were observed [85]. The role of HDACi-supplementation in reprogramming demonstrates the pivotal role of epigenetic modification in the de-differentiation process and indicates the importance of the open chromatin architecture characteristic of ESCs [52].

Conclusions

A picture of the influence of epigenetic modifications on the mechanisms controlling stem cell fate and differentiation is developing, which critically involves DNMT and HDAC enzyme function, cell maturity and environmental signals. Further elucidation of the role of these enzymes is required to understand the influence of epigenetic modifications on pluripotency, their influence on stem cell fate and role in regenerative responses prior to any proposed clinical application. Regenerative tissue engineering approaches require SCs, signalling mediator molecules and a scaffold within which the cells can operate [86]. Therapeutically, HDAC and DNMT inhibitors could epigenetically manipulate the self-renewal capacity of SCs in vitro or indeed in vivo in the implanted stem cells. Additionally, there are opportunities for pharmacological epigenetic inhibitors to improve the efficiency of somatic cell reprogramming to iPSCs for subsequent craniofacial tissue regeneration, as availability of sufficient SCs may be limited. Finally, the availability of DSCs, their isolation, expansion, and epigenetic manipulation with HDACi could, at the very least, lead to a better understanding of DSC biology, which will enable their future clinical use within regenerative medical strategies.

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Dental Stem Cells and Growth Factors

Paolo Ghensi

Stem Cells, Scaffolds and Growth Factors

The aim of the regenerative medicine and tissue engineering is to regenerate and repair the damaged cells and tissues in order to establish the normal functions [1]. The regenerative medicine involves the use of scaffolds, growth factors and stem cells [2]. Regeneration of the tissues exists naturally due to the presence of stem cells with the potential to self-regenerate and differentiate into one of more specialized cell types [3].

Stem cells are immature and unspecialized cells with the ability to renew and divide themselves indefinitely through "self-renewal" and able to differentiate into multiple cell lineages [4]. In the last decade, several improvements have been produced in the comprehension of stem cells properties in view of the fact that these cells have an important role in the repair of every organ and tissue [3].

In general, the stem cells are divided into three main types that can be utilized for tissue repair and regeneration: (1) the embryonic stem cells derived from embryos (ES) [5, 6]; (2) the adult stem cells that are derived from adult tissue [7]; and (3) the induced pluripotent stem (iPS) cells that have been produced artificially via genetic manipulation of the somatic cells [8]. ES and iPS cells are considered pluripotent stem cells because they can develop into all types of cells from all three germinal layers. On the contrary, adult stem cells are multipotent because they can only differentiate into a restricted number of cell types. Adult stem cells, also termed postnatal stem cells or somatic stem cells, are discovered in a particular area of each tissue named "stem cell niche" [3].

P. Ghensi (✉)
Centre for Integrative Biology (CIBIO), University of Trento, Trento, Italy

Department of Neurosciences, University of Padova, Padova, Italy
e-mail: dr.ghensi@gmail.com

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Different type of postnatal stem cells resides in numerous mesenchymal tissues and these cells are at the same time referred to as mesenchymal stem cells (MSCs) [7, 9]. Different studies have showed that MSCs can be isolated from different tissues, such as peripheral blood, umbilical cord blood, amniotic membrane, adult connective, adipose and dental tissues [10–14].

Today, every cell population which has the following characteristics independently of its tissue source, is usually referred as MSCs: (1) they adhere to plastic and have a fibroblast-like morphology; (2) they have the capacity of self-renewal and could differentiate into cells of the mesenchymal lineage such as osteocytes, chondrocytes and adipocytes. In addition, MSCs also can also differentiate, under appropriate conditions, into cells of the endoderm and ectoderm lineages such as hepatocytes and neurons, respectively [15, 16].

Research related to MSC from oral origin began in 2000 [17] and every year numerous investigations have demonstrated that oral tissues, which are simply available for dentists, are a rich source for mesenchymal stem cells [18, 19].

Today numerous types of MSCs have been isolated from teeth: in 2000 MSCs were first isolated by Gronthos et al. from dental pulp (DPSCs) [17, 19]. Subsequently, MSCs have been also isolated from dental pulp of human exfoliated deciduous teeth (SHEDs) [20]. The periodontal ligament is another adult MSCs source in dental tissue, and periodontal ligament stem cells (PDLSCs) were isolated from extracted teeth [21]. Moreover, MSCs have been also isolated from developing dental tissues such as the dental follicle (DFPCs) [22] and apical papilla (SCAPs) [23].

These cells all share a number of features with the well-studied bone marrow-derived mesenchymal stem cells, including their ability to differentiate, albeit with varying efficacy, into cells of the major mesenchymal lineages: osteoblasts, chondrocytes, and adipocytes. However, these cells seem to differ in their propensity to differentiate into functional odontoblasts [24]. Further, some of these cells, such as the SCAPs, are isolated from specific developmental stages (in this case the apex of a developing tooth), and thus, do not serve as practical sources of cells for autologous cell-based transplantation [25].

Dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHEDs), SCAPs, and PDLSCs have all been successfully isolated and characterized both in vitro and in vivo. Because of the nature of the deciduous teeth, guiding the development of permanent teeth and eventual replacement by the permanent teeth, deciduous teeth may act as a very practical and easily accessibly reservoir for autologous stem cells and hold the most value in stem cell therapy [20].

Much excitement has recently surrounded the identification and application of these stem cells with therapeutic value, but equally important is the development of delivery methods for transplantation of these cells and the promotion of their efficient engraftment. Traditionally, stem cells are introduced into the body via injection into the site of interest or into the circulation. However, the efficacy of this methodology is questionable, since studies have shown poor cell survival, engraftment, and unpredictable differentiation in vivo [25].

The potential efficacy of stem cell delivery and differentiation may be improved with the adoption of tissue-engineered scaffolds for cell delivery and structural

support. Delivery of dental stem cells can potentially be supported by scaffolds that provide both mechanical and molecular (growth Factors, integrin receptor engagement) cues for differentiation [26].

Properties of scaffolds include providing cell adhesion, enabling cell proliferation and differentiation, and mimicking microenvironment observed in natural tissues and organs. Unlike injection-based delivery, scaffolds allow for superior control for stem cell delivery and allow for impregnation with time-release growth factors, modulation of stiffness, pore size, and cell–substrate interaction. Some evidence suggests that in addition to directly participating in tissue regeneration, transplanted stem cells can also regulate tissue regeneration by secreting trophic factors [27]. These two properties of stem cells suggest that the proper local distribution of stem cells is critical, making bioscaffold-based delivery one of the most promising strategies in cell-based regenerative dentistry [25].

Apart from stem cells and an appropriate scaffold, regenerative medicine and tissue engineering also require the use of growth factors and ECM molecules that induce specific differentiation pathways and maintain the cellular phenotype.

Incorporation of growth factors and/or cytokines has been shown to increase the efficacy of cell-based regeneration, both in the context of exogenous cell delivery, and recruitment of patients' endogenous stem cells from the local environment in vivo [25]. Growth factors enhance dental stem cell activities by promoting the migration of endogenous cells and the subsequent proliferation, differentiation, angiogenesis and neuronal growth. Growth factors and cytokines are either autocrine or paracrine in nature and modulate cellular behavior by mediating intracellular communication [25]. Kim et al. recently discussed the effects of some growth factors in pulp-dentin regeneration [28]. These growth factors include platelet-derived growth factors (PDGF), transforming growth factor β (TGFb) family, vascular endothelial growth factor (VEGF), bone morphogenic proteins (BMPs), fibroblast growth factor (FGF) and etc. When delivered alone or with stem cells to sites of interest, they share common properties of promoting dentinogenesis, cellular proliferation and odontoblastic differentiation. PDGF, for example, normally released by platelets, induces chemotaxis and proliferation of stem cells by promoting angiogenesis and cell proliferation [29]. Rizk and Rabie demonstrated chondrogenesis of DPSCs transplanted into nude mice on poly-L-lactic acid/polyethylene glycol electrospun fiber scaffolds. These DPSC transplants were more efficient at chondrogenesis when transduced with TGFb3 virus, further supporting the notion that codelivery of growth factors can stimulate basal levels of stem cell differentiation upon transplantation in vivo [30].

Definition and Mechanism of Action of Growth Factors (GFs)

The term growth factors denominates a group of polypeptides of approx. 6–45 kD which have a role in controlling biological processes, such as cell growth, proliferation, differentiation and morphogenesis of tissues and organs during

embryogenesis, postnatal growth and adulthood. GFs cannot pass through a cell's membrane; they must bind to high-affinity cell receptors in order to take effect. Many GFs stimulate several cell populations, while others are less versatile and specific to a particular cell line [31].

Growth factors can act as mitogenes in that they enhance proliferation of certain cell types. Some growth factors are also morphogenic in that they change the phenotype of their target cells. Growth factors act in an autocrine manner by directing the secreting cell itself and in a paracrine fashion by affecting neighbouring cells. For some growth factors, an endocrine effect is assumed or even established because of elevated serum levels. Many growth factors are deposited in the extracellular matrix where they are released during matrix degradation and act as part of a complex network of signals with mutual effects during tissue remodelling and regeneration. The effect of growth factors is mediated through surface receptors on the target cells by activating intracellular phosphorylating enzymes, which in turn induce an intracellular signalling pathway by aggregation of co-factors and other proteins, which migrate to the nucleus. Together with other transcription factors they activate a set of genes, which then exert the specific changes in cellular activity or phenotype. In vivo, the effect of growth factors is regulated through a complex system of feed back loops, which involve other growth factors, enzymes and binding proteins [31].

Postnatal therapeutic application of growth factors for repair of damaged tissue or an organ is used to accomplish regeneration or generation of tissue by reinducing the developmental process that had created this organ or body part during foetal or postnatal growth [31]. Several authors have described the importance of growth factors in tissue repair processes, in fact, they are important elements for new tissue production, moreover, they perform feedback controls on inflammatory processes within the tissue graft, in cases of regenerative surgery [32–35].

As growth and development are subject to a complex regulation that is also essential for the suppression of malignant growth, there are concerns that unreflected enhancement of isolated signals in this complex system might initiate a process of uncontrolled growth. This is particularly true for factors that are also associated with the development of malignant tumours. Consideration of the use of growth factors, therefore, also has to include aspects of growth control and interactions with suppressing and activating co-factors [31].

Dental Stem Cells and Growth Factors

Human Recombinant and Autogenous Growth Factors

The identification of the genetic code of growth factors has enabled the production of human recombinant proteins more than 20 years ago. This has repeatedly raised hopes that effective clinical application is just around the corner. Unfortunately, despite extensive research and successful preclinical testing, the clinical use of

human recombinant growth factors has not yet reached a level of application that would reflect the numerous successful experimental reports that have been published for more than 15 years [36]. Costs and regulatory issues as well as the complexity of mimicking physiological dosage levels at the site of delivery have rendered the introduction of human recombinant growth factors into clinical use more difficult than it has been anticipated [36, 37].

An alternative strategy that has been applied more frequently than the application of recombinant proteins is the use of patients' own growth factors. Autogenous growth factors are present in rather large quantities in platelets and are easily available from platelet enriched centrifugation products of whole blood. Different concepts and preparation methods have been developed, that vary in the content of platelets, leukocytes and fibrin and hence the condition of application (liquid vs. gel) [38].

Platelet concentrates (PCs) contain a combination of growth factors. The concentration may vary between individual patients and according to the method of production [39–41]. The growth factors are contained in the alpha granules of the platelets. They can be released by addition of calcium chloride, allogeneic thrombin or by autologous fibrin depending on the preparation technique [38, 39, 42–45]. Other than individual recombinant growth factors, PCs convey a number of signals that may vary in their relative strength according to the dose levels present in the individual preparations. According to the nature of the growth factors contained in PCs, they are supposed to enhance mesenchymal and epithelial proliferation as well as angiogenesis.

Whitman [46] and Marx [47] published the first studies on the use of growth factors contained in platelet gel, called Platelet-Rich Plasma (PRP). Thanks to Marx's studies, it was possible to verify that the platelet concentrate is a very effective tool for the modulation of wound healing and tissue regeneration [3].

However, the PRP showed a number of disadvantages, such as the need of having to run a complex and expensive protocol for its production. To overcome some of these problems, the PRGF (Plasma Rich in Growth Factors) was introduced in the list of platelet concentrates. The PRGF is considered an evolution of the PRP and it allows a higher concentration of growth factors in platelet preparation [41, 48].

Among the advantages of the PRGF, we can cite the lesser amount of blood taken for the preparation and a procedure relatively faster, while, among the disadvantages we can mention the rapid clot formation, which require speed in its surgical use [3].

In 2001, Choukroun et coll. have instead proposed an alternative technique: the PRF (Platelet Rich Fibrin). PRF is derived from a simple preparation protocol that does not require alteration of the blood; it is a platelet concentrate rich in GFs that contains a three-dimensional matrix of autologous, elastic and flexible fibrin [36].

Dohan et al. have shown that platelet cytokines (PDGF, TGFbeta1 and IGF-1) are present in three-dimensional fibrin matrix derived from these platelet concentrates; moreover, PRF matrix traps glycosaminoglycans such as heparin and hyaluronic acid, which have considerable affinity with some peptides present in the bloodstream and therefore show strong ability of chemotaxis and diapedesis, useful for the healing of tissue damaged, for example, by trauma [3, 49].

Nowadays use of autogenous proteins is hardly impaired by regulatory issues and is available at low costs. The ease of production and the almost unrestrained use has fuelled a widespread clinical application of PCs in regenerative procedures [32, 36, 50].

Effect of Growth Factors on Dental Stem Cells

Fundamental to our understanding of regenerative medicine is the knowledge of growth factors that effect on a broad range of cellular activities including migration, proliferation, differentiation and apoptosis of cells, including stem/progenitor cells. Growth factors and cytokines may act as signaling molecules that modulate cell behavior by mediating intracellular communication. Growth factors are polypeptides or proteins that bind to specific receptors on the surface of target cells [51, 52]. They can initiate a cascade of intracellular signaling and act in either an autocrine or paracrine manner [53]. Cytokines are typically referred to as immunomodulatory proteins or polypeptides [54]. Cytokines are often used interchangeably with growth factors because many cytokines share similar actions as growth factors. As opposed to systemic effects by hormones on target cells, growth factors or cytokines typically act locally on target cells [28]. The effects of various growth factors on dental stem cells and how they may participate in dental pulp-dentin regeneration will now follow.

Platelet-Derived Growth Factor (PDGF)

Platelet derived growth factor (PDGF) is released by platelets, and has potency in promoting angiogenesis and cell proliferation [28, 55–58]. PDGF is a highly basic dimeric glycoprotein of 30 kD consisting of two disulphide bonded polypeptides encoded by different genes [30]. There are three isoforms characterized by the combination of A- and B-chains featuring two homodimeric (PDGF-AA and PDGF-BB) and one heterodimeric isoform (PDGF-AB) [59–61]. PDGF-BB and PDGF-AB are systemically circulating isoforms contained in alpha granules of platelets from where they are released after adhesion of platelets to injured sites of vessel walls, whereas PDGF-AA is secreted by unstimulated cells of the osteoblastic lineage [60–62]. PDGF stimulates specific target cells by binding to cell surface receptors with tyrosine kinase activity. PDGF plays an important role in the development of many tissues and organs in the growing embryo. It is essential not only for mesoderm induction and mesenchymal-epithelial interactions during organ development but also for development of the neural crest and patterning of limbs and myotomes in early embryogenesis [30, 63, 64].

The chemotaxis and proliferation of mesenchymal stem cells can be induced by PDGF in the injury site. In trauma, hemorrhage is followed by blood clot formation in dental pulp. Platelets in the blood clot release α-granules containing PDGFs and

attract neutrophils and macrophages [56]. These cells play key roles in early wound healing by producing other signaling molecules for the formation of granulation tissues. However, PDGFs appear to have little effects on the formation of the dentin-like nodule in dental pulp stem cells isolated from rat lower incisors although PDGF-AB and -BB isoforms stimulate the expression of dentin sialoprotein (DSP) [65]. PDGFs stimulate cell proliferation and dentin matrix protein synthesis but appear to inhibit alkaline phosphatase (ALP) activity in dental pulp stem cells in culture [66–68]. DSP expression is inhibited by PDGF-AA, but enhanced by PDGF-AB and PDGF-BB although the mineralized tissue formation is inhibited, suggesting diverging effects of PDGFs on odontoblastic differentiation depending on dimeric form [65]. PDGF senhance the proliferation of fibroblasts in human dental pulp [66]. PDGF-BB may increase the expression of VEGF in osteoblasts and promotes angiogenesis at the site of dental pulp injury [69]. In vivo, PDGF promotes de novo formation of dental-pulp-like tissues in endodontically treated human teeth that are implanted in rats [28, 70].

Transforming Growth Factor-β (TGFβ)

Transforming growth factor β (TGFβ) is among the most widespread and versatile cytokine and plays an important role in the formation and development of many types of tissue [30]. There are more than 30 proteins, which belong to the TGFβ-superfamily such as TGFβ itself, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), Anti-Mullerian hormone (AMH), activins and nodal [28, 71, 72].

TGFβ is composed of ~390 amino acids, which are released mainly from platelets, macrophages, and bone [73]. This inactive polypeptide undergoes proteolytic cleavage to create the active C-terminal 112-amino acid form. The active form of TGFβ dimerizes to form 25 kD homodimers [74]. The name transforming growth factor is derived from the fact, that TGFβ belong to a group of growth regulators, that were originally detected in tumour extracts and thought to induce or maintain the neoplastic or transformed cell type [75]. Although TGFβ suppresses proliferation of epithelial cells, it is overexpressed in a number of epithelial malignancies.

The three isoforms present in mammals, which are TGFβ1, TGFβ2, TGFβ3, are detected also in human dentin [76].

The effect of TGFβ is highly variable and dependent on the type of cells and tissues. TGFβ1 regulates a wide range of cellular activities, such as cell migration, cell proliferation, cell differentiation, and extracellular matrix synthesis [28, 77–81]. TGFβ1 has been shown to increase cell proliferation and production of the extracellular matrix in dental pulp tissue culture [81], and promotes odontoblastic differentiation of dental pulp stem cells [82]. The effect of TGFβ1 can be synergistically upregulated by fibroblast growth fatocr-2 (FGF2), as evidenced by the increased ALP activity, the formation of mineralized nodule, and the expression of DSP and dentin matrix protein-1 [82]. The dentinogenic ability of dental pulp stem cells in the mechanically exposed dental pulp of dog teeth is shown to be induced by

exogenous TGFβ1 [83]. TGFβ is chemotactic on dental pulp stem cells in vitro [84] TGFβ1 also plays an important role in the immune response during the dental pulp injury [28, 85, 86].

Bone Morphogenetic Protein (BMP)

Bone morphogenic proteins (BMPs) are homodimeric proteins of approx. 30 kD with the two identical strands linked by a cysteine binding group [30]. Nearly 20 modifications of BMPs with slightly different modifications in small secondary structure elements [87] have been identified so far [88], among which growth differentiation factors (GDFs) were included. The content of BMPs in bone has been estimated to be 1 μg/g bone tissue[30]. BMP2–BMP9 belong to the TGFβ superfamily with a high degree of homology with the TGFβs [30]. TGF β and BMP have a common scaffold with the cysteine knot motif and two double-stranded beta sheets [87]. BMPs activity is mediated through a transmembraneous heteromeric receptor complex on the cell surface that is identical to that of TGFβ. The difference in signalling pathway is characterized by the SMAD proteins that are activated by BMPs [30].

BMPs play important roles in the development and remodeling of the bone, and they can promote the chemotaxis and aggregation of cells into osteogenic site in different ways and facilitate the differentiation into osteoblasts. In addition, these proteins can also promote the angiogenesis, regulate the activity of some growth factors and affect the production of these growth factors, which is helpful for the osteogenesis. BMPs have been considered as the most potent growth factors that can promote the bone regeneration. To date, more than 20 BMPs have been identified and BMP-2, -4, -6 and -7 [osteogenic protein 1 (OP1)] have found to the osteogenic potential [89–93]. BMP9 is also known as growth differentiation factor 2 (GDF-2) and mainly expressed in the liver [94]. BMP9 can induce and maintain the cholinergic differentiation of embryonic neurons, regulate the metabolism of glucose and fatty acid, modulate the dynamic balance of iron and exert other important biological functions [95–97]. However, the role of BMP9 in the osteogenesis and bone regeneration is poorly understood.

Human recombinant BMP2 stimulates the differentiation of dental pulp cells into odontoblasts [98], inducing mRNA expression of dentin sailophosphoproteins (DSPPs) and higher ALP activity upon BMP2 application, but no effect on cell proliferation. DSPP expression and odontoblastic differentiation are regulated likely via BMP2-induced nuclear transcription factor Y signaling [99]. BMP2 also stimulates the differentiation of dental pulp stem cells into odontoblasts in vivo and in vitro [100]. Bovine dental pulp cells treated with BMP2 and BMP4 differentiate into preodontoblasts [101]. BMP7, also known as osteogenic protein-1, promotes dentin formation when placed over amputated dental pulp in macaque teeth [102, 103]. The dentinogenic effect of BMP7 on amputated dental pulp has been shown in several animal models including rats [104], ferrets [105], and miniature swine [106]. Dental pulp cells transfected with BMP11, also known as GDF11, yields

mineralization [107]. Dentin matrix protein 1, ALP, DSPP, enamelysin, and phosphate-regulating gene are highly expressed in BMP11-tranfected cells [107]. Transplantation of BMP11-transfected cell pellets induces formation of dentin-like tissue on amputated dental pulp in dogs [107]. Ultrasound-mediated gene delivery of BMP11 stimulates odontoblastic differentiation of dental pulp stem/progenitor cells in vitro and reparative dentin formation in vivo [108].

Detection of alkaline phosphatase (ALP) and calcium deposition showed dental follicle stem cells transfected with BMP9 gene could significantly promote the osteogenesis. In addition, SB203580 and PD98059 were employed to block the p38 mitogen-activated protein kinase (p38MAPK) and extracellular signal-regulated kinase (ERK1/2), respectively. Detection of ALP and calcium deposition revealed the BMP9 induced osteogenic differentiation of dental follicle stem cells depended on MAPK signaling pathway [109].

BMP9 can effectively induce odontogenic differentiation of the stem cells from mouse apical papilla (SCAPs). Using a reversible immortalization system expressing SV40 T flanked with Cre/loxP sites, it was demonstrated that the SCAPs can be immortalized, resulting in immortalized SCAPs (iSCAPs) that express mesenchymal stem cell markers. BMP9 upregulates Runx2, Sox9, and PPARc2 and odontoblastic markers, and induces alkaline phosphatase activity and matrix mineralization in the iSCAPs. The in vivo stem cell implantation studies indicate that iSCAPs can differentiate into bone, cartilage, and, to lesser extent, adipocytes upon BMP9 stimulation. Results demonstrate that the conditionally iSCAPs not only maintain long-term cell proliferation but also retain the ability to differentiate into multiple lineages, including osteo/odontoblastic differentiation [110].

Fibroblast Growth Factor (FGF)

Fibroblast Growth Factor (FGF) plays key roles in cell migration, proliferation and differentiation during embryonic development [111] and wound healing [112]. Currently 22 members have been identified in humans [113], of which FGF2 appears to be significant in regeneration of the pulp-dentin complex [28]. FGF2 is a basic FGF (bFGF), while FGF1 is acidic. Basic FGF (bFGF) is known to be a critically important factor for maintaining the self-renewal ability of human embryonic stem (hES) cells in cultures and is a potent mitogen for cultured neural progenitors [114, 115].

bFGF dramatically induces the mRNA expression of dentin sialophosphoprotein (DSPP) and bone sialoprotein (BSP) in immature adult rat incisor dental pulp cells [116], suppresses alkaline phosphatase (ALP) activity and osteogenesis differentiation in human dental pulp cells (hDPCs), and inhibits osteogenesis in mouse adipose tissue-derived stromal cells, whereas bFGF sustains their proliferative and osteogenic potential state [117–119]. bFGF induces the migration of dental pulp stem cells [120]. Using a transwell migration assay, significantly more dental pulp cells are recruited by bFGF (FGF2) into a 3D collagen gel than controls without cytokines and BMP7 [120]. bFGF also stimulates the proliferation of dental pulp

stem cells without differentiation, whereas bFGF combined with TGFβ1 induces differentiation of dental pulp stem cells into odotoblast-like cells, and synergistically upregulates the effect of TGFβ1 on odontoblast differentiation [82].

bFGF stimulation in the osteogenic differentiation period decreased the in vitro osteogenic differentiation ability of DPSCs. One week pre-treatment with bFGF increased the in vitro osteogenic differentiation ability of DPSCs, whereas 2 weeks pre-treatment with bFGF decreased the in vitro osteogenic differentiation ability of DPSCs. The pre-treatment period was vital for the osteogenic differentiation of DPSCs in vitro. The in vivo results were similar to the in vitro results. bFGF affected the osteogenic differentiation of DPSCs in a treatment-dependent manner both in vitro and in vivo [121].

In vitro studies showed that different timing of the presence of bFGF might either suppress osteogenic/dentinogenic differentiation of SCAP or enhance osteogenic/dentinogenc differentiation [122]. These findings indicate that the effects of bFGF are differentiation stage specific and that bFGF might modulate cell differentiation by acting at distinct stages of cell maturation [123]. Under certain conditions, therefore, bFGF enhances SCAP stemness by up-regulating stem cell gene expression, increasing proliferation ability, and potentiating differentiation potency [122]. Results showed that short-term bFGF treatment enhanced OCT4, REX1, and NANOG mRNA expression as well as colony forming unit ability in stem cells isolated from human exfoliated deciduous teeth (SHEDs). The FGFR inhibitor pre-treatment was able to attenuate the influence of bFGF on pluripotent stem cell marker expression, confirming bFGF function. In addition, cells cultured in high passage number had decreased in cell proliferation, colony forming unit capacity, and pluripotent stem cell maker mRNA expression. However, bFGF supplementation in culture medium enhanced both pluripotent stem cell marker expression and colony forming unit capacity in later passage, though the effect was not robust. Together, these results indicate that high passage number may attenuate pluripotent properties of SHEDs and bFGF supplementation could be the beneficial approach to maintain SHEDs' stemness properties [124].

Insulin-Like Growth Factor (IGF)

Insulin-like growth factors (IGFs) are single chain peptides that exist in two isoforms (IGF-I (70 amino acids) and IGF-II (67 amino acids)). IGFs have approximately 40–50 % homology between themselves and with insulin. Despite this significant homology between insulin and IGFs, all three have unique binding sites to their receptors [125]. In contrast to many other growth factors, which act merely on a local or regional level, IGFs are also factors with a general both metabolic and growth promoting activity in many cell and tissue types. IGFs, comprising IGF-1 and IGF-2, contribute to odontogenesis and dental tissue repair by cell proliferation and differentiation. [28, 126]. Of the two isoforms, IGF-1, also known as somatomedin C, has potency in growth and differentiation of dental pulp cells [127]. IGF-1 induces proliferation and differentiation of dog dental pulp cells into

odontoblast-like cells in serum-free medium [127]. IGF-1 with PDGF-BB has a synergistic effect on the proliferation of dental pulp cells in vitro [128]. IGF-1 and IGF-1R have a higher level of expression in dental pulp tissue from teeth with complete root development than teeth with incomplete root formation, suggesting that IGF-1 stimulates mineralization and cell differentiation [128–130]. IGF-1 could promote proliferation and osteogenic differentiation of human dental pulp stem cells (DPSCs) via mTOR pathways, which might have clinical implications for osteoporosis [131].

IGF-1 treated stem cells from apical papilla (SCAPs) present the morphological and ultrastructural changes. Cell proliferation, alkaline phosphatase (ALP) activity and mineralization capacity of SCAPs are increased by IGF-1. Western blot and quantitative RT-PCR analyses further demonstrate that the expression of osteogenic-related proteins and genes (e.g., alkaline phosphatase, runt-related transcription factor 2, osterix, and osteocalcin) is significantly up-regulated in IGF-1 treated SCAPs, whereas the expression of odontoblast-specific markers (e.g., dentin sialoprotein and dentin sialophosphoprotein) is downregulated by IGF-1. In vivo results reveal that IGF-1 treated SCAPs mostly give birth to bone-like tissues while untreated SCAPs mainly generate dentin-pulp complex-like structures after transplantation. IGF-1 can promote the osteogenic differentiation and osteogenesis capacity of SCAPs, but weaken their odontogenic differentiation and dentinogenesis capability, indicating that IGF-1 treated SCAPs can be used as a potential candidate for bone tissue engineering [132].

Vascular Endothelial Growth Factor (VEGF)

Vascular Endothelial Growth Factor (VEGF) is a heparin-binding protein with specific affinity to endothelial cells and plays a keyrole in angiogenesis [133]. The functions of VEGF involve the proliferation of endothelial cells and their enhanced survival [134], stimulating neovascularization in the area of injury [28].

VEGF, belonging to a family of homodimeric proteins consisting of six members (VEGF-A, -B, -C, -D, -E; placenta growth factor). In particular, VEGF-A exists in five different isoforms among which VEGF-A like other isoforms, binding specific co-receptor proteins, named Neuropilin-1 and -2, activates two different tyrosine kinase receptors VEGFR-1 (named also Flt-1) and VEGFR-2 (KDR/Flk-1), the stimulation of which triggers different signals in a large number of cells. For a long time, VEGF has mainly been considered as a potent mitogenic factor for vascular endothelial cells, involved in the modulation of physiological angiogenesis, vascular permeability and also the occurrence/of tissue inflammation. More recently, VEGF has been recognized as a positive regulator of bone development, skeletal growth and fracture repair, also stimulating proliferation and differentation of bone-derived osteoblasts [135, 136].

VEGF is produced by human dental pulp cells, in physiological and even more in pathological conditions [137, 138], causing in these cells a potent induction of chemotaxis, a marginal activation of proliferation and an increase in the alkaline

phosphatase (ALP) expression [137]. Recently, D'Aquino et al. [139] showed that human DPSC comprise at least two types of cells expressing different immunophenotypic profiles, which spontaneously diffrentiate, both in vitro and in vivo, into osteoblasts and, to a lesser extent, into endothelial cells over a long period. The spontaneous commitment of DPSC towards vascular phenotype cells may be accelerated by plating DPSC on an appropriate substrate (Matrigel) and exposing them to VEGF for 8 days [140].

VEGF appears to induce the differentiation of human dental pulp stem cells into endothelial cells [140]. Dental pulp cells become positive for CD29, CD44, CD73, CD105, CD166 but negative for CD14, CD34, CD45 after VEGF treatment [140]. VEGF increases the expression of VEGFR1 (fms-like tyrosine kinase, Flt-1) and VEGFR2 (kinase-insert domain containing receptor, KDR) and microvessel formation in a three-dimensional fibrin mesh seeded with dental pulp stem cells [28, 140]. MSC obtained from human dental pulp (DPSC) of normal impacted third molars, when cultured in lineage-specific inducing media, differentiate into osteoblasts and adipocytes (evaluated by Alizarin Red S and Red Oil O stainings, respectively), thus showing a multipotency. DPSC, grown under undifferentiating conditions, are negative for hematopoietic (CD45, CD31, CD34, CD144) and positive for mesenchymal (CD29, CD90, CD105, CD166, CD146, STRO-1) markers, that underwent downregulation when cells were grown in osteogenic medium for 3 weeks. In this condition, they also exhibit an increase in the expression of osteogenic markers (RUNX-2, alkaline phosphatase) and extracellular calcium deposition, whereas the expression of receptors (VEGFR-1 and -2) for vascular endothelial growth factors (VEGF) and related VEGF binding proteins was similar to that found in undifferentiated DPSC [136]. Exposure of DPSC growing under undifferentiating or osteogenic conditions to VEGF-A165 peptide (10–40 ng/ml) for 8 days dose- and time-dependently increased the number of proliferating cells without inducing differentiation towards endothelial lineage, as evaluated by the lack of expression of specific markers (CD31, CD34, CD144). Additionally, exposure of DPSC cultured in osteogenic medium to VEGF-A165 for a similar period enhanced cell differentiation towards osteoblasts as evaluated after 14 and 21 days by Alizarin Red S staining and alkaline phosphatase activity quantification. These findings may have clinical implications possibly facilitating tissue repair and remodeling [136].

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Nano Surface and Stem Cells for Implants

G.K. Thakral

Introduction

Nanotechnology can be defined as the science and engineering involved in the design, synthesis, characterization and application of materials and devices whose smallest functional organization, in at least one dimension is on the nanometer (nm) scale or one billionth of a meter [1].

The technology involves increasing the complexity of the surface topography with the addition of nanoscale molecules (Fig. 1) [2].

Nanotechnology therefore, involves materials that have a nano-sized topography or are composed of nano-sized materials ranging between 1 and 100 nm [3]. Nanobiotechnology can be described as a hybrid science that has evolved by blending two predominant technologies—nanotechnology and biotechnology. It essentially involves the further improvement and enhancement of the physical and chemical properties of biomaterials for their better and advantageous use in the medical science [4]. Since, bio-molecules are present in nanometer dimensions, nano-sized biomaterials are expected to integrate and assimilate well in biomedical devices and procedures [5].

An evaluation of nano-mechanical properties of the surrounding bone by nanoindentation revealed that while both implants exhibited similar bone-to-implant contact, the nano-indentation demonstrated that the tissue quality was significantly enhanced around the hydroxyapatite (HA)-coated implants [6]. Since osteoblasts readily adhere to this novel surface, dental implants coated with TiO_2 nanotubes could significantly improve healing following dental implant surgery (Fig. 2) [2].

Nanotechnology may involve one-dimensional concepts (nanodots and nanowires) or the self-assembly of more complex structures (nanotubes). Materials are

G.K. Thakral (✉)
Department of Dentistry, SGRR Institute of Medical & Health Sciences, Dehradun, India
e-mail: gkthakral@yahoo.com

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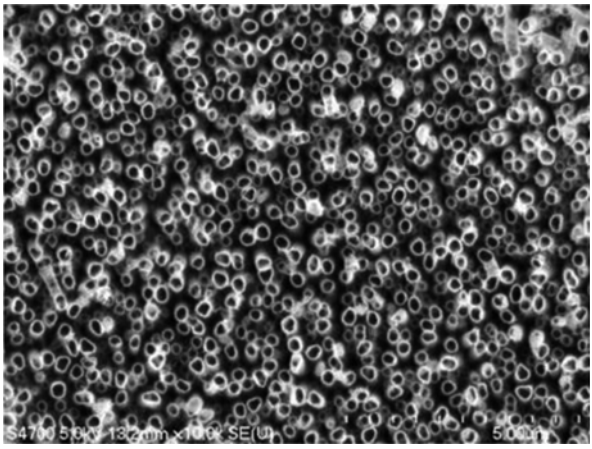

Fig. 1 A cluster of titanium dioxide nanotubes [2]

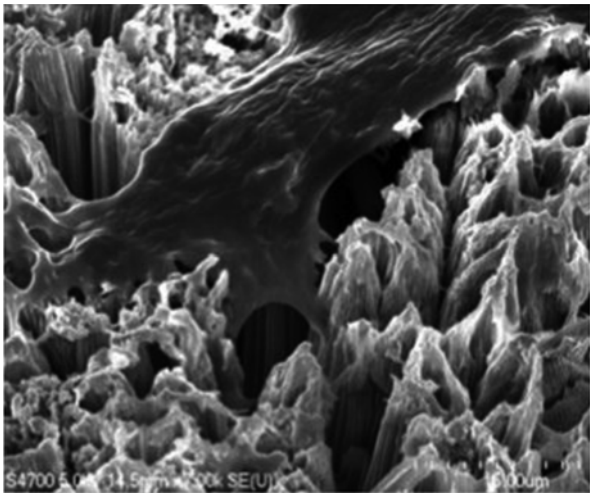

Fig. 2 Bone cell anchoring to a surface of titanium dioxide nanotubes [2]

also classified according to their form and structure as nano-structures, nanocrystals, nano-coatings, nano-particles, and nano-fibers [3]. Nano-scale modification of the titanium endosseous implant surface may lead to alteration in the topography as well as chemistry of the surface. Therefore, the goal of nanoscale modification should be a specific chemical modification of commercially pure (cp) titanium (grade 4 and 5). A distinct implication associated with nano-scale manipulation of any material is that it also leads to inherent chemical changes on the material surface.

The topographic changes invariably create an increased surface area and nanoscale surface roughness leading to better biological responses of osteogenic cells and effective tissue-implant mechanical interlocking, apart from making the implant surface high wear resistant. These qualities of the nano-biomaterials make it more

favourable for implant procedures as compared to other materials. Nano modifications in biomaterials can be undertaken as under:

- Biomaterial Surface Modifications
  - Alteration of Surface Topography
  - Alteration of Surface Chemistry
- Scaffolds involving Nano Materials
  - Nano-ceramic Composites
  - Nano-fibre Composites

These surface modifications enhance solubility and stability of nano materials in aqueous media and make them biologically more compatible. The surface modifications can be achieved by employing physical, chemical and biological methods. Physical modifications can be achieved through molecular coating, molecular adsorption, surface entrapment, plasma spraying and ozone ablation etc. Whereas, chemical modifications may involves procedures like silanisation, surface oxidation and self-assembled monolayers etc.

The advantages of nano-topography based implants or nano-material based implants are significant and may include improved mechanical properties, wear resistance, bone-to-implant contact, protein adsorption, cell response and osseointegration [7, 8].

Dental implants closely mimic natural teeth, but lack in tissue dynamics associated with periodontal tissues. Stem cells offer a promising source for tissue regeneration due to their peculiar quality of proliferation, differentiation and natural plasticity. A positive and desirable interaction of stem cells with various biomaterials offers an enormous scope for their use in various therapeutic applications. Dental stem cells derived from exfoliated deciduous teeth as well as from wisdom teeth are an easy and rich resource for tissue regeneration including osseointegration of implants.

Biomaterials

Biomaterials can be defined as the materials that are well tolerated and accepted when placed inside or adjacent to the living tissues in a body. Biocompatibility therefore, refers to a positive response a material generates in close proximity to the living tissues. The placement of a material in close proximity to the living tissues creates an interface. The material and the tissues come face to face at this interface leading to some reaction or response from both sides. This response is not merely a static reaction but a dynamic one. This reaction or interaction between the material and the living tissues may lead to certain specifically outcomes like:

- The degeneration of tissues affecting the properties of the material
- The degeneration of the material affecting health of tissues
- Regeneration of the tissues and survival of the material

Table 1 Classification of implant biomaterials

Classification	Response	Effect
Bio-tolerant	Formation of thin connective tissue capsules that does not adhere to the implant surface	Rejection of the implant, leading to implant failure
Bio-active	Formation of bony tissue around the implant material and strongly integrates with the implant surface	Acceptance of the implant leading to success of implant
Bio-reabsorbable	Replaced by the autologous tissue	Acceptance of the implant leading to success of implant

It is this dynamic interaction that determines both—the biological response of the tissues to the material and survival of the material in the biological environment against corrosion and degeneration. That is the basic concept of biocompatibility of a material. Biocompatibility therefore, is not merely a property of the material. Biocompatible activity of a material at the interface of material and the tissue depends on factors like—site of the material placement, its duration in the biological environment, properties of the material and health of the host. Therefore, a material that is biocompatible as a crown and bridge material may not necessarily be compatible as an implant material and vice-versa. Health conditions of the host also make an impact on the biocompatibility response of a material. For that matter, biocompatibility of a young person may be different from that of an older person. The biological response of the same material may vary from a healthy young adult to a young adult affected by some debilitating disease or conditions that hinder or inhibit tissue response.

Therefore, it is important to evaluate and understand the physical, chemical and biological properties of basic components of a material before any biological applications, since these properties determine the success or the failure of an implant material in a long term use.

Broadly speaking, the implant biomaterials can be classified into three groups based on their interaction with the tissues (Table 1).

Bio-tolerant: The term refers to the behavior of a material that depicts minimal interaction with its surrounding tissue when placed in the body. The tissue response may be limited to formation of some connective (scar) tissue at the bone implant interface without any integration to the implant surface.

Bio-active: It refers to a material that actively interacts with the surrounding bone and soft tissues. The bio-active materials may either initiate or promote some regenerative activity once placed adjacent to the living tissues. The tissue response leads to formation of bony tissue at the bone implant interface that strongly integrates with the implant surface. This is the most widely used bio-material category for dental implants.

Bio-resorbable: It refers to that property of a material that upon placement within the human body makes it to gradually dissolve and get replaced by adjacent tissues. The tissue response creates formation of autologous tissue leading to implant success. These types of bio-materials are more frequently used in orthopedic implants.

Desired Properties of Biomaterials

Development of biomaterials is a highly skilled job requiring interdisciplinary collective involvement of professionals like pathologists, clinicians, material scientists, material engineers, biomedical scientists etc. The basic properties that ensure success of an implant adjacent to human tissues can be summarized as—

Mechanical Properties

The mechanical properties of a material play a decisive role in its selection for fabrication of implants. Some of the important properties include hardness, tensile strength, modulus of elasticity and elongation. The response of the material to repeated occlusal loads is dependent on the fatigue strength of the material and determines the long term success of implant under recurring occlusal stresses. The modulus of elasticity of bone varies in magnitude from 4 to 30 Gpa depending on the type of bone and the direction of measurement [9, 10].

The material used for implants should have modulus of elasticity equivalent to that of the bone. Inadequate strength or any mismatch in mechanical properties between implant material and the bone is bound to lead to fracture of implant making it biomechanically incompatible. At the same time, an implant material that has a strength much higher than the bone will prevent transfer of the stress to the adjacent bone leading to bone resorption around the implant and implant failure. The biomechanical incompatibility that leads to death of the bone cells is known as 'Stress Shielding Effect [11]. Therefore, the material of choice for dental implants has to have a combination of high strength and low modulus of elasticity closer to the bone.

Biocompatibility

The materials used for implants are expected to be well accepted and tolerated by the human tissues without causing any adverse effect or reactions in the body. This quality of a material is known as biocompatibility [12]. Interaction and some reaction start between implant surface and adjacent tissues immediately upon placement of implant in the human body. The reaction involves blood coagulation and adhesion of blood platelets to biomaterial surface and encapsulation of the biomaterial by fibrous tissues.

The biological compatibility of implant materials is directly dependent upon their chemical composition, design of the implant, topographic mechanics and wettability of the implant material. Use of nanotechnology may lead to the development of dental implants with controlled topography and chemistry leading to the development of implant surfaces with predictable and favourable tissue response during osseointegration [13, 14].

High Corrosion and Wear Resistance

The implants should be developed using materials with high wear and corrosion resistance in order to ensure longevity of the dental implant under biological conditions. The longevity of the implant is usually determined by its resistance to abrasion and wear in the mouth. The implant materials having low wear and corrosion resistance release non-compatible metal ions in the body fluids. The released ions cause allergic and toxic reactions in the body [15].

A low resistance to wear leads to formation and deposition of wear debris at the implant-tissue interface causing several chemical and biological reactions in the tissues with ultimate failure of the implant due to its loosening [16].

Osseointegration

Osseointegration is a term founded by Professor Per-Ingvar Brånemark after his important breakthrough in the 1950s when he discovered that bone can integrate with titanium components. Osseointegration has been described as a direct structural and functional bone to implant contact under load [17]. Professor Brånemark named his discovery after Latin word 'os' that means bone, and 'integrate' that means make whole. When conjoined together it points out to interactive coexistence. It was observed that when a screw-shaped implant fixture made of titanium was carefully placed in the bone, the genetic code that usually makes bone reject a foreign material was not activated. The bone cells were able to attach to the titanium surface resulting in a firm and permanent anchorage for a prosthetic reconstruction. It actually promotes bone regeneration that fills the micro gaps between the implant surface and the adjacent bone. The term has been used to explain the success or failure of the implants in medical and dental science ever since. In the absence of osseointegration, fibrous tissue is formed between the bone and the implant surface. The implant therefore, is not well integrated into the bone resulting in implant loosening and subsequent implant failure [18]. Surface topography, surface roughness, surface material and surface chemistry, all play a significant role for a successful osseointegration.

Impact of Nano Topography in Implants

Titanium and Titanium alloys Ti_6Al_4V have over the years emerged as the most biocompatible material for dental implants due to their excellent corrosion resistance property. Resistance to corrosion in Titanium occurs due to the formation of biologically inert oxide layer on the surface of the implant body. This oxide layer spontaneously converts into tenacious surface oxide on exposure to air or physiologic saline. This surface oxide transformation leads to the formation of three types

of oxides on the implant surface; namely TiO (Anastase), TiO_2 (Rutile) and Ti_2O_3 (Brookite). Of the three oxides, TiO_2 (Rutile) is the most stable and the most frequently formed oxide layer that has the potential to regenerate instantly. For that matter, if implant surface gets abraded or scratched during implant placement procedure, this oxide layer can recreate itself spontaneously.

Majority of dental implants today are made of cp titanium (1–4 grades) or titanium alloys like Ti_6Al_4V. Factors that contribute to the ultimate success of an implant include—

1. Physiological conditions of the patient
2. Implant placement procedure
3. Implant material
4. Implant design, and
5. Implant surface

Albrektsson and Wennerberg [19] divided implant surface quality into three categories—

1. Mechanical properties,
2. Topographic properties, and
3. Physico-chemical properties.

They pointed out that these characteristics are inter-related and a change in any of these groups affects the others as well. This significant observation is quite relevant to the study of nano-surface modifications of the endosseous cp titanium implant surface.

The success of dental implantology to a great extent depends on two basic eventualities. The first is to achieve osseointegration and maintain it through the life of implants. This biological bonding of the implant with the surrounding bone ensures a sound mechanical anchoring in situ. The second is an excellent adaptation and blending of the gingival tissues at the neck of dental implant. This ensures a good sealing of the implant in the soft tissue and isolation of implant body from oral environment and thereby preventing any bacterial lodgment in the region and subsequent peri-implantitis.

This initial tissue response to osseointegration renders primary stability to the dental implant that gradually decreases to pave the way for a secondary tissue response of biological union at the implant tissue interface. The implant material, implant design and implant surface play a very significant and crucial role in the success of secondary tissue response for a lasting osseointegration of the dental implant and may be preceded by a transient phase of decreased implant stability. Surface topography of the dental implants plays a significant role on the quality and quantity of osseointegration post implantation. In order to have favourable osseointegration, the implant surface should promote adsorption of proteins, the initial adhesion followed by differentiation of cells and homogenous tissue integration.

It has been established through studies that Calcium Phosphate (CaP) coatings provide titanium implants with an osteo-conductive surface. The CaP coatings get dissolved in the peri-implant region after implant placement leading to increased

ionic strength and blood saturation that in turn initiates precipitation of biological apatite nano-crystals on the implant surface. The inherent proteins in the biological apatite layer promote adhesion of osteo-progenitor cells that generate extracellular matrix of bony tissues [20]. Studies have also revealed that osteoclasts that are capable to resorb the bone cells cause degradation of the CaP coating through enzymatic reactions and create resorption pits on the CaP coated implant surface [21].

CaP particles on the implant surface serve as a catalyst and promote almost immediate osseointegration of implants into the adjacent bone. It is significant to create CaP coating on the implant surface that would disintegrate at a rate similar to apposition of bone so that a direct bone contact may develop on the surface of implant.

A favourable biological interactions between implant and bone interface are greatly dependent upon the topography, chemical compositions and wettability of the implant surface. An implant surface that allows predictable, controlled and guided tissue regeneration is more likely to promote contact osteogenesis [22, 23].

Moreover, nano-surface modifications substantially increase the total surface areas at the interface for osteoapposition leading to better osteointegration of the implant. Introduction of Ti nanotubes (300 nm) resulted in significant increase of up to 3.1 times in the strength of bone-titanium interface in rat femur [22].

Methods of Imparting Nano-Features

General modification in implant materials may be broadly categorized into—physical modifications, chemical modifications and biological modifications and can be summarized as under:

1. Physical Modification
   (a) Molecular Coating
   (b) Surface Entrapment
   (c) Plasma Spraying
   (d) Ozon Ablation
2. Chemical Modification
   (a) Surface Oxidation
   (b) PEG (Poly-ethylene-Glycol) Chemistry
   (c) Silane (Silicone-Based) Chemistry
   (d) Self-assembled Monolayers
3. Biological Modification
   (a) Antibody–Antigen
   (b) Receptor–Ligand (Ligand is a molecule like an antibody, hormone or drug that binds to a receptor)
   (c) DNA–DNA Hybridization

Table 2 Methods for creating nano-features on cp titanium implants [24]

Methods	Characteristics
Self-assembly of monolayers	The exposed functional end group could be a molecule with different functions (an osteoinductive or cell adhesive molecule)
Compaction of nanoparticles	Conserves the chemistry of the surface among different topographies. Not readily applied over implant surfaces
Ion beam deposition	Can impart nanofeatures to the surface based on the material used
Acid etching	Combined with other methods (sandblasting and/or peroxidation) can impart nanofeatures to the surface and remove contaminants
Peroxidation	Produces a titania gel layer. Both chemical and topography changes are imparted
Alkali treatment (NaOH)	Produces a sodium titanate gel layer allowing hydroxyapatite deposition. Both chemical and topographic changes are imparted
Anodization	Can impart nanofeatures to the surface creating a new oxide layer (based on the material used)
Sol–gel (colloidal particle adsorption)	Creates a thin-film of controlled chemical characteristics. Atomic-scale interactions display strong physical interactions
Discrete crystalline deposition	Superimposes a nanoscale surface topographical complexity on the surface
Lithography and contact printing technique	Many different shapes and materials can be applied over the surface. Approaches are labor intensive and require considerable development prior to clinical translation and application on implant surface

There are various methods to create nanoscale features at the implant surface (Table 2) [24].

These methods include:

1. Physical methods, like self-assembly of mono-layers, compaction of nano particles and ion beam deposition;
2. Chemical methods, like acid etching, peroxidation, alkali treatment (NaOH) and anodization;
3. Nano particle deposition like sol–gel (colloidal particle deposition) and discrete crystalline deposition; and
4. Lithography and contact printing technique

Influence of Implant Surface on Osseointegration

One of the main concerns related to coating the implant surface is the risk of coating detachment and resultant toxicity of related debris. An evaluation of relationship of particle size and cell viability and proliferation compared to micron-particles revealed that nano-particles of titanium and alumina had less negative impact in cell viability and proliferation as compared to conventional particles. There may be an advantage to nano-scale modification of surfaces using sol–gel coating methods. The quantum interaction of high electron density at the atomic level can enforce high bond strength between the substrate and nano-scale coating [25].

Studies reveal that the addition of a nanometer-scale calcium phosphate treatment to a dual acid-etched implant surface appeared to increase the extent of bone development after 4 and 8 weeks of healing. It was observed that this rapid accrual of bone at the implant surface expedites the implant healing period and supports early loading protocols [26].

Nanotechnology and Cellular Activity

The observation that a micron-scale rough surface prepared by grit blasting and subsequent acid etching was capable of rapid and increased bone accumulation further strengthened an earlier report that a TiO_2 grit blasted surface also supported more rapid and increased bone accrual at cp titanium implants [27].

The study also pointed to a significant fact that the cp titanium surface could be modified to enhance bone accumulation and suggested that cp titanium was not only "bioinert" or "biocompatible", but was also capable to influence cellular activity or tissue responses leading to better and greater osteogenesis and thereby promoting better osseointegration. Nano-topography seems to influence cell interactions at surface of the material being used. It also leads to changed cell behavior when compared to conventional sized topography. The cellular protein adsorption is altered by nanoscale modification of bulk material. Depending on the nano-architecture, cell spreading may be increased or decreased. The present undefined mechanisms indicate that cell proliferation appears to be enhanced by nanoscale topography. Several investigators have shown that nanoscale topography enhances osteoblast differentiation [28–30].

Protein Adsorption (Surface Wettability) in Nano-Surface

Alteration in initial protein surface interaction is believed to control osteoblast adhesion, a critical aspect of the osseointegration process [31]. When implant comes in contact with a biological environment, the initiation of protein adsorption (e.g. plasma fibronectin) promotes subsequent cell attachment and proliferation. Change in surface energy or wettability of a biomaterial corresponds to a typical way of altering cell interactions with the surface. Nano-scale topography is now an established way of altering protein interactions within a surface. An increased vitronectin adsorption has been observed on nano-structured surfaces when compared to conventional surfaces [32, 33]. The study also suggested an increased osteoblast adhesion when compared to other cell types, such as fibroblasts, on the nanosurfaces [33].

Cell Adhesion, Spreading and Motility in Nanosurface

Irrespective of the surface-adsorbed proteins, cells are remarkable in their ability to sense nanostructure. Nano-features of a surface affect both cell adhesion and cell motility. Both of these cell traits are attributed in part, to the function of integrins

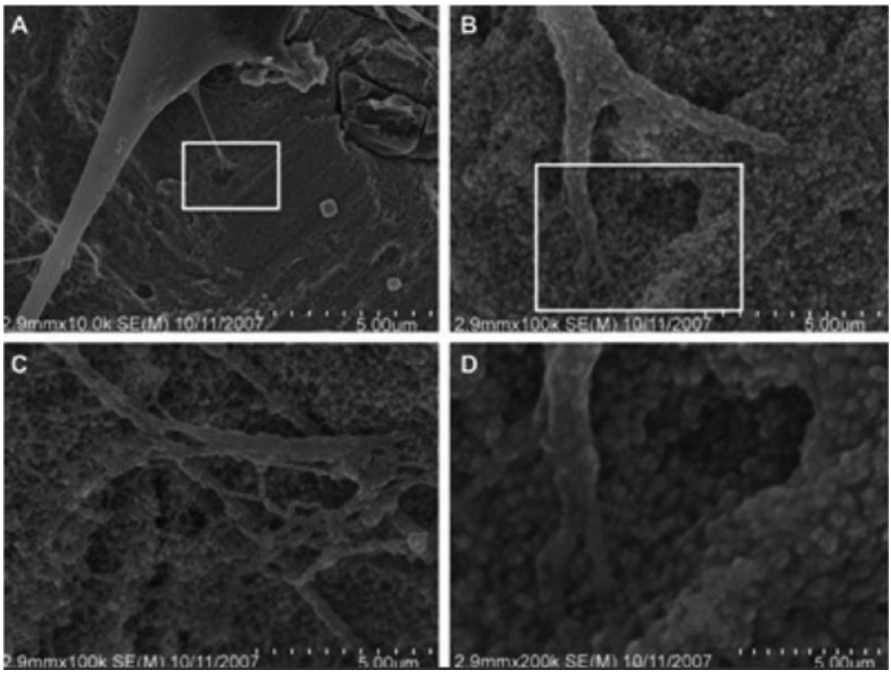

Fig. 3 Nanoscale topography-cell interactions on a nano-surface produced by H_2O_2/H_2SO_4. Treatment (**a**) 10,000 image of adherent cell, (**b**, **c**) represent 100,000 images of the same adherent cell and (**d**) 200,000 magnification of the cell with nano-features. (**b**) Higher magnification of the *rectangle* in (**a**). (**d**) Higher magnification of the *rectangle* in (**b**) [33]

[33, 34]. Underlying substratum topography influences cell behaviors by both direct and indirectinteractions. In 20–40 nm features produced by H_2O_2/H_2SO_4 treatment there were definitive interactive points for lamellipodia of spreading cells (Fig. 3) [33]. Cell behavior is affected by both, the dimension and the density of the nano structures.

Proliferation in Nano-Surface

Nanosurface modifications promote adherent cell proliferation. Zhao et al., utilizing three distinct methods—electrochemical machining, anodization and chemical etching to produce reproducible submicron-scale structures on titanium surfaces reported an opposing relationship between cell proliferation and cell differentiation with increase in micro scale of surface features.

It has further been supported by studies that observed an increase in the osteoblast proliferation on nano scale materials like alumina, titanium and hydroxyapatite [35, 36]. However, the mechanism involved is still not clear as to how nano-structured surfaces modulate the adherent osteoblast response.

Selectivity of Adhesion in Nano-Surface

Selectivity of cell adhesion is an interesting feature attributed to nanoscale topographic surfaces. Several studies have revealed a relative lowering of fibroblast adhesion compared to osteoblast adhesion on evaluation of nano and micron-structured surfaces [37, 38]. The affinity ratio between osteoblasts and fibroblasts was 3 to 1 on nanosized materials as compared to the conventional materials depicting a ratio of 1 to 1 [33]. Studies on other cell types such as smooth muscle cells and chondrocytes have also reported similar results [39].

All these observations may lead to some major implications in specification of tissue response at bone and mucosal surface of the dental implant/abutment assembly.

Differentiation

A rapid differentiation of adherent mesenchymal cells along the osteoblast lineage is as significant for the process of osseointegration as supporting osteoblast-specific adhesion and adherent cellular proliferation. Studies have revealed that alkaline phosphatase synthesis and calcium mineral content increased in cell layers formed on nano sized materials after 21 and 28 days [40, 41] (Table 3).

Nano-Surface and Bacterial Proliferation

Another significant finding with nano-surfaces has been found to be a diminished bacterial adhesion and proliferation [70]. There was a marked decrease in bacterial colonization on nano structured TiO_2 and ZnO_2 irrespective of the fact that these surfaces promote osteoblast adhesion and differentiation. These initial observations may suggest the need for further exploration of the implant abutment surface with focus on biofilm accumulation and peri-implantitis.

Nano-Surface and Surface Reactivity

Endosseous implant surface reactivity may get influenced by nano-surface modifications. Insignificant bone bonding occurs at endosseous titanium implants, especially during the initial phases of bone formation [71]. Nano-scale topographic modifications tend to change the chemical reactivity of materials and presence of bone on implant surface during early stages [72].

Bone bonding seems to be an advantage associated with titanium implants with nano-scale surface modifications. Advantages of nano-topography on biomaterials have been demonstrated as early as 1999 [30].

Table 3 Summary of the osteochondro-inductive nanostructures presented and the cell behavior outcomes observed [42]

Nanostructured materials	Fabrication techniques	Cell types	Cell behaviors
TiO_2 nanotubes with Ta coating [43]	Two electrode setup anodization and vacuum-deposited	Osteoblasts	Improvement of viability and faster Mineralization
TiO_2 nanotubes [44]	Two electrode setup anodization	Chondrocytes	Promotion of chondrogenesis
Carbon nanotubes-nanocomposite of CHI fibers + HA crystals [45]	Arc discharged method, freeze-drying and lyophilization	Osteoblasts	Improvement of adhesion and proliferation
Graphene oxide (GO) + CHI + HA [46]	Covalent liaison of CHI and GO in solution	MC3T3-E1 fibroblastic cells	Improvement of adhesion, proliferation and osteogenic differentiation
Graphene oxide with PMMA [47]	Chemical vapor deposition	Mesenchymal stem cells	Improvement of osteogenic differentiation
Collagen-GAG scaffolds with biomolecular pattern (fibronectin) [48]	Direct photolithography	MC3T3-E1 fibroblastic cells	Improvement of the speed of cell attachment
Nanostructured alumina surfaces [49]	Chemical vapor deposition	Osteoblasls	Induction of osteogenic differentiation
Polycrystalline titanium nanostructured surface with conformal bioactive calcium phosphate thin films sputter [50]	RF magnetron sputter deposition	Bone marrow mesenchymal stem cells	Improvement of adherence, proliferation and osteogenic differentiation
Electrochemically grooved nanostructured stainless steel implant with pre-adsorption of Protein [51]	Phase reversion-induced nanograined structure	Osteoblasts	Improvement of osteoblastic function and activity
Micro- and nanopatterned transplantable poly(lactic-co-glycolic-acid) polymer [52]	Capillary force lithography with a surface micro-wrinkling	Mesenchymal stem cells	Improvement of adhesion, osteogenic differentiation and pattern-controlled bone regeneration
Patterned silicon topographically-patterned surface [53]	Nanolithography	Mesenchymal stem cells	Specific size scale of topographic cue promotes osteogenic differentiation wilh or without osteogenic agents
Carbon nanotubes-reintorced HA coating on titanium implants [54]	Plasma-spray	Osteoblasts	CNT addition improves osseo integration

(continued)

Table 3 (continued)

Nanostructured materials	Fabrication techniques	Cell types	Cell behaviors
Nanofibrous CHI-nanocrystalline HA scaffolds with single-walled carbon nanotubes [45]	Freeze-drying and lyophilization	Osteoblasts	Improvement of cytocompatibility for osteoblasts adhesion and proliferation
Nanofibrous PCL with BMP-2 nanoreservoirs [55]	Electrospinning and layer by layer deposition	Osteoblasts	Improvement of osteogenic gene expression and mineralization
Nanofibrous gelatin [56]	Thermally induced phase separation and porogene-leaching	Osteoblasts	Improvement of migration, proliferation and mineralization
Collagen scaffold and heparin-binding peptide amphiphiles with nanofiber-heparan sulfate [57]	Peptide synthesis	In vivo implantation without cells	Large volumes of regenerated bone
Nano-fibrous Poly(l-lactic)acid scaffolds [58]	Freeze-drying and lyophilization	Osteoblasts	Improvement of osteoblast phenotype, mineralization and earlier differentiation
Supra. molecular self-assembled nanofibers of peptide amphiphiles [59]	Standard solid phase methods and self assembly	Mesenchymal stem cells	Improvement of viability and chondrogenic differentiation
Peptide hydrogel KLD12 ($[KLDL]_3$) and RAD16-1 ($[RADA]_4$)	Self assembly	Chondrocytes [60] Bone marrow stromal cells [61]	Promotion of chondrogenesis Promotion of chondrogenesis
Poly (lactic acid-co-glycolic acid) nanocapsules with bone morphogenetic protein BMP-2 and poly(3-Hydroxybutyrate-co-3-hydraxyvalerate) nanocapsules with BMP-7 embedded in CHI scaffold [62]	Co-electrospinning	Bone marrow stromal cells	Improvement of osteogenic differentiation (ALP activity)
Nanofibrous CHI, silk Fibroin (SF) and CHI/SF [63]	Electrospinning	Bone marrow mesenchymal stem cells	Promotion of proliferation and osteogenic differentiation

Nanostructured materials	Fabrication techniques	Cell types	Cell behaviors
Nanofibrous PCL with BMP-2 nanoreservoirs [64]	Electrospinning and layer by layer deposition	Osteoblasts	Promotion of mineralization and proliferation
Nanofibrous PCL trilaminarcomposite scaffolds [62]	Electrospinning	Chondrocytes	Support chondrogenesis and higher mechanical properties
Aligned nanofibrous PCI [65]	Electrospinning	Chondrocytes	Higher resistance to damage
Bilayered nanofibrous PCL [66]	Electrospinning	Chondrocytes correct topology	Promotion of chondrogenesis
Nanofibrous Polyurethane (PU) and PU-HA composite [67]	Electrospinning	Osteoblasts, embryonic mesenchymal progenitor cells	Higher mechanical properties and improvement of bone matrix formation
Oriented and aligned nanofibers of biodegradable poly-Dl-lactide with embedded multi-walled carbon nanotubes [68]	Electrospinning	Osteoblasts	Improvement of osteoblast functions
Interspersed PLA and gelatin fibers [69]	Co-etectrospinning	Chondrocytes	Improvement of proliferation and differentiation

Biomechanics of Stem Cells and Nano Surface

Nano Topography Supports Stem Cell Differentiation

The quality of stem cells to have extensive expansion and differentiation capability has led to their use as a reliable source for tissue engineering. Nano-fibrous scaffolds used for stem cell culture have been found to support basic processing of stem cells like proliferation and multi-differentiation [73]. Since there are no ethical or legal issues involved with dental stem cells coupled with their ease of retrieval and availability from deciduous teeth as well as permanent third molar, they are emerging as one of the most suited sources of stem cells for tissue engineering.

Bone has a distinct structure and chemical composition that makes it suitable to support external loads and the capability to absorb shocks associated with external loading. The bone consists of two distinct layers of different density and biological quality—the outer layer composed of compact bone and the inner layer of cortical bone. The two different cell types in the bone render it the potential to constantly undergo a dynamic remodeling. The osteoblasts continuously keep on building new bone whereas osteoclasts keep on digesting the old bone. MSCs have the capability to differentiate into various connective tissue cells in order to replenish them. Bone tissue engineering employs the use of osteoblasts, osteoprogenitor cells and stem cells. The stem cells when seeded with electrospun nano-fibrous scaffolds promote and allow osteogenic differentiation in vitro as well as in vivo [74, 75]. The addition of calcium carbonate resulted in increased mineralization in the cellular assemblies, a prelude to bone formation.

The osteo-conductivity of calcium carbonate and composite scaffold along with osteogenic promotion of nano-fibers is successfully being used in guided bone regeneration therapy to repair alveolar bone defects.

Interactions Between Implant Surface and Mesenchymal Stem Cells

Immediately following implantation procedure, the MSCs are attracted to the implant surface and play a specific role in the healing at the implant-tissue interface. The complete healing process encompasses certain stages of tissue reaction and transformation as under:

1. Tissue Migration,
2. Tissue Adhesion,
3. Tissue Proliferation, and
4. Tissue Differentiation

An instant favourable interaction between the implant surface and the adjacent osseous and gingival tissues defines the successful osseointegration of dental implant. The implant surface should have the potential to guide cells colonization

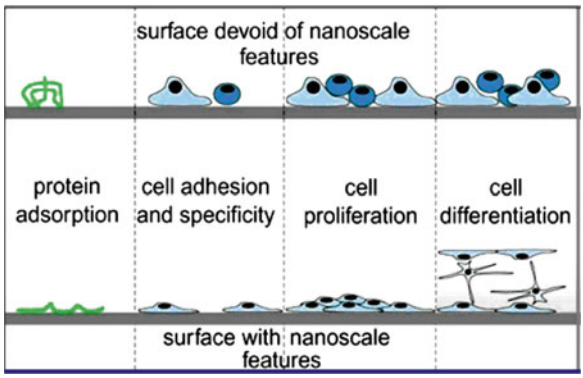

Fig. 4 Depiction of broad range of nano-scale topography effects observed in cellular protein adsorption in bulk materials. Both cell specificity and extent of cell adhesion are altered. Depending on the nano-architecture cell spreading may be increased or decreased [30]

and differentiation. Cell migration, adhesion, and proliferation on implant surfaces are a prerequisite to initiate the tissue regeneration (Fig. 4) [30].

Successful osteogenesis is directly proportional to factors like, growth of specific cells like progenitor cells and osteoblasts, development of a mineralized ECM, presence of soluble bioactive molecules like growth factors, ions, vitamins and hormones etc. and mechanical stimulus. These factors play a significant role right from initiation to the complete bone formation. All these factors contribute to differentiation of MSCs towards osteogenic lineage, a pre-requisite to oseointegration of dental implants.

Studies have revealed that some biological factors present in the tissues, released during the inflammatory phase have the capability to attract MSCs to the site of trauma [76, 77]. It is an established fact that the platelet factors initiate and stimulate the proliferation of Mesenchymal stem cells [78]. In addition, the plasma clot adjacent to the implant surface serves as a three-dimensional microporous structure, thus allowing the diffusion of regulatory factors [79, 80].

The migration and adhesion of the MSCs to the implant surface and the extracellular matrix follows a substantial proliferation so as to generate new tissues. Needless to say, nano-level topography of the implant surface is a strong determinant parameter to the biological response. The plasma clot at the site of injury acts as a storehouse of fibrin molecules that in turn release various bioactive factors including growth factors that initiate migration and subsequent differentiation of MSCs into specific lineages [81–83]. These factors stimulate MSCs to differentiate into osteoblastic cells adjacent to bone and into fibroblastic cells adjacent to the gingival tissues. The fibroblastic adhesion and subsequent differentiation in the gingival portion of the dental implant is highly desirable. Studies have shown this fibroblastic adhesion and proliferation to be considerably lower on the nano-scale surface as compared to the conventional surface [84]. The findings point out to the fact that nano-scale topography is neither required nor advisable at the neck of dental implants. The nano-surface of dental implants restricts/decreases fibrous tissue adhesion and proliferation at the implant body and favours adhesion and proliferation of osteoblastic cells, leading to success of the implant [85, 86]. In fact, it serves as a deterrent to fibrous tissue proliferation on the main implant body.

Interaction of Stem Cells to Nano Surface Dental Implants

The size, shape and surface texture offers three-dimensional surface topography to the dental implants and serves as one of the most significant parameters to influence cellular reactions at the implant-bone interface. Osteoblasts from the mesenchymal progenitor cells are the main architect of osseointegration of nano-structured implants with the adjacent bone leading to osteoid calcification and formation of hydroxyapatite. Osteoblasts further differentiate into osteocytes upon being encircled by mineralized bone. However, as on date the topography of the various implant surfaces has been categorized on the basis of their manufacturing procedure and not on the basis of the precise measurements of the topographic modifications [87, 88].

Cellular Attachment and Adhesion

The very first response of a biomaterial in the biological environment is the rapid adsorption of proteins to its surface [89]. The amount, type and composition of the adsorbed proteins guides and determines the subsequent process of cell adhesion, protein exchange [90–92], cell migration, proliferation and cell differentiation. The capability of a biomaterial to adsorb proteins is dependent upon its physiochemical qualities like surface energy or hydrophobicity and also on the surrounding environment like *p*H, concentration of ions, composition and functional groups of proteins, strength of solution, temperature etc. (*Vroman Effect*) [93].

The protein adsorption on a biomaterial surface takes place in two phases. In the first phase small rapidly diffusing proteins get attached to the implant surface. The second phase comprises of a progressive replacement of the smaller proteins by larger proteins having high affinity to the substrate. In the later phase, proteins containing sequences such as fibronectin or vitronectin having chemotactic or adhesive properties to bone cells act as cell receptors. These peptides also have strong impact on matrix maturation and bio-mineralization [94–96]. The conditioning of the naked biomaterial by protein adsorption promotes a rapid cell attachment on the protein coated surface of the implant material [97]. Studies have revealed that the contact angle (CA), a significant parameter for wettability of cpTi surfaces increases linearly with the average roughness having angles more than 45°, whereas angles less than 45° resulted into a decrease in linearly [98].

Nano-tubes and nano-particle surface of Ti created by anodization has been found to promote osteoblast adhesion up to 3 times as compared to non-anodized surface [99]. This enhancement in osteoblastic adhesion could possibly be explained due to increase in the surface area created by nano topography of the biomaterial. Enhanced protein and cell binding properties have also been found in porous HA materials due to a larger surface area and increased degree of roughness of the implant surface [100].

Helical Rosette Nano-tubes (HRN), capable of building self-assembly surface structures explains the significance of surface topography for protein binding and osteoblastic adhesion. Significant change of HRN coverage upon heating has been found to enhance protein binding and osteoblastic adhesion quality of Ti surface [101, 102]. The initially enhanced protein deposition and cell adhesion due to surface topography favourably modifies the surface properties of the implant surface leading to more advantageous interaction between the biomaterial and the tissues.

Cellular Migration and Proliferation

Structured nano surfaces exhibit a predictable osteoblastic orientation and migration that eventually leads to well defined cell colonization during directed tissue formation [103, 104]. Titanium Oxide with nano topography has been found to promote cellular spreading and to induce osteoblastic differentiation [105, 106]. The rate of cell migration also depends on the type of cells and the stage of its differentiation. A lower level of osteoblastic differentiation leads to a higher migration rate. The cells with low motility have been found to have the potential to form stronger focal contacts (FC). Cellular adhesion through FC may strengthen the linkage between the cell and extracellular matrix (ECM) and may also influence the rate of migration [107].

Cellular Differentiation, Gene Expression and Protein Synthesis

Different cell phenotypes exhibit different levels of sensitivities upon adhesion to nano-topography biomaterials [108, 109]. Osteoblasts have been found to be present in the surfaces as low as up to 10 nm dimensions, the size of a single collagen fibre [110]. The topography and the physio-chemical surface of the biomaterials significantly influence the qualitative and quantitative kinetics in gene and protein expression [111].

It has also been observed that micro-level Discontinuous Edge Surface (DES), having square boxes with a depth of 10 μm led to alteration of osteoblast adhesion and migration but enhanced cell multi-layering, matrix deposition and mineralization in comparison to the smooth surface control groups [112].

In vitro experiments have shown that nano-surface biomaterials were able to substantially enhance not only calcium and phosphorus deposition by osteoblasts but also encouraged calcium and phosphorus precipitation from culture media without osteoblasts in contrast to micro-surface Ti6Al4V and CoCrMo [113].

A marked difference in proliferation of MSCs on various sizes of TiO_2 nanotubes has been demonstrated. The MSCs appeared to be more round, stationary and devoid of any noticeable filopodia extensions and cellular propagation. Whereas, on nano-tube surfaces of larger diameters a large number of prominent filopodia and

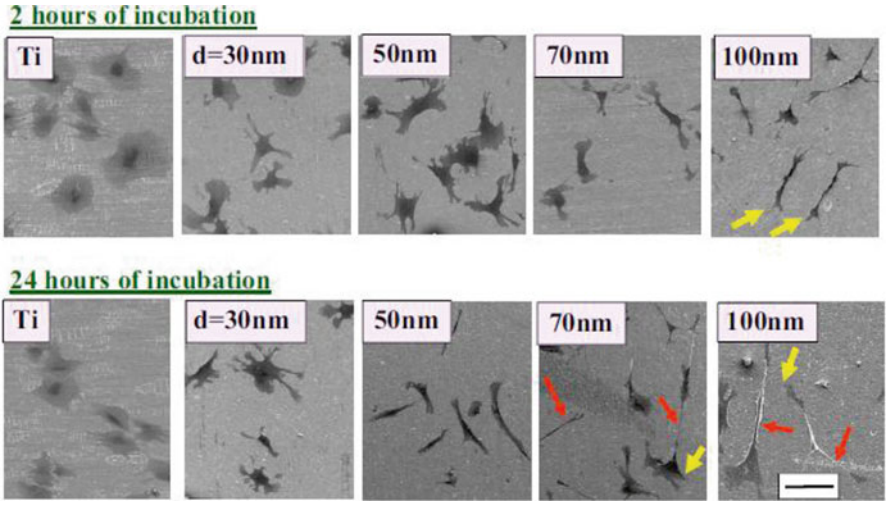

Fig. 5 SEM micrographs of human mesenchymal stem cells (hMSCs) on flat Ti and 30-, 50, 70-, and 100 nm diameter TiO_2 nanotube surfaces after 2 h of culture. (Scale bar, 100 m.) Cells are flat, spread out, and round-shaped on the flat Ti substrate, they are somewhat flat and rounded on 30-nm nanotubes, and they become progressively elongated as the nanotube diameter is increased to 50-nm diameter and beyond. Extraordinary cell elongation is induced on nanotubes with diameters of 70 and 100 nm (see *red arrows*), especially after the 24 h culture. More mobile morphologies are indicated by the presence of somewhat elongated leading edges of lamellipodia (*yellow arrows*) seen on 70 and 100 nm nanotubular surfaces. The cell shapes suggest that cells are more elongated on the bigger TiO_2 nanotubes [114]

unidirectional lamellipodia extensions were evident even after 2 h culture (Fig. 5, Upper). The proliferation became more visible after 24 h of culture and there was significantly pronounced when nano-tube diameter was increased (Fig. 5, Lower) [114].

Higher magnification SEM images of TiO_2 nano-tube surface after 2 h of culture revealed the adhesion of many round protein aggregates modifying the nano-surface with a protein coating for further adhesion and proliferation of MSCs. Comparatively much less protein aggregates with sparse distribution was observed on flat Ti samples (Fig. 6) [114].

Conclusion

Nano-surface modifications in biomaterials, tissue engineering and effective use of stem cells still requires further research and analysis in order to achieve the goal of successful osseointegration of dental implants. Further studies on stem cell behavior and interactions between ECM and various nano-surface scaffolds may pave the way for functionally advantageous tissue engineering and a more effective use of

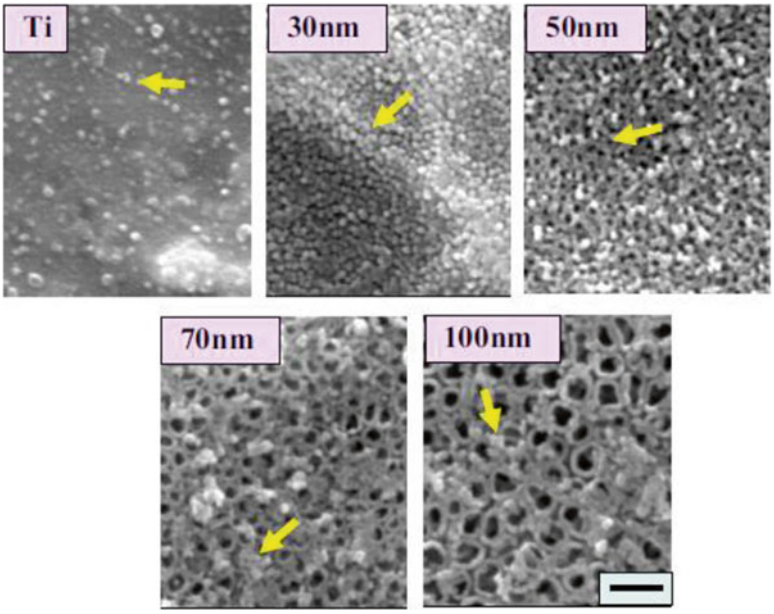

Fig. 6 SEM micrographs showing extracellular matrix aggregates on the surfaces of flat Ti and 30-, 50-, 70-, and 100-nm diameter TiO_2 nanotubes after 2 h of hMSC culture. (Scale bar, 200 nm.) Note that the presence of protein aggregates is infrequent on Ti, abundant on 300-nm nanotubes, and much less on the larger-diameter 70- and 100-nm nanotubes [114]

the bio-technology in dental implantology. Current information pointing to a favourable tissue growth of stem cells on nano-surface of various bio-materials may serve as a platform to discover a more conclusive physical, chemical and biological technology in the future for more assuring and lasting implants in the oral cavity. The outcome in the coming years may completely revolutionize the implant selection, implant procedure, their success and longevity of the oral implants.

Application of nano-topography in biomaterials and use of stem cells in bioengineering is passing though the phase of infancy and much needs to be explored and achieved in order to take full advantage of the technology.

A positive bone response on nano-surface biomaterials is already an established fact. Up to what extent the stem cell tissue engineering and nano-topography of biomaterials can be used to improve tissue and abutment interface bonding remain to be seen. Commercially available current implants using nano-topography may not have acquired the level of microstructure desired for the ultimate goal of perfection in the area. Further research and study in the field may open up new horizons of a more favorable use of stem cells and titanium with nano-surface in implant dentistry. The development may well make dental implants last for life in the oral cavity like natural teeth with practically no failures.

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Dental Pulp Stem Cells and Hydrogel in Pulp Regeneration

Waruna Lakmal Dissanayaka and Chengfei Zhang

Dental Pulp Stem Cells

The major functional cell population residing in the dental pulp is called odontoblasts, a subpopulation of mesenchymal cells which differentiated due to epithelial-dental papilla interactions during tooth morphogenesis. These cells deposit distinct mineralized matrix, dentin, during development of the tooth. Although, once formed, dental hard tissue do not undergo remodelling, it was shown that even after tooth eruption reactionary dentin formation continues in response to dentin destruction due to caries, erosion or mild chronic trauma [1]. Several studies have suggested that a precursor population that can proliferate and differentiate into odontoblasts reside somewhere in the adult dental pulp [2]. However, until recently only a little was known about the characteristics of such precursor cells.

The isolation and characterization of an odontogenic progenitor population named dental pulp stem cells (DPSCs) from adult dental pulp tissue was first reported by Gronthos et al. in 2000 [3]. In this study, a clonogenic and highly proliferative cell population was isolated and showed the capability of regenerating a dentin/pulp-like structure after xenogenic transplantation.

DPSCs can easily be isolated from discarded permanent teeth (3rd molars, supernumerary teeth, orthodontically unwanted teeth) with no serious ethical issues. This technique is minimally invasive and safe compared to that of other mesenchymal stem cell sources; and can be used to obtain large pools of autologous cells. For these reasons, DPSCs is considered as a promising population of stem cells in regenerative medicine. DPSCs share many similar characteristics with bone marrow mesenchymal stem cells (BMMSCs) including a similar gene expression profile [4]. Interestingly, DPSCs show a higher proliferation rate compared to that of BMMSCs [3].

W.L. Dissanayaka (✉) • C. Zhang
Comprehensive Dental Care, Faculty of Dentistry, The University of Hong Kong, Hong Kong, Hong Kong
e-mail: warunad@hku.hk

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There are mainly two methods to isolate DPSCs from the dental pulp; namely, enzymatic digestion method and the explant outgrowth method. It was shown that both methods give heterogenous populations of mesenchymal stem cells composed of more than one stem cell population [5]. Studies which aimed to characterize the differentiation capacity of DPSCs found that single colony derived DPSC strains vary from each other with regards to their odontogenic differentiation capacity [6]. These results further suggested that dental pulp contain a hierarchy of stem cells including a minor population of highly-proliferative, self-renewing, multipotent stem cells, among more committed population of progenitors.

Therefore, to select more defined clonal subset of stem/progenitor cells, several studies have used different surface markers for immunoselection by flow cytometry or magnetic-activated cell sorting. The markers used are STRO-1 [7–10], c-kit/CD117 [7, 11], CD34 [12], low-affinity nerve growth factor receptor (LANGFR/p75/CD271) [13], CD105/endoglin [14, 15], stage specific embryonic antigen 4 (SSEA-4) [16], and chemokine receptor type 4 (CXCR4) [17]. Alternatively, isolation and enrichment of a side population fraction based on the efflux of DNA binding fluorescent dye Hoechst 33342 has been used to isolate more potent stem cells [18–21]. However, one major challenge in utilizing DPSCs in clinical setting is to generate clinical grade human DPSCs. Immunoselection is not considered as a safe procedure to isolate cells for clinical application. Therefore, heterogenous DPSC populations still play a major role as the likely cell population which can be used clinically. Nevertheless, standardization of the isolation procedure is important to optimize the reproducibility and safety of clinical grade DPSC population in order to be used in regenerative endodontics.

Since DPSCs were isolated and reported, many studies have emerged to describe its multipotentiality and possible use under different conditions. These studies showed that DPSCs not only can differentiate into odontogenic lineage but also to osteogenic [11, 12], chondrogenic [22], adipogenic [6, 18, 22], endothelial [23], myogenic [24–26] and neurogenic lineages [27]. Many other studies subsequently showed that DPSCs share similar characteristics with mesenchymal stem cell populations of different origins, such as bone marrow and adipose tissue.

Over the past few years, there has been a significant revolution in the understanding of the functionality of mesenchymal stem cells (MSCs) as a source of cell replacement. More recently MSCs have been utilized for mediating tissue regeneration by release of "paracrine" factors and this in turn may forward investigations into more productive directions. Emerging data suggest that paracrine factors secreted by MSCs exert their effects to induce tissue recovery on many different ways, including activation of endogenous progenitor/stem cells, inhibition of apoptosis of susceptible cells, remodelling of extracellular matrix, and induction of neo-vasculogenesis.

Scaffolds

Engineering of three dimensional tissues requires cells to seed into an artificial structure capable of supporting their survival, proliferation and differentiation. These structures, generally known as scaffolds, should be able to recapitulate the

in vivo milieu of the differentiating cells and should allow cells to manipulate their own microenvironments. Scaffolds often serve as carriers, to which the cells and/or growth factors can attach and migrate. They also act as vessels that enable diffusion of nutrients and metabolic products. Further, scaffolds provide mechanical and biological support to modulate the performance of the cells.

A scaffold of interest must meet certain requirements in order to accomplish the goal of tissue regeneration. Biodegradability is one of the crucial factors since scaffolds should be degraded allowing the regenerating tissue to unite with the in situ environment. The degradation rate has to be well-matched with the rate of tissue development, while the scaffold providing structural integrity until the cells/tissue fabricate their own natural matrix which can take over the mechanical load. Biocompatibility is also of paramount importance to avoid any unwanted reactions. To facilitate the cell seeding, attachment and diffusion of cells and nutrients, optimum porosity and an adequate pore size of the scaffold components are necessary. In addition to provision of structural support, ECM is also responsible for mediating cell-to-cell crosstalk via integration of cell signalling. This modulates the cellular behaviour, phenotype and function. Being able to injectable into an in vivo tissue is an added advantage, specifically when it comes to clinical use.

Scaffolds in Dental Pulp Tissue Engineering

Among the various types of scaffold materials that have been examined in pulp regeneration studies both in vitro and in vivo, natural biomaterials such as reconstituted collagen [14] and synthetic biomaterials such as poly(lactic) acid (PLA) [28] and poly(glycotic) acid (PGA) [29] are the most commonly investigated so far. However, neither of these materials tested are proven to have all the properties, and the structure of an ideal material, which should closely resemble the natural extracellular matrix (ECM) of the pulp [30]. Recently, hydrogels have been introduced to the field of pulp regeneration due to their favourable biological and mechanical properties.

Hydrogels

Hydrogels are known as water insoluble, crosslinked, three-dimensional networks of polymer chains plus water that fills the voids between polymer chains [31]. Hydrogels consist mostly of water with a much greater mass fraction than that of the polymer. Crosslinking enables insolubility in water and therefore, provides necessary mechanical strength and physical integrity. Hydrogels are mainly classified based on the method of cross-linking and the origin of the gel precursor molecules. Naturally occurring materials such as collagen, fibrin and matrigel are commonly used for their ability to provide crucial signals that are present within the natural

cellular niche. In contrast, synthetic gels are not capable of providing specific cues for cellular functions, but they are readily optimized to allow a permissive niche for essential cell functions to occur. Polyethylene glycol (PEG), polyhydroxyethyl methacrylate (PHEMA), polyvinyl alcohol (PVA), and polyacrylic acid (PAA) are few macromolecules that are used in hydrogel synthesis.

DPSCs and Hydrogels in Dental Pulp Regeneration

Dental pulp is a loose, highly vascularized, soft connective tissue occupying the pulp chamber and root canals of the tooth. Histology of the dental pulp reveals that although it is composed of relatively less number of specific cell types, structural organization of these elements is quite distinct. Furthermore, due to the unique structure of the tooth with a pulp space encased within hard dentin, it gets a single blood supply only from a small apical opening located at the apex of the root canals. Additionally, varying size and contour of the root canals play a critical role and should get into serious consideration in the context of pulp regeneration.

Development and application of injectable hydrogel systems for pulp tissue engineering have been considered a promising approach in recent years, as they can be easily incorporated with growth factors and cells by simple mixing and can be adapted to the varying contour of the pulp chamber and root canals, following injection [30]. As the cross-linking of hydrogel precursors occur in situ after being injected into the target site, it can be injected to any irregular-shaped defect eliminating the necessity for custom-made scaffold designs.

Table 1 gives a summary of different types of hydrogels that have been utilized to encapsulate DPSCs for the purpose of investigating their potential in differentiating towards odontogenic lineage in vitro and engineering dental pulp tissue in vivo. Peptide-amphiphile, self-assembling peptide nanofibers/PuraMatrix, PEGylated fibrin/fibrinogen, collagen, and glycol-chitin are the predominant types of hydrogels that are used to encapsulate DPSCs in pulp regeneration studies. In these approaches, all the aspects of classical tissue engineering triad i.e. stem/progenitor cells, scaffold and growth factors, have been manipulated and tuned to augment the conditions for pulp regeneration.

In cell based regenerative approach, in order for pulp regeneration to occur, the stem/progenitor cells must proliferate and produce new matrix. In addition, there must be stimulation of the odontoblasts to proliferate and produce new dentin. Over the past few years there have been a significant advancement in dentin-pulp regeneration technology by stem cell based approach. Several studies, using small (mouse/rat) animal models, have shown that DPSC encapsulated hydrogel constructs can result in pulp/dentin tissue regeneration partially or completely in the root canals with enlarged apical openings of 0.7–3.0 mm.

Irrespective of the type of target tissue/organ, following implantation, an engineered three-dimensional tissue construct requires to develop rapid vasculature in order to meet the oxygen demand. However, immediately after implantation in vivo,

Table 1 Hydrogel types predominantly used as scaffolds in dental pulp regeneration

Type of hydrogel	Promising features	Experimental approach	Result	References
Peptide-Amphiphile (PA)	• Self-assemble into 3D networks • Easy to handle • Can be introduced into small defects	• Cell adhesion sequence RGD and an enzyme-cleavable site had been incorporated to tailor the cell–matrix interactions • DPSCs had been cultured in PA hydrogels for 4 weeks using osteogenic supplements	• DPSCs differentiated to osteoblast-like phenotype • Expressed osteoblast marker genes, and deposited mineral	Galler et al. [38]
PEGylated fibrin	• Injectable • PEGylation decelerates rapid degradation of fibrin	• Constructs of DPSC encapsulated PEGylated fibrin were transplanted in immunocompromised mice for 5 weeks and examined for new tissue formation	• Histologic analysis revealed vascularized soft connective tissue similar to dental pulp with degradation of fibrin and production of a collagenous matrix and mineral deposition	Galler et al. [39]
Self-assembling peptide nanofibers	• Customised for stem cell delivery • Growth factors can be incorporated • Ease of synthesis • Injectable • Biocompatible • Biodegradable	• DPSCs had been encapsulated together with fibroblast growth factor basic, transforming growth factor β1, and vascular endothelial growth factor via heparin binding • The hydrogel within dentin cylinders had been transplanted into immunocompromised mice	• A vascularized soft connective tissue similar to dental pulp was formed	Galler et al. [40] Galler et al. [30]

(continued)

Table 1 (continued)

Type of hydrogel	Promising features	Experimental approach	Result	References
Alginate	• Nontoxic • Biocompatible • Permeable to small molecular-weight proteins	• Exogenous TGFβ1 loaded alginate hydrogel constructs on human dental pulp repair in vitro	• Dentin matrix secretion • Odontoblast-like cell differentiation • Secretion of tubular dentin matrix	Dobie et al. [35]
		• DPSCs and HUVECs encapsulated within RGD-bearing alginate framework in 1:1 ratio was supplemented with VEGF and FGF • The contour of the construct was made to replicate the shape of gutta-percha points	• Both DPSCs and HUVECs showed high cell viability within alginate • The combination of vascular endothelial growth factor and fibroblast growth factor synergize to significantly up-regulate cell proliferation	Bhoj et al. [36]
Glycol Chitin-based Thermoresponsive (GC-TR) Hydrogel	• Biocompatible • Injectable • Conform more easily to the varying contours • Easy to manipulate as it maintains a sol phase unless the temperature reaches the body temperature	• Effects of GC-TRH on DPSC viability, expression of odontogenic/osteogenic differentiation markers were analysed	• Dentin sialophosphoprotein and dentin matrix protein-1 were expressed by cells cultured in GC-TRS at a higher level than that in cells cultured in collagen	Park et al. [41]
Collagen type I	• Biocompatible	• Radiolabelled rat pulp cells were added to polymerizing type I collagen hydrogel and was implanted in the emptied pulp chamber space in the upper first rat molar • Labelled cells were then tracked for a period of 3 weeks	• Histological analysis showed mitotically active fibroblastic cells as well as neo-angiogenesis and nervous fibres in pulp equivalents seeded with entire cells	Souron et al. [42]

PEG-fibrinogen (PF)	• Injectable • Mechanical properties can be tuned by the degree of cross-linking • The precursor solution can be injected into the dental pulp chamber before rapid gelation by photo-polymerization	• DPSCs were cultured within PF hydrogels with varying degree of cross-linking • Cell morphology, viability, odontogenic gene expression and mineralization were examined	• DPSCs cultured within the highest cross-linked hydrogel remained mostly rounded in aggregates and demonstrated the greatest enhancement in odontogenic gene expression and mineralization	Lu et al. [43]
Peptide Hydrogel (PuraMatrix)	• Biocompatible • Injectable	• DPSCs suspended in PuraMatrix was injected into the human tooth slices to verify DPSC differentiation, as measured by expression of DSPP and DMP-1	• After 21 days in tooth slices containing Puramatrix, DPSC cells expressed DMP-1 and DSPP and exhibited cytoplasmic elongations	Cavalcanti et al. [44]
		• DPSCs and HUVECs co-encapsulated in PuraMatrix was injected into the roots of human premolars and transplanted subcutaneously in SCID mice	• Formation of pulp-like tissue with higher vasculature and mineralizing capacity in prevascularized group compared with that of DPSC-alone group	Dissanayaka et al. [37]

tissue constructs depend solely on the oxygen supply diffuse from the nearest capillary, which could be only up to 200 μm away [32, 33]. If the distance form a capillary exceeds 200 μm, the majority of the cells undergo apoptosis [34]. Therefore, the survival of implanted tissue constructs of greater size requires the formation of a capillary network of its own which can deliver necessary nutrients for the cells. Although host blood vessels start to invade the implanted tissue construct, partly in response to the angiogenic factors secreted by the cells undergoing hypoxia, this process happens very slowly, growing only few tenths of micrometers per day [32]. Use of angiogenic growth factor incorporated hydrogel scaffolds [30, 35, 36] and co-culture of DPSCs with endothelial cells within a hydrogel scaffold [37] are two main approaches that have been investigated to promote rapid vascularization following implantation of a tissue/cellular construct.

In a similar study, we used the peptide hydrogel PuraMatrix as a scaffold system to investigate the role of DPSCs in prompting angiogenesis and the potential for regenerating vascularized pulp in vivo [37]. Human umbilical vein endothelial cells (HUVECs), DPSCs, or co-cultures of both cell types were encapsulated in three-dimensional PuraMatrix. The peptide nanofiber microenvironment supported cell survival, cell migration, and capillary network formation in the absence of exogenous growth factors (Fig. 1). Further, we demonstrated that DPSCs enhanced early vascular network formation by facilitating the migration of HUVECs and by increasing vascular endothelial growth factor (VEGF) expression. Both the DPSC-monoculture and co-culture transplanted groups exhibited vascularized pulp-like tissue with patches of osteodentin after transplantation in immunocompromised mice (Fig. 2). Interestingly, the co-cultured groups showed pulp-like tissues with more extracellular matrix, vascularization, and mineralization than that of the DPSC-monocultures in vivo. Findings of this study highlighted the crucial role of DPSCs in initial angiogenesis. Furthermore, this study convincingly showed the PuraMatrix hydrogel as a promising scaffold with a microenvironment to support cell–cell interactions and cell migration, which contribute to successful dental pulp regeneration.

Summary

DPSCs are considered as a promising population of stem/progenitor cells in regenerative medicine for their ready availability, ease of harvesting and stemness properties. Furthermore, DPSCs is the predominant type of stem cells that holds the potential to be used in dental pulp regeneration. Encapsulation of DPSCs within an injectable hydrogel scaffold provides a favourable approach for pulp regeneration as it can be injected to any irregular-shaped defect eliminating the necessity for custom-made scaffold designs. However, regeneration of a pulp-dentin complex that resembles the natural structural organization of cellular elements is yet to be achieved. With regards to this aspect, it is important to develop multiphase hydrogel systems that integrate different cell signalling and differentiation pathways.

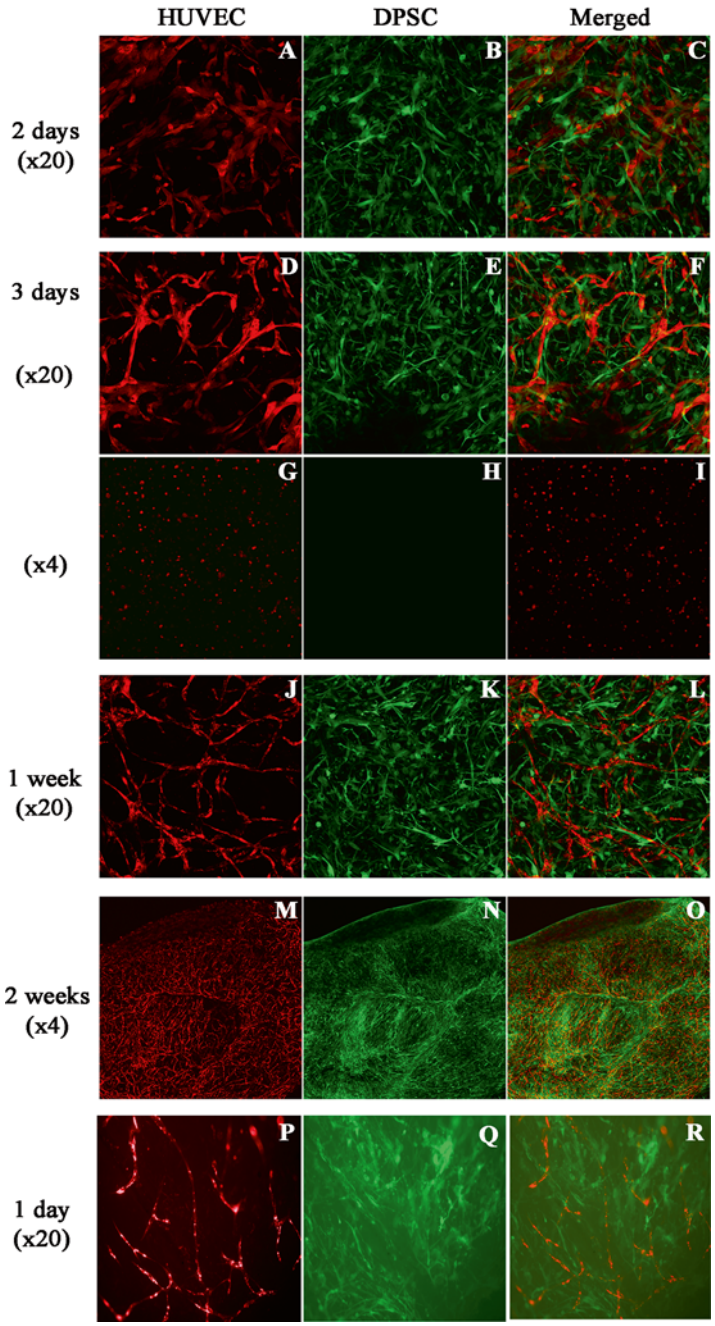

Fig. 1 Vessel-like structure formation by HUVECs that were cocultured as PuraMatrix constructs (Green: green fluorescent protein-expressing cells, Red: red fluorescent protein-expressing cells). (**a–c**) Coordinated cell migration was observed in the cocultures 48 h after seeding. (**d–f**) Vessel-like structure formation by HUVECs was observed in the cocultures 3 days after seeding, with DPSCs surrounding the nascent HUVEC networks. (**g–i**) HUVEC monocultures did not exhibit cell migration or vessel structure formation. (**j–o**) Vessel-like networks continued to be remodelled and to stabilize for 2 weeks. (**p–r**) Vascular structure formation was observed after 24 h when cocultured PuraMatrix was injected into root canals of root segments [37]

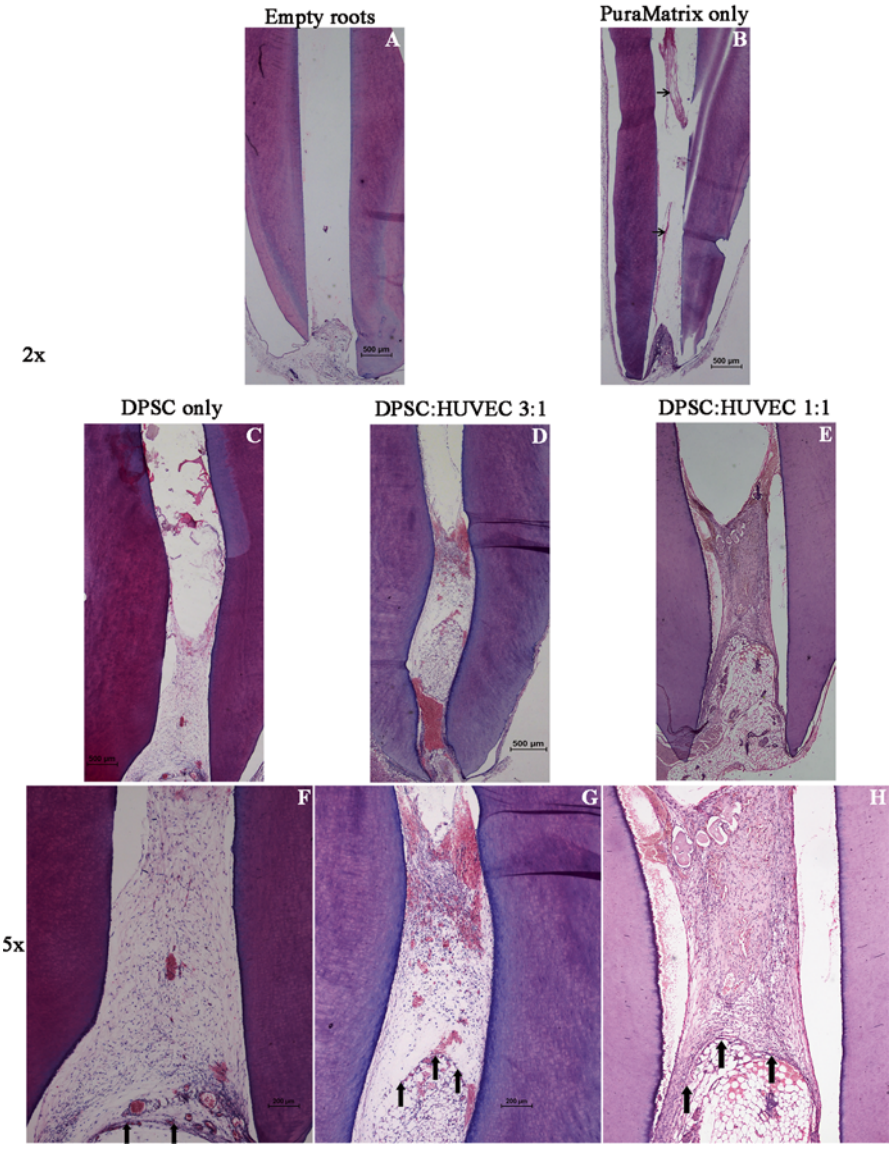

Fig. 2 Pulp regeneration in cell/PuraMatrix constructs in vivo 4 weeks after transplantation. (**a**) Empty roots, (**b**) PuraMatrix-alone in root fragments (*black arrows* indicate the remnants of PuraMatrix) (**c**, **f**) DPSC-alone in PuraMatrix, (**d**, **g**) DPSC: HUVEC 3:1 in PuraMatrix, (**e**, **h**) DPSC: HUVEC 1:1 in PuraMatrix. *Vertical black arrows* indicate the border between the transplanted tissue and host tissue [37]

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Dental Stem Cells for Pulp Regeneration

Nileshkumar Dubey, Kyung-san Min, and Vinicius Rosa

Introduction

Dental pulp is a highly vascularized connective tissue encapsulated in mineralized structure formed by enamel, dentin and cementum. It is responsible to keep the homeostasis of the tooth organ and possesses highly responsive sensory nervous system that detects unhealthy stimuli [1]. The apical foramen is the opening at the apex of the root that provides the dental pulp with blood supply for oxygen, nutrients and cells for defence. This limited accessibility and inflexible surroundings makes it difficult for the immune system to eradicate infections in the tissue owing to the lack of a collateral blood supply [2].

Clinically, odontoblasts may survive and continue to produce dentin to protect the pulp in cases of injuries by mild caries, moderate attrition or erosion [3]. On the other hand, dental pulp exposure due to fracture, caries in deep dentin or where the progression of the lesion is aggressive, the survival of primary odontoblasts is frequently compromised demanding root canal treatment. This therapy often involves total pulp extirpation and disinfection followed by compaction of a material within the root canal [4]. Though this is a well-established approach, it is based on the substitution of host tissues for synthetic, and in most of the cases, inert materials [5]. In immature teeth, the routine root canal treatment can allow for infection control, but often does not facilitate the completion of root formation or protects against external root resorption [6]. In these teeth, the root canal treatment is even more

N. Dubey • V. Rosa (✉)
Discipline of Oral Sciences, Faculty of Dentistry, National University of Singapore, Singapore, Singapore
e-mail: denvr@nus.edu.sg

K.-s. Min
Department of Conservative Dentistry, School of Dentistry, Chonbuk National University, Jeonju, South Korea

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challenging due to large apical opening which can have a divergent configuration and does not provide the mechanical stop necessary to confine the filling material within the root canal [6].

Although the success rate of endodontic treatments is relatively high [7], this modality of treatment present some shortcomings such as the high sensitivity to obstruction of the root canal space, breakage of instruments in the canals and perforations [8, 9]. The root canal instrumentation removes significant amount of tooth structure that results in thin dentin walls and large pulp chamber making the remaining structure weaker. Moreover, the devitalization may increase root brittleness increasing the chances of postoperative fracture [10, 11]. In addition, pulpless teeth do not have the ability to sense the aggressions arising from caries. In fact, endodontically treated teeth loss is higher than non-treated ones due to secondary caries [12].

The shift towards the regenerative endodontics may have started with the advent of vital pulp therapy. Clinically, it is based on the indirect pulp treatment in cases of deep dentinal cavities and direct pulp capping where the pulp tissue is exposed [13]. Indirect pulp capping is the procedure wherein the deepest layer of affected dentin is covered with a thin layer of protective agent in order to prevent pulp exposure [13]. In the direct pulp capping, the exposed vital pulp is covered with a protective dressing (such as calcium hydroxide and mineral trioxide aggregate) directly applied over the exposure site to prevent further injury and to stimulate dentin-pulp complex regeneration [14, 15].

One of the methods to treat the pulp of immature teeth is the apexification. This relies on the formation of an apical barrier to close the open apex [4]. For this, calcium hydroxide ($Ca(OH)_2$) must be inserted in the root canal and replaced periodically to stimulate the formation of a calcified structure. Although this strategy has been used clinically for long time, the outcomes are largely unpredictable and fracture rates of nearly 25 % have been reported after long-term $Ca(OH)_2$ treatment due to changes in mechanical properties of dentin [14]. Alternatively, apexification strategies include use of mineral trioxide aggregate (MTA) that is a material with osteogenic, cementogenic and odontogenic potentials that can be used for perforation repairs, pulp capping and pulpotomy [4]. Unfortunately, apexification by either $Ca(OH)_2$ or MTA may prevent further development of the root [14, 15]. This is specially complex in immature tooth where the apexification does not stimulate the reestablishment of a functional pulp tissue or the continued root development [16].

In existing endodontic treatment, teeth lose substantial amount of sound structure and their ability to detect the secondary infections. There has been no superior synthetic material to replace natural pulp and dentin in terms of chemical composition, physical, mechanical and biological properties. Thus, current research in endodontics has extended its focus on the development of biological therapies that are more biologically effective and mechanically reliable than traditional pulp therapies. Hence, the regeneration of pulp-dentin complex via tissue engineering has the potential to restore tooth functions compromised by the loss of pulp vitality.

Dental Pulp Tissue Engineering

Dental Stem Cells

Stem cells are undifferentiated cells the can differentiate into specialized cells. These cells are responsible for normal tissue renewal, healing and regeneration after injuries [5, 17]. Multipotent stem cells are able to cross lineage boundaries and differentiate into multiple, but limited number of cell types. For instance, a multipotent blood stem cell is a hematopoietic cell that can differentiate into several types of blood cell types (such as neutrophils and lymphocytes) but cannot differentiate into brain cells, bone cells or other non-blood cell types [17].

The oral environment harbours several types of stem cells [5, 17, 18]. The stem cells from oral sources can be considered a population of mesenchymal stem cells (MSCs) as they present positive expression of STRO-1, Nanog, Oct4, CD73, CD90, CD105, CD106, CD146 and others [19–25]. These cells are capable to differentiate into many types of specialized cells such as adipocytes, neurons, osteoblasts and myocytes. For dental pulp regeneration, the potentially suitable cell are those directly derived from pulp tissue or from the precursor of dental pulp [17, 18, 20, 26, 27].

The apical papilla refers to the soft tissue at the apices of developing permanent teeth that is source of undifferentiated cells in the process of root development [24]. This population of stem cells from the apical papilla (SCAP) can be only obtained in a limited window of time as after the formation of the crown the dental papilla becomes the dental pulp [24, 28]. The apical portion of the dental papilla is loosely attached to the apex of the developing root and it is separated from the differentiated pulp tissue by a cell rich zone [29]. As the apical papilla is poorly vascularized, the perivascular niche does not appear to be a major source of SCAP [24, 28].

It is possible that SCAP are derived from neural crest cells or at least associated with them [24]. They present positive expression of CD73, CD90, CD105, CD146 and other MSCs markers [24, 28, 30]. SCAP are capable to express positive staining for neural markers such as neurofilament M, neuronal nuclear antigen, nestin and βIII tubulin after being cultured with neurogenic medium for 4 weeks [24]. These cells can also accumulate calcium when cultured under osteogenic medium. Moreover, subpopulations of SCAP with positive expression of STRO-1–positive co-expressed several dentinogenic markers such as bone sialoprotein (BSP), osteocalcin (OCN) and matrix extracellular phosphoglycoprotein (MEPE) after stimulation with odontogenic conditioning medium [24].

Dental pulp stem cells (DPSC) were firstly derived by enzymatic digestion of dental pulp from adult human impacted third molars in 2000 [31]. These highly proliferative cells have their niche localized in the perivascular and perineural sheath areas and are negative for the odontoblast-specific marker dentin sialophosphoprotein, suggesting an undifferentiated phenotype [23, 31, 32]. DPSC have gene expression profiles similar to bone marrow stem cells (BMSC) for more than 4000 genes [33]. In fact, both BMSC and DPSC present similar expression for several

markers such as fibroblast growth factor 2, alkaline phosphatase, type I collagen, osteocalcin, α-smooth muscle actin, vascular cell adhesion molecule 1 and others. Nonetheless, some markers such as bone matrix protein, bone sialoprotein is positive at low levels in BMSC and negative in DPSC [31]. DPSC also express neuronal and glial cell markers, which may be related to their neural crest-cell origin [34].

DPSC can differentiate into multiple cell lineages (e.g. adipocytes, chrondocytes, β cells of islet of pancreas) making these cells a versatile and interesting model for tissue engineering and regeneration [31, 35–38]. DPSC also have the potential to induce angiogenesis/vasculogenesis as a subfraction of a side population was identified to be positive for CD31, CD146, CD34 and vascular endothelial growth factor-2 (VEGF-2). The transplantation of these cells into mouse ischemia resulted in high density of capillary formation and increase in the blood flow into the graft [39]. DPSC express various neural markers making them an interesting model for neural differentiation [38]. The differentiation of DPSC into glial cells and neurons was also confirmed by the upregulation of neural gene markers (GFAP and βIII-tubulin) in the presence of FGF and epidermal growth factor [40]. DPSC presented neuron-like morphological changes and increased gene (Nestin and Tub3) and protein (MAP2) expression after being cultured for 5 days in serum- and growth factor-free medium [41]. After in vivo transplantation into the mesencephalon of chicken embryo, DPSC not only acquired a neuronal morphology but also express neuronal markers [42].

Although both DPSC and SCAP are located in anatomically adjacent sites, they are originated in distinct developmental stages and present differences regarding their growth potential and biochemical characteristics [24, 29, 31]. Morphologically, SCAP are smaller in size, fibroblast-like or stellate in shape, with numerous cytoplasmic processes [43]. They also present higher number of STRO-1–positive cells when compared with DPSC. Compared to DPSC, SCAP present higher expression of the anti-apoptotic protein surviving and human telomerase reverse transcriptase (hTERT) activity, a catalytic subunit of the enzyme telomerase that maintains the telomere length [30]. Considering the proliferation rate, SCAP cultures can present more than double the number of cells compared to DPSCs after an observation period of 4 days [43]. In spite of dentinogenic markers expressed by SCAP after ex vivo expansion, DPSC present higher expression levels of dentin sialoprotein (DSP), matrix extracellular phosphoglycoprotein (MEPE), fibroblast growth factor receptor 3 (FGFR3), transforming growth factor beta receptor II (TGF beta RII) and others [24]. Although both DPSC and SCAP present remarkable characteristics making them attractive models for dental pulp regeneration, the first present a wider window of opportunity to be isolated from fully developed teeth while SCAP can only be retrieved from teeth under development [5, 24, 31, 43].

Stem cells from human exfoliated deciduous teeth (SHED) were firstly isolated in 2003 from normal exfoliated human deciduous incisors and present positive expression of MSCs markers (STRO-1 and CD146), embryonic stem cells markers (Oct4, Nanog), stage-specific embryonic antigens (SSEA-3, SSEA-4) and tumour recognition antigens (TRA-1-60 and TRA-1-81) [22, 44]. The ability that these cells have to cross lineage boundaries expands the potential use of SHED for therapies

involving a large number of tissues. SHED can differentiate into adipogenic, chondrogenic, neurons and endothelial cells [6, 22, 27]. SHED can also differentiate into osteoblasts and are positive for TGF-β, FGF and VEGF receptors [27, 45].

SHED present a remarkable advantage over DPSC as they can be retrieved from naturally exfoliated teeth, which are one of the only disposable post-natal human tissues [6, 22, 26, 46]. Although both DPSC and SHED cells are originated from the dental pulp, deciduous teeth are different from permanent teeth with regards to their developmental processes, tissue structure and function. Therefore, it is not a surprise to find out that SHED are distinct from DPSC with respect to their higher proliferation rate, increased cell-population doublings, sphere-like cell-cluster formation and osteoinductive capacity in vivo. SHED apparently represent a population of multipotent stem cells that are perhaps more immature than DPSC [18, 19, 22, 26, 27, 31, 33, 36]. For example, during osteogenic differentiation, SHED present higher levels of alkaline phosphatase activity and osteocalcin production than DPSC [36]. However, the ability to regenerate a dentin/pulp-like complex found in DPSC is also observed in SHED [19, 22, 27, 31].

Dental Stem Cells-Based Pulp Tissue Engineering

The use of synthetic restorative materials as substitutes for dental structures is a practice nearly as old as dentistry itself. To date, most of the procedures performed in dentistry are limited to the replacement of damaged tissues for biocompatible materials that may not present chemical, biological or physico-mechanical characteristics similar to the host tissues. These discrepancies, together with the hostile environment of the oral cavity, can result in frequent need for re-treatment [5]. Particularly in endodontics, despite of the development and introduction of new techniques, instruments and medicaments for the clinical management of the dental pulp, the fundamental principles of clinical practices are not drastically different than those of the time when first root canal instruments and gutta-percha were introduced in the 1800s [26]. Most of the endodontic treatments rely on the disinfection of the root canal followed by a hermetic three-dimensional obturation. Though this is a well-established approach it is based on the substitution of a living tissue for synthetic and, in most of the cases, inert materials.

In regards to biological-based therapies for dental pulp there cell-free and cell-based approaches. The first relies on the chemo attraction of host cells into the root canal [47, 48]. The second is based on the transplantation of responsive stem cells using a suitable scaffold into the root canal to promote tissue formation and regeneration [5, 49–51].

Cell-free approaches can make use of functionalized scaffolds or release of dentin-derived chemotactic factors to induce the migration of host cells from surrounding areas to regenerate the tissue [52]. One example of clinically viable cell-free therapy is the use of the blood clot as a scaffold to attract and maintain periapical (or circulating progenitor) cells inside the root canal [52–54]. The root

canal revascularization via blood clotting depends on the establishment of bleeding into the canal system via over instrumentation of the apex [54]. This is an attractive alternative to be performed directly in clinical appointments and eliminate the needs of retrieving, culturing and transplanting cells as required in cell-based approaches [5, 26, 52, 54]. Interestingly, by promoting the bleeding inside the root canal the level of stem cells markers in the blood clot can be up to 600 times higher as compared with levels found in the systemic blood [55]. In fact, revascularization was reported to effectively induce complete root development of immature permanent teeth with apical periodontitis [53]. Other approaches involve the use of functionalized scaffolds and bioactive materials for chemo attraction of periapical cells [56, 57].

Though cell-free approaches are a clinical reality [53], host cells need to migrate long distances inside the root canal space through the apical foramen that narrows with time [52]. Moreover, the types and concentration of cells and composition of the fibrin clot are unpredictable [54]. Nonetheless, the revascularization approach can lead to dentinal wall thickening, apical closure and increased root length [14, 15].

Cell-based strategies aim to develop clinically approachable techniques that induce the regeneration of a functional dental pulp that is capable to deposit mineralized and organized matrix contributing for the development of the root structure. Dental pulp tissue engineering requires the fine orchestration of three elements: cells, scaffolds and morphogenesis/signalling pathways [5, 26, 52]. In this new paradigm, it is rather exciting that stem cells from oral sources play the focal role moving forward the field of regenerative endodontics.

Apart of all stem cells that can be obtained in the oral environment, three of them, namely SCAP, DPSC and SHED have great potential to be used for dental pulp tissue engineering. The first can undergo dentinogenic differentiation in vitro under proper stimulation. Moreover, when SCAP were mixed with hydroxyapatite/tricalcium phosphate (HA/TCP) scaffolds simulating a root canal and implanted in vivo, they presented the ability to regenerate vascularized dental pulp–like tissues in the hollow space and to produce new dentin-like tissue on the walls [24, 29, 30].

DPSC were shown to have great potential to induce pulp regeneration since they were firstly isolated and extensively characterized in the landmark paper from Gronthos et al. [31]. The DPSC isolated were expanded in vitro, mixed with HA/TCP ceramic powder and transplanted into the dorsum of immunocompromised mice. Remarkably, after 6 weeks from implantation, the surface of the ceramic particles was coated by an odontoblast-like layer and dentin-like structure (Fig. 1). The mineralized tissue formed was composed of a highly ordered collagenous matrix containing mainly collagen type I which is the major fibrous component of the dental pulp besides collagen type III [58]. Notably, there was also the formation of a pulp-like tissue permeated with blood vessels and the transplants were positive for human-specific markers for bone sialoprotein, osteocalcin and dentin sialophosphoprotein (DSPP) [31].

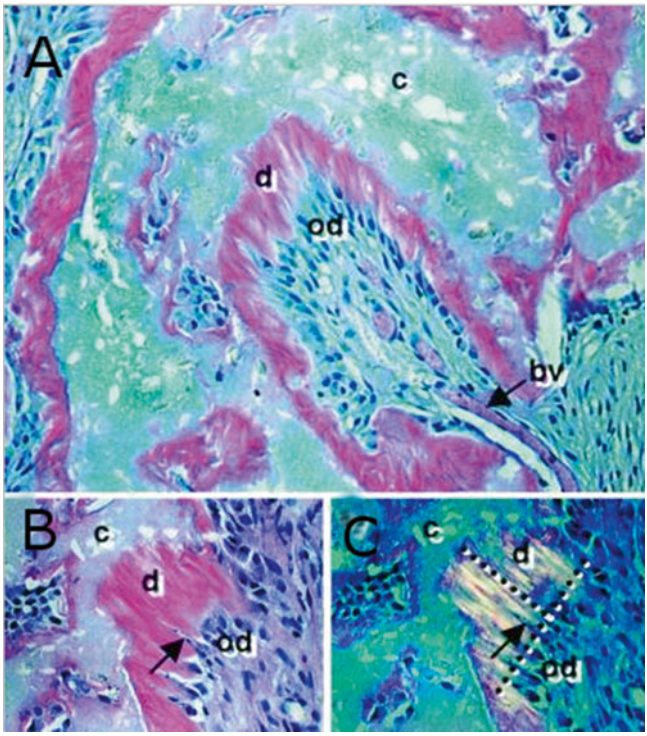

Fig. 1 Developmental potential of DPSC transplanted with HA/TCP carrier in vivo. (**a**) After 6 weeks, DPSC generated a dental pulp-like tissue within the HA/TCP carrier (c) with the presence of dentin-like matrix (d), odontoblast-like cells (od) and blood vessels (bv). (**b**) A magnified view of the dentin matrix (d) highlights the odontoblast-like layer (od) and odontoblast processes (*arrow*) (**c**). Polarized light demonstrates perpendicular alignment (*dashed lines*) of the collagen fibers to the forming surface. Adapted from Gronthos et al. [31] (Copyright (2000) National Academy of Sciences, U.S.A.)

The residual pulp of exfoliated deciduous teeth contains stem cells (SHED) that can regenerate a dental pulp-like tissue that can deposit mineralized tissue [6, 22, 27, 45]. These cells showed their potential to be used for dental pulp tissue regeneration in 2003 in a seminal paper published by Miura et al. There, SHED mixed with HA/TCP were capable to differentiate into odontoblasts after 8 weeks from implantation in immunocompromised mice. Nonetheless, the cells failed to regenerate a complete dentin–pulp-like complex in vivo as previously observed for DPSCs (Fig. 2) [22, 31]. Nonetheless, this ability was reported later [6, 27, 59].

Although SCAP is one of the candidates to be used in dental pulp tissue engineering, the majority of the research for such purpose focus on the use of stem cells obtained from the dental pulp itself (DPSC and SHED). Although SCAP are capable of forming odontoblast-like cells to produce dentin in vivo, they are likely to be the source of primary odontoblasts for the root dentin formation. This limits their

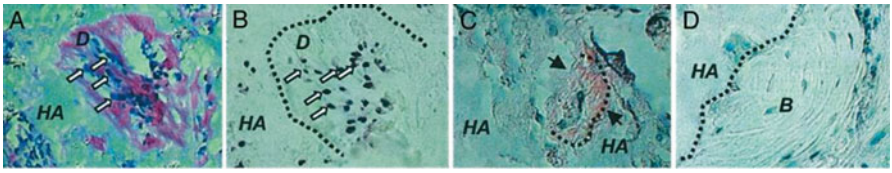

Fig. 2 (**a**) After 8 weeks of transplantation, SHED were able to differentiate into odontoblasts (*open arrows*) that were responsible for the dentin-like structure (D) formation on the surfaces of the HA/TCP scaffold (HA). (**b**) In situ hybridization showed that the tissue formed had human origin (*open arrows*). (**c**) The black dashed line represents interface between newly formed dentin. (**d**) bone (B) generated by host cells in the same SHED transplant shows no reactivity to the DSPP antibody. Adapted from Miura et al. [22] (Copyright (2003) National Academy of Sciences, U.S.A.)

isolation to the period when the root is not yet fully formed [24]. Thus, third molars are the most attractive source of SCAP as their root completion is only achieved by ages from 18 to 25. DPSC can be obtained from the dental pulp of permanent tooth. Here, pre-molars that are extracted due to orthodontic reasons are also an interesting source of DPSC. SHED can be retrieved from the only naturally disposable postnatal human tissue. This creates a window of approximately 11 years to obtain these stem cells mainly from deciduous central incisors and canines.

The success of cell-based regenerative endodontic strategies depends on techniques that will allow clinicians to create a functional pulp tissue within cleaned and shaped root canal systems [5, 17, 20, 52]. One of the first signs that this could be achieved was reported in 1996. The team led by David Mooney engineered pulp-like tissues in vitro utilizing fibroblast obtained from human adult dental pulp seeded in a synthetic extracellular matrix made of polyglycolic acid fiber-based meshes. Histological analysis performed after 60 days from implantation showed that the cells were capable to proliferate and form a new tissue with general appearance and cell density similar to native dental pulp [60]. In 2004, autogenous transplantation of pulp cells stimulated with bone morphogenetic protein 2 (BMP-2) into amputated pulp of dogs induced the formation of reparative dentin formation [61]. These and other studies shed a light that cell-based regenerative endodontics could be on the horizon of clinicians in the future. Since then, a series of proof-of principles research have been published showing the feasibility of regenerating functional human dental pulp using tissue engineering principles and dental stem cells.

To prove the potential of dental stem cells to induce pulp regeneration in vivo, several reports use the Tooth Slice/Scaffold of Dental Pulp Tissue Engineering model. This strategy allows for the generation of a dental-pulp-like tissue via the transplantation of human dental pulp stem cells seeded in a biodegradable scaffold cast within the pulp chamber of human tooth slices [62]. This model was used to show the ability of SHED to generate dental pulp-like tissue in vivo by the Nör's lab in 1996. There, SHED were seeded into poly-L-lactic acid (PLLA) scaffolds cast in 1 mm thick tooth slice and transplanted into the subcutaneous space of immuno-

compromised mice. After 4 weeks, a well-vascularized pulp-like tissue was formed in the pulp chamber space. Notably, there was a layer of odontoblast-like cells expressing dentin sialoprotein (DSP) lining the dentin surface. Although these cells were morphologically similar to odontoblasts, including the eccentric polarized position of the nucleus at the basal part of the cell body, no new dentin deposition was observed [46].

The capability of SHED to differentiate into fully functional odontoblasts capable of generating tubular dentin in vivo was observed later (Fig. 3). For this, SHED were seeded a poly-L-lactic acid (PLLA) scaffold cast in a tooth slice and implanted subcutaneously into the dorsum of mice, which received one injection of tetracycline hydrochloride every 5 days. After 32 days from implantation, the dentin disks presented well-defined fluorescent lines originated from the chelation of calcium ions in the newly deposited dentin. The dental pulp generated displayed positive protein expression for dentin sialophosphoprotein (DSPP) and dentin matrix protein 1 (DMP-1). Interestingly, the rate of dentin formation by SHED was far superior (14.1 μm/day) as compared to the one observed for the human dental pulp used as control (3.3 μm/day) [27]. Growth rates of 4–15 μm/day have been reported for primary dentin depending on the stage of development and age of the tooth [63].

DPSC were also used to regenerate pulp-like tissue using a similar approach. By seeding DPSC in a collagen-based scaffold along with a growth-factor (DMP-1) it was possible to engineer pulp-like tissue after 6 weeks from subcutaneous transplantation in mice. However, in this study, no odontoblast-like cells were found on the surface of pre-existing dentin surface and the quality of the pulp-like tissue obtained appeared to be less promising as the ones obtained using PLLA scaffold [64]. These drawbacks can be related to the contraction that collagen scaf-

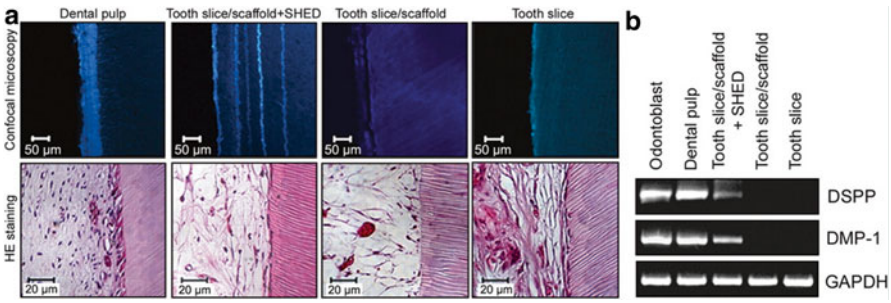

Fig. 3 SHED differentiate into functional odontoblasts that generate new tubular dentin in vivo. (**a**) The pictures show samples retrieved from mice after 32 days after implantation. (**b**) RT-PCR analysis showed the high expression of DSPP and DMP-1 on tissues engineered on tooth slice/scaffolds seeded with SHED. Reprinted from Journal of Dental Research, 89/8, VT Sakai, Z Zhang, Z Dong, KG Neiva, MA Machado, S Shi, CF Santos and JE Nör, SHED differentiate into functional odontoblasts and endothelium, 791–796, Copyright (2010), with permission from SAGE Publications

fold experiences leading to a size reduction proportional to the density of cells seeded in it [65].

Although these studies provide an exciting panorama for regenerative endodontics, the use of tooth substrates with few millimetres in thickness does not fully represent the challenges of regenerating a pulp tissue with in complete roots [6]. The larger dimension of the tissue that has to be engineered in addition to the fact that vascularization can be available only through the apex, pose important obstacles to be overcome. Hence, some studies aimed to prove that pulp regeneration can be achieved in three-dimensional environments.

An important step towards stem cells-based regenerative endodontics was obtained by a team of researchers who have seeded swine DPSC into the root segments with 5–6 mm in length and implanted into the jawbone of the adult minipigs. After 6 weeks from implantation, the proliferating cells were filling the space once left by the scaffolds made of collagen or poly(lactic-co-glycolic) acid (PLGA) undergoing degradation. At that time, new extracellular matrix had been occasionally deposited in a polar predentin-like pattern on the canal dentinal walls. After 10 weeks, a continuous layer of cells with columnar or spindle-shaped morphology along with the presence of newly formed organic matrix was observed [66]. An interesting fact about this study was that the swine DPSC used were cryopreserved for 1 year prior to implantation showing that the DPSC do not lose their potential to regenerate dentin/dental pulp-like tissue even after being cryopreserved.

Dental pulp tissue formation using DPSC was also achieved within tooth slices as long as 7 mm in length. Cells mixed with poly-D, L-lactide and glycolide (PLG) scaffolds were inserted into the canal with internal space ranging from 1 to 2.5 mm and one end sealed with MTA. Four months after the implantation into the subcutaneous of mice, the emptied canal was filled with regenerated pulp-like tissue. The entire pulp-like tissue was vascularized with a uniform cell density resembling the natural pulp. Though the odontoblast-like cells were not well organized and did not present characteristics of natural odontoblasts, a layer of mineralized tissue was deposited onto the dentinal walls and the MTA cement used to seal the canal [67]. However, as MTA is known to release calcium ions that induce the formation of calcified barriers [68] it was not possible to determine if DPSC alone could induce such extent of calcified tissue deposition. Nonetheless, that paper offered great perspectives on the capability of using dental stem cells to induce de novo synthesis of vascularized human pulp-like tissue capable to deposit dentin-like tissue onto the surface of root canals [67].

SHED are also capable to regenerate dental pulp within full-length root canals in vivo (Fig. 4). Cells were injected in empty roots using either a self-assembling hydrogel or recombinant human collagen type I. The roots were implanted in the subcutaneous of mice and after 28 days a human pulp-like tissue was observed throughout the extent of the root canals. More excitingly, the engineered pulp was capable of generating new dentin at a rate of approximately 10 μm/day. The high activity of the functional odontoblasts was supported by a high concentration of blood vessels close to the dentinal walls (Fig. 4b) [6]. One fact that deserves to be

highlighted in this study is that cells were delivered inside the root canals using injectable scaffolds. As injection is one the most practiced procedures by dentists, the learning curve can be shortened for clinicians to apply this technology in patients.

Future Challenges

Stem cells-based therapies offer new perspectives targeting dental pulp regeneration and further development of root structure [17]. Besides that, the two branches of the tissue engineering triad (the development of suitable scaffolds to deliver cells and fine tuning of signalling pathways) still offer several challanges to be solved in order to improve the outcomes.

Scaffolds are frameworks that provide the support for cells to proliferate, differentiate and generate the desired tissue [5, 6]. Ideally, a scaffold must allow cell attachment and migration, permit the localized and sustained delivery of growth factors and enable the influx of oxygen to maintain the high metabolic demands of cells and tissues in formation [5].

The scaffolds physical and mechanical characteristics must be compatible with the surrounding tissues to support specific demands in vivo [69–71]. It is widely known that stiffness of the substrate play an important role in differentiation of stem cells thus. In fact, the physical and mechanical properties of the scaffolds can have direct impact in cell differentiation through mechano transduction [5, 69, 72]. Interestingly, the increase of scaffold' stiffness favours the differentiation of MSCs into neurons, myoblasts, and osteoblasts in that order [72]. DPSC seeded on soft matrices present upregulation of collagen I but downregulation of markers such as DSPP, DMP-1 [73]. In addition, physical features of the scaffolds (*e.g.* quantity and extension of pores) change the specific surface modifying its permeability and mechanical properties, influencing cell differentiation and tissue formation [5, 25, 74]. Noticeably, higher number and extension of pores can contribute significantly for cellularity but compromise scaffold strength [74, 75]. Pore interconnectivity also plays a crucial role to sustain tissue growth [75, 76]. Also, the rate of scaffold degradation is important to achieve success in tissue engineering therapies. The scaffold should ideally reabsorb once it has served its purpose of providing a template for tissue regeneration. The degradation ought happen at a rate compatible with the new tissue formation [5, 27, 71, 73, 76]. The by-products generated cannot be toxic and must be easily cleared or resorbed to minimize the risk of inflammatory response [77].

The third pillar to be tuned for successful tissue engineering is cell signalling [5, 52]. This is a complex system of communication that governs cell activities individually and it can be dramatically changed upon cellular interactions or external stimulation. This could be observed when proteins present in dentin disks [78], dentin extract in EDTA [79] or a tooth-germ conditioned extract [80] were found

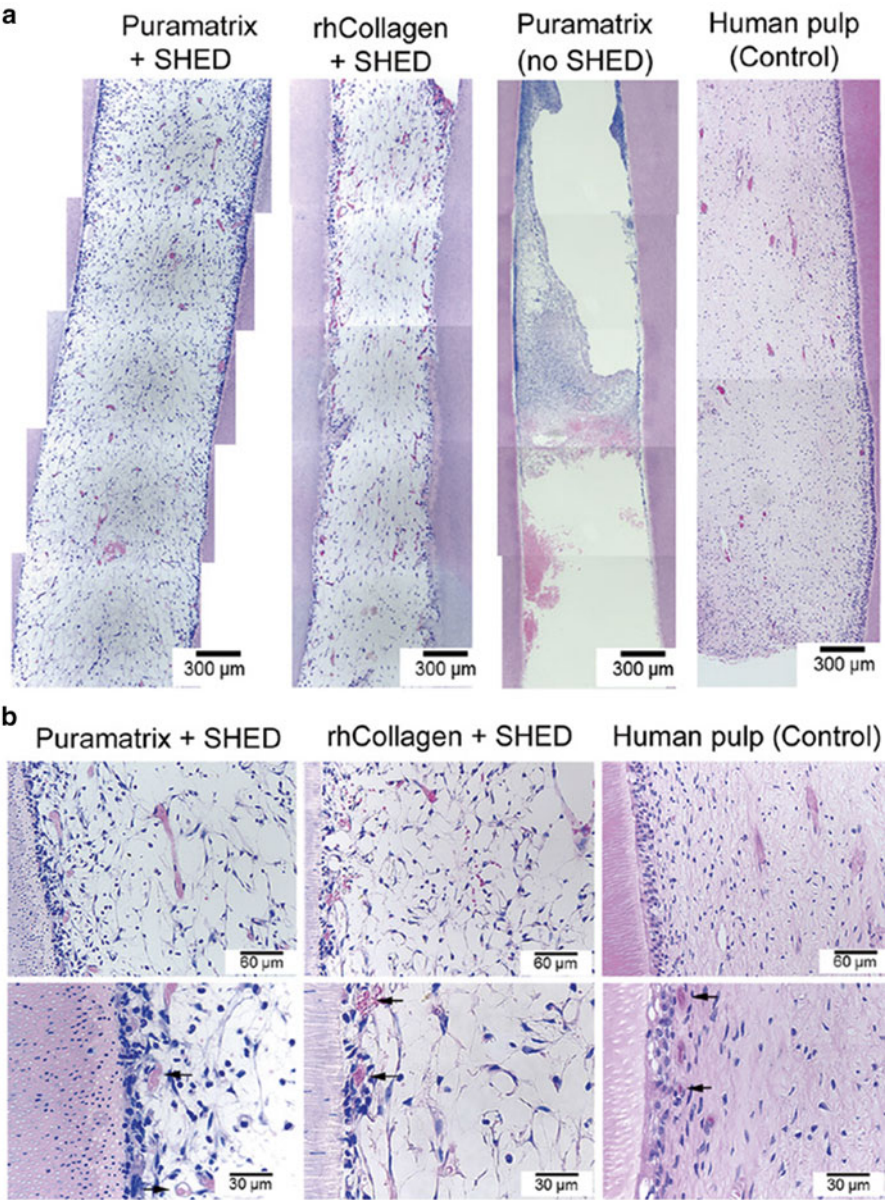

Fig. 4 Dental pulp tissue engineering with SHED injected into human root canals and transplanted into immunodeficient mice. (**a**) Low-magnification and (**b**) high-magnification images of tissues formed when SHED mixed with scaffolds (Puramatrix™, rhCollagen type I groups) were injected into full-length root canals of human premolars. A vascularized connective tissue occupied the full extension of the root canal. Cell densification and many blood vessels were observed along dentin walls. Scaffolds (Puramatrix™) injected into the root canals without cells were used as controls for SHED. Freshly extracted human premolars were used as tissue controls. *Black arrows* point to blood vessels close to the odontoblastic layer. Reprinted from Journal of Dental Research, 92/11, V Rosa, Z Zhang, RHM Grande and JE Nör, Dental Pulp Tissue Engineering in Full-length Human Root Canals, 970–975, Copyright (2013), with permission from SAGE Publications

to supplement the scaffolds as a mechanism of cellular induction. For example, there is evidence that the TGF-1 is released from the dentin after injuries [81] and dentin itself can induce the odontogenic differentiation by releasing embedded growth factors like transforming growth factor-β1 (TGF-β1) [82]. Moreover, BMP's are involved in the odontoblastic differentiation processes [59, 83] and both BMP-2 and 7 present inductive effects in reparative dentinogenesis [84, 85]. Nonetheless, SHED undergoing odontoblastic differentiation responds strongly to BMP-2 as compared to BMP-7 [59]. This complex system where molecules can trigger different responses creates a very challenging environment for researchers developing cell-based therapies.

Other challenge that needs to be addressed is the cost of therapies when available at large. Comparatively to routine root canal treatments, regenerative endodontics requires additional procedures and qualified manpower in order to isolate, expand, preserve and prepare cells for use. Undoubtedly, these add extra cost to the process that eventually will be directed to patients preventing popularization of the treatments. There are lots of research efforts to optimize process and minimize the number of procedures impacting the economic burden. History has shown that most of the revolutionary technologies became vertiginously popular as they also became more affordable. Hopefully, this also can be true for tissue engineering in endodontics (Fig. 5).

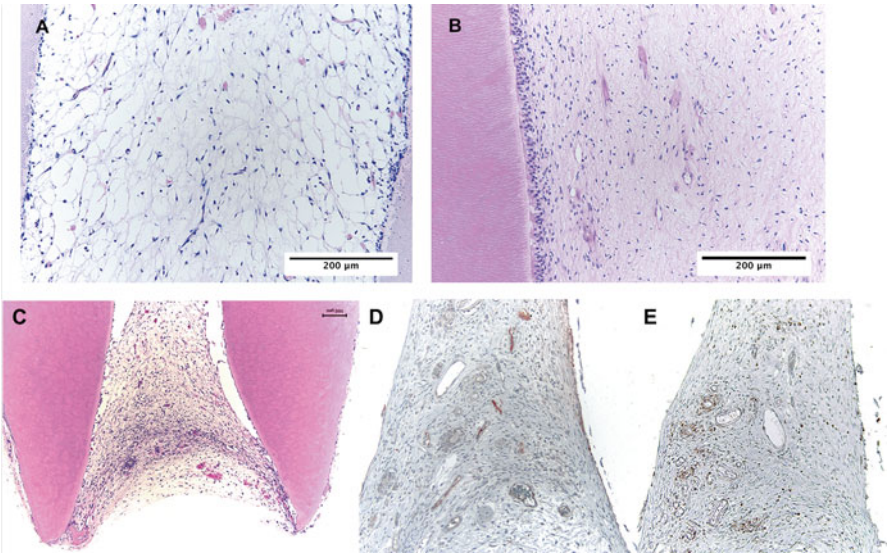

Fig. 5 Dental pulp tissue engineered inside root canal using SHED cells (**a**) and natural dental pulp from a young premolar (**b**). The engineered tissue occupies the apical portion (**c**). Immunohistochemistry with PCNA (proliferating cell nuclear activity) and Factor VIII show a proliferative tissue with established blood network (**d**, **e**). Reprinted from Dental Materials, 28/04, B Cavalcanti, A Della Bona and JE Nör, Tissue engineering: From research to dental clinics, 341–348, Copyright (2012), with permission from Elsevier

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Stem Cells for Periodontal Regeneration

Giorgio Pagni

Background

The Importance of Maintaining an Adequate Oral Health Status

A poor oral health status has been associated with systemic diseases, such as diabetes and risk factors for oral diseases, such as tobacco use, are shared with other systemic diseases. Poor oral health also impacts diet and nutrition and affects social activities, such as school and work [1]. Significant disadvantages are consequently associated with oral diseases and tooth loss.

As an indirect indicator of oral health conditions, we know that since the 1970s, fewer adults have experienced tooth loss and the prevalence of complete edentulism among adults has been consistently declining; however, important variations remain among subgroups of the population. Age is strongly related to tooth loss, gender is not related after adjustment for age, and race-ethnicity is consistently after adjustment for age and gender [1]. While the prevalence of edentulism continues to decline, the rate of aging of the western civilization and the longer life expectancy will keep increasing the need for dental treatment in order to prevent tooth loss.

Periodontal Diseases

Periodontal diseases include a variety of pathologies affecting the supporting structures of teeth and eventually leading to tooth loss. Periodontal disease affects almost half of the population in western countries [2] and severe periodontitis is the sixth

G. Pagni (✉)
Private Practice, Via Lamarmora 29, 50121 Florence, Italy

University of Milan, Milan, Italy
e-mail: giorgio.pagni@gmail.com

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most prevalent disease of mankind [3]. More than 70 % of the population over 65 years old is affected by periodontal disease. The prevalence is higher in males than females and highest in Mexican–Americans than in African–Americans and in African–Americans in relation to Caucasians. In smokers the prevalence of periodontal disease is 64.2 %. Research has shown that periodontal disease is associated with other chronic inflammatory diseases as diabetes and cardiovascular disease [2]. Periodontitis is also one of the main causes of tooth loss followed by tooth decay [4–6].

Chronic Periodontitis, Aggressive Periodontitis, Periodontitis as a Manifestation of Systemic Diseases, Necrotizing Periodontal Diseases, Abscesses of the Periodontium, Periodontitis Associated With Endodontic lesions and Other Developmental and Acquired Deformities are the main groups of pathologies possibly affecting the attachment apparatus [7]. All these conditions may have different etiology and contributing factors, but they all share loss of clinical attachment as the main characteristic bringing them together. Once the etiological and contributing factors are controlled the clinician often finds himself in the difficult position of correcting the deformities caused by the disease in order to facilitate oral hygiene maintenance and reducing the chance for disease recurrence and tooth loss [8].

Challenges in Periodontal Tissue Regeneration

The tooth attachment apparatus is a complex organ including cementum, periodontal ligament (PDL) and boundle bone. Embryologically speaking, all these structures have ectodermal origin making them different in relation to bone tissues normally originating from the mesoderm, later on we'll discuss on the possible importance of this different origin. The cementum is a thin layer of a mineralized tissue covering the tooth root dentin and in which the PDL fibers are inserted. The PDL is the tooth shock absorber system. It is made of collagen fibers called Sharpey's fibers but it also includes vessels and nerves bringing nourishment and Proprioceptivity to the surrounding structures. The boundle bone is the layer of bone, which is closer to the root surface. Sharpey's fibers are inserted in it, which make it different in relation to the alveolar bone. Many teeth positioned outside the alveolar bone housing are covered by boundle bone only and not any alveolar bone [9].

When attachment loss occurs, the PDL and boundle bone are resorbed by tissue enzymes and the cementum is left of the tooth surface and covered by bacterial plaque and calculus [10, 11]. Removal of plaque and calculus results in the closure of the periodontal pocket via formation of a long junctional epithelium [12]. Other traditional surgical approaches showed different repair models.

In 1976 Melcher [13] hypothesized that the repair model was dictated by the proliferation speed of different cell populations. In other words once the surgical trauma occurred and bacterial plaque was removed, a competition between soft tissue cells and hard tissue cells would take place. Being epithelial cells the fastest they would colonize the periodontal defects first.

Nyman and coworkers decided therefore to exclude soft tissue cells from this competition by attempting the first human case of guided periodontal tissue regeneration using a Millipore filter between the flap and the previously diseased root surface [14]. They showed, for the first time in humans, the regeneration of all of the three attachment apparatus structures: cementum, a functionally oriented PDL and boundle bone. After their pioneering work several authors have researched on guided tissue regeneration procedures.

Following studies shifted the attention from the competition theory to the importance of blood clot stabilization: that is that if the adhesion between the blood clot and the root surface is preserved epithelial cells would not infiltrate between the clot and the root surface resulting in periodontal regeneration [15, 16].

Periodontal Regeneration: State of the Art

Modern approaches to periodontal regeneration demonstrated better clinical results when adopting surgical techniques minimizing surgical invasiveness by reducing flap extension and mobilization and preserving the soft tissues as much as possible. Papilla preservation techniques were first introduced in 1985 [17] and then refined in 1995 [18], 1999 [19] and 2001 [20]. Later on surgical techniques using a single flap approach were introduced [21–24] and other flaps boosting the biological performance of blood clot stabilization have been developed [25].

Biomaterials in Periodontal Regeneration

Following Nyman's first guided tissue regeneration case with a Millipore filter several biomaterials have been evaluated with the intent of enhancing the clinical performance of periodontal regeneration surgeries.

Barrier membranes have been developed attempting to maintain soft tissue separated from the underlying structures during wound healing. Non-resorbable e-PTFE membranes were widely adopted either with or without a titanium frame reinforcement. Soon after resorbable membranes were introduced to overcome the need for a second surgical intervention to remove the e-PTFE membrane. Synthetic materials as polylactic acid, polyglycolic acid and combinations or xenogenic collagen membranes with different resorption rates have been used and evaluated widely in recent decades. Other than the barrier effect, membranes maintain the capability to support and stabilize the underlying tissues thus improving the clinical outcome of the regeneration procedure.

Autogenous bone and bone substitutes as synthetic materials, xenogenic bone substitutes and allogenic bone substitutes such as freeze-dried bone allograft (FDBA) and demineralized freeze-dried bone allograft (DFDBA) have also been thoroughly evaluated. Autogenous bone has been considered the gold standard as it

maintains all the characteristics required for a grafting material: a scaffolding effect, an osteoconductive effect, presence of inductive proteins as the Bone Morphogenic Proteins capable of inducing the differentiation of neighboring undifferentiated cells toward the osteoblast pathway (osteoinduction) and the presence of cells which could directly initiate bone formation (osteogenic capability). Other bone substitutes have demonstrated good osteoconductive capabilities but only DFDBA has shown limited osteoinductive capabilities.

The clinical efficacy of membranes and biomaterials including autogenous bone has been widely documented when using extended flaps. With the advancements in flap manipulation and surgical techniques the use of biomaterials have been questioned in contenitive defects as their impact on clinical performance may be limited [26, 27]. Biomaterials are still considered important when treating larger non-contenitive defects. Moreover, tissue engineering approaches are currently evaluated in order to facilitate, enhance and speed up periodontal regeneration and possibly make it more predictable even when treating more challenging defects.

Tissue Engineering in Periodontal Tissue Regeneration

Tissue engineering is a broad term that describes very different applications. In a comprehensive manner MacArthur and Oreffo defined it as the understanding of the principles of tissue growth, and its application to produce functional replacement tissue for clinical use [28]. In its common view though tissue engineering is closely associated with the repair or regeneration of portions or whole tissues. In the medical field its weight is directly related to the importance that the ability of regenerating an organ or a tissue has to the survival or quality of life of an individual. In dentistry tissue engineering techniques find practical applications in the regeneration of lost bone, periodontal ligament, dental pulp or keratinized mucosa.

Regenerative strategies can be categorized in three major classes: Conductive approaches utilize biomaterials in a passive manner to facilitate the growth or regenerative capacity of existing tissue (guided tissue regeneration in periodontal dentistry is an example of this approach). Another tissue engineering strategy is Induction. The original concept lying behind this mechanism was explored with the discovery of bone morphogenetic proteins (BMPs), molecules that are able to promote the differentiation of uncommitted mesenchymal cells through the osteoblastic pathway inducing the formation of bone even in an ectopic (nonmineralizing) tissue. Cell transplantation is the third class of tissue engineering strategies. The collaboration of surgeons and bioengineers is critical for the delivery of cells that will interface with the host cells for the regeneration of the new natural tissue [29].

As previously mentioned most bone substitute materials used nowadays are adopted for their conductive capability in the regeneration of periodontal tissues; other inductive approaches as protein therapy and gene therapy and of cell transplantation have been evaluated and are currently studied in the attempt to generate better grafting solution for periodontal regeneration.

Protein Therapy: Growth Factor Delivery

The incorporation of bioactive molecules as Growth Factors (GFs) into scaffolding materials has been used to stimulate precursor cells and enhance wound healing and bone formation. The concept lying behind the use of GFs is related on the knowledge about cell-to-cell and platelets-to-cells biochemical interactions during wound healing.

A variety of factors have been studied that we can mainly group into two categories: *growth factors* act primarily through increasing cell proliferation (mitogenic) and recruitment of cells (chemotactic) while *morphogens* act through the alteration of cellular phenotype. BMPs are an example of morphogens; they have one of the most outstanding inductive properties in tissue regeneration: the ability of induce the differentiation of stem cells into bone formatting cells in a process known as *osteoinduction* [30]. We will now focus on proteins that have shown the most promising result in periodontal and implant related tissue engineering.

Platelet-Rich Plasma

The first attempt of using growth factors in tissue regeneration was by the use of platelet-rich plasma commonly known with its acronym: PRP.

Platelets are one of the body's best sources of growth factors. When activated they release α-granules containing, between others, Platelet Derived Growth Factor (PDGF), Transforming Growth Factor-β (TGF-β) Epidermal Growth factor (EGF) and Endothelial Cell Growth Factor (ECGF). These factors initiate several pathways during wound healing in all tissues. PRP was originally used for treatment of diabetic ulcers, decubitus ulcers, treatment of sternal wounds after cardiac surgery, non-healing fractures and it was then introduced its use in the dental field in 1998 [31]. PRGF is a technique similar to the production of PRP using although only one centrifugation and depleting the plasma from all red blood cells [32]. Other techniques have been described with significant variations in centrifugations protocols and final product indications.

A review by Boyapati and Wang critically evaluated the available literature on animal and human application of PRP. The authors concluded that due to the paucity of critical scientific data regarding the effects of PRP in sinus augmentation procedures and the poor study design of the available trials the use of PRP cannot be supported in sinus augmentation procedures [33]. A systematic review from Plachokova et al. came to similar conclusions about the materials and methods of the analyzed articles. When considering periodontal tissue regeneration although the results seem to be a little more encouraging [34].

With centrifugation techniques increases in platelet concentration between 2 and 10 times can be achieved. Protein therapy approaches have been introduced which are able to provide growth factors concentrations of thousands times higher than that carried by the blood.

Platelet Derived Growth Factor (PDGF)

Platelet-Derived Growth Factor (rh PDGF-BB) is one of the numerous growth factors, or proteins that regulate cell growth and division. In particular, it plays a significant role in blood vessel formation (angiogenesis), the growth of blood vessels from already existing blood vessel tissue. Mitogenic and chemotactic effects have been shown on calvarial osteogenic cells, periosteal cells from long bones, trabecular bone cells and bone marrow stromal cells. PDGF also increases expression of angiogenic molecules such as VEGF, hepatocyte growth factor/scatter factor, and of inflammatory factors as IL-6. PDGFs also increase osteogenic cells' responsiveness to BMPs [30].

The use of PDGF-BB in combination with other growth factors as the insulin-like Growth Factor-1 (IGF-1) has shown increased bone density and alveolar bone formation when compared to controls in periodontal defects and increased bone to implant contact (BIC) when used around implants immediately placed in extraction sockets [35–38]. When used in combination with allograft it has also shown encouraging results on the treatment of Class-II furcations and infrabony interproximal pockets [39, 40]. Recombinant human platelet-derived growth factor (rhPDGF-BB) mixed with a synthetic beta-tricalcium phosphate (beta-TCP) has been favorably used in the treatment of periodontal osseous defects [41, 42].

Although good results have been showed in eliciting bone regeneration some authors have hypothesized that suboptimal tooth-supporting tissue regeneration is related to the short half-life of PDGF in vivo. This could be a result of proteolytic degradation, rapid diffusion, or the solubility of the delivery matrix within periodontal lesions [43]. Increasing the substantivity of the molecule in vivo may result in improved periodontal regeneration.

Bone Morphogenetic Proteins (BMPs)

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β superfamily, have been shown to be responsible for post-fetal bone induction, including normal bone remodeling healing and repair. BMPs have been shown to be able to induce heterotopic bone formation [44]. Although all BMPs are involved in bone and cartilage formation by stimulating cellular events and mesenchymal progenitor cells, only a subset of BMPs (i.e. BMP-2, -4, -7 and -9) has osteoinductive properties [45]. New bone growth following use of BMPs results from the differentiation of pluripotent mesenchymal cells along osteoblastic pathways [46]. RhBMP-2 in combination to an absorbable collagen sponge have been tested in maxillary sinus elevation and for local alveolar ridge preservation or augmentation [36, 37, 46–48]. Lee et al. have tested ceramic barriers impregnated with BMP-2 greatly to improve craniofacial bone regeneration in vivo [49]. Bone repair has been documented in periodontal defects when using rhBMP-2/ACS although ankylosis was a frequent observation and only occasionally was a regeneration with functionally oriented PDL fibers observed [50]. Bone morphogenetic protein-7 (BMP-7), is

a multifunctional member of the BMP family with multiple effects on bone formation and regeneration. BMP-7 stimulates bone regeneration around teeth [51], around endosseous dental implants [52], and in maxillary sinus floor augmentation procedures [53, 54]. Wikesjo reported in an animal study that while rhBMP-2/ACS is able to induce more robust bone regeneration rhBMP-12/ACS can induce more proper PDL regeneration with periodontal fibers inserting perpendicularly to the root surface into the newly regenerated cementum and supporting bone [55].

One of the limitations related to the use of BMPs in oral applications is the super-physiological dose of BMPs implanted in the wound site in certain trials resulting in a dose-dumping of potent morphogens [47, 48, 54]. There is a concern that this delivery method could lead to systemic toxicity in example by diffusion of potent morphogens through the bloodstream. The effect and safety of BMPs in vivo may benefit from a more controlled delivery method able to provide optimal concentration of these molecules through an adequate period of time.

Gene Therapy

In vivo growth factors have a transient effect since the half-life of these molecules is in the order of minutes to hours. Therefore, the induced production of growth factor via gene transfer is could potentially increase the effect of GFs by releasing them in a sustained manner. The latter is called Gene therapy and consists of the insertion of genes into individual's cells either directly or indirectly with a matrix to promote a specific biological effect. Gene therapy typically aims to supplement a defective mutant allele with a functional one in a therapeutic approach but can also be used to induce a more favorable host response. Targeting cells for gene therapy requires the use of vectors or direct delivery methods to transfect them [56]. Tissue engineering of alveolar bone using gene therapeutic approaches may offer potential for optimizing the delivery of growth-promoting molecules such as bone morphogenetic proteins (BMPs) at implant osteotomy sites [43, 57].

The most commonly employed vectors for gene transfer are viruses as retrovirus, lentivirus, adenovirus or adeno-associated virus [58]. A possible concern of the use of viral vectors is the possibility of an accidental generation of replication competent virus during vector production and packaging, the mobilization of the engineered vector by endogenous retrovirus present in the human genome and the random insertion or mutagenesis of the vector leading to cancer or germ cell alteration resulting in vertical inheritance of the acquired genes [59].

Non-viral vectors include plasmid DNA and synthetic or natural polymers. These vectors are safer than the viral vectors but they have low and unpredictable gene-transfer efficiency and allow only a limited size of DNA insert to penetrate in the targeted cells.

Both viral and non-viral vectors can be used in in vivo or ex vivo. In vivo approaches require the delivery of the vector directly into the host where the gene-transfer will happen. This method is less safe, more unpredictable but less invasive

than ex vivo approaches. In ex vivo applications the gene-transfer happens in vitro of previously harvested host cells or in cell lines. The efficiency of the gene-transfer can be controlled and the infected cells are then re-implanted in the host. Ex vivo gene therapy has been widely adopted in combination to stem cell therapy.

In vitro studies have shown promising results when using recombinant adenoviral vectors encoding for the PDGF-A gene on cloned cementoblasts [60]; Chen and Giannobile evaluated the effects of PDGF on cell signaling via gene therapy when applied to tissue engineering of skin and periodontal wounds. The results of this study showed a sustained activation of the transfected genome by ligand binding resulting in a prolonged production of PDGF-AA and a sustained stimulation of several signaling pathways [61]. Syngenic dermal fibroblasts (SDFs) transduced ex vivo with adenoviruses were transplanted into large mandibular defect in a rat model with significantly improved results compared to controls [62]. Sustained and targeted delivery of BMP-7 to large alveolar bone defects associated with dental implant fixtures in a rat model also demonstrated that in vivo gene transfer promotes bone regeneration [53].

In a rat model periodontal defects where treated with either a collagen matrix alone or with a matrix containing adenoviruses encoding luciferase, PDGF-A (PDGF-1308) or (4) PDGF-B. At 14 days the control groups showed limited bone bridging while a fourfold increase in bridging bone and sixfold increase in tooth-lining cemental repair was observed the Ad-PDGF-B-treated sites that presented greater evidence of vascularization [63].

Chang et al. demonstrated that the use of adenovirus encoding the PDGF-B gene (AdPDGF-B) delivered in a collagen matrix to periodontal lesions in rats exhibited acceptable safety profiles; AdPDGF-B was well contained within the localized osseous defect area without viremia or distant organ involvement [64]. The same group evaluated the efficacy of the gene therapy approach in two concentrations of Ad-PDGF-B, a control Ad encoding luciferase or the protein therapy with rhPDGF-BB. Bone repair was found to be accelerated by gene and protein therapy compared with control, with the high dose of Ad-PDGF-B more effective than the low dose. Interestingly the protein therapy approach was found to be more effective than the gene therapy approach. Typically, high concentrations of growth factors are required to promote tissue regeneration [65]. A possible explanation for this unexpected result is that the concentrations of growth factor released during healing in the Ad-PDGF-B treated sites remained below the effective levels of the rhPDGF-BB treatment.

Cell Therapy

To summarize a few concepts described above we can identify the needs for tissue regeneration in the following categories: cells, growth factors, scaffold, and new vessels [56]. In conventional regeneration therapies cells need to reach the area where the new tissue has to be created from the periphery of the grafted site.

Scaffolds are used both as a support for their migration and as a frame for cells to start building up the tissue matrix. The migration and the differentiation of the cells is regulated and stimulated by *growth factors* that modulate the cellular activity and provide stimuli to cells to differentiate and produce matrix toward the developing tissue. GFs also stimulate the production of angiogenic signals inducing the formation of a new *vascular network*, which is essential for working cells as a source of nutrients and oxygen and as a pathway for the removal of catabolites.

Cell therapy approaches provide an additional source of cells in the area of interest with the intent to be used as grafted cells (which will integrate into the patients body) or, when not intended for integration, as a source of growth factors.

Both cultivated somatic cells and stem cells have been studied and tested in different branches of contemporary medicine and periodontology. Dogan et al. seeded cells derived from R to promote the regeneration in artificial furcation defects of a dog model and found this method to be superior than controls [66]. Cultivated fibroblasts have also been safely used for the treatment of interdental papillary insufficiency following a papilla priming procedure by McGuire and coworkers with satisfactory results [67]. Cell therapy has also been tested in mucogingival surgery [68–73]. Cloned cementoblasts have also demonstrated potential in periodontal therapy [74, 75]. Skin fibroblasts transduced by the BMP-7 gene promoted the tissue engineering of periodontal bone defects including new bone, functional PDL and tooth root cementum [62].

Stem Cells

Stem cells are cells that maintain an increased potential for renewal and differentiation in relation to cultivated cells. The benefit in the use of stem cells is that once transplanted in the grafted site they may differentiate in strictly osteogenic cells as well as in "supportive osteogenic cells". Supportive osteogenic cells are defined, as cells that do not directly create bone but that facilitate bone depositions creating structures that are needed to allow this process (i.e. vascular network) [76, 77]. Mesenchymal stem cells also play a role in preventing inflammation in the grafted site [78] allowing faster regeneration of new tissues.

Mesenchymal stem cells (MSCs) were first discovered in bone marrow and then in other adult tissues such as liver, adipose tissue, and muscle, but the marrow stroma remains the most widely used sources of stem cells [79]. They are characterized by elevated renewal potency and the ability of growing bone, cartilage, midollar adipocytes, miocytes, fibrous tissues from a single colony forming unit-fibroblast (CFU-F) when transplanted in vivo [80]. In the differentiation pathway, limitative or inductive differentiation stimuli may lead to cells progressively characterized by lower renewal capacity and by an augmented degree of differentiation [79]. This path can be reversed so that an adult adipocyte may de-differentiate back to levels with higher generative capabilities and then differentiate through the osteogenic pathway [81].

Given the inconvenience of obtaining stem cells from adult tissues including the use of invasive harvesting techniques, researchers have succeeded in harvesting mesenchymal stem cells from dental-derived tissues. Dental Stem Cells (DSCs) are adult stem cells derived from dental-related tissues such as the periodontal ligament of extracted teeth [82], gingiva [83], dental follicles [84], dental pulp [85], apical papilla [86], and human exfoliated deciduous teeth [87, 88].

Dental stem cells populations still maintain similar properties as bone marrow derived stem cells [85, 89–96]. Although they are reported to derive from the neural crest during tooth development [90], in mature individuals they demonstrate stem cells properties similar to MSCs rather than neural crest cells [97]. Jo and coworkers isolated stem cells from dental pulp, periodontal ligament, periapical follicle, and the surrounding mandibular bone marrow and found that they were able to differentiate into osteoblasts, adipocytes, and other kinds of cells with varying efficiency [98]. Because of their MSCs-like characteristics DSCs have been used in the regeneration of distant tissues and have been suggested as excellent candidates for cell-based therapy to treat liver diseases [99].

It has to be considered though that despite having similar properties each single stem cell type is different to the other. One important feature of dental pulp stem cells (DPSCs), in example, is their odontoblastic differentiation potential making them ideal to be seeded onto dentin and for dentin regeneration strategies. In endodontic therapy interesting results come from the autogenous transplantation of the BMP2-treated pellet culture of pulp progenitor/stem cells onto the amputated pulp. The treatment stimulated reparative dentin formation in a dog model [100]. Stem Cells from Human Exfoliated Deciduous Teeth (SHED) proliferate faster with greater population doublings than DPSCs and BMMSCs; unlike DPSCs, SHED are unable to regenerate a complete dentin-pulp-like complex in vivo. They showed to maintain the ability to differentiate into osteoblasts and induce bone formation, generate dentin and dental pulp [92, 101, 102]. Their ability to differentiate into bone-forming cells is not a property attributed to DPSCs following transplantation in vivo [90]. Similar to DPSCs and SHED, ex vivo expanded Stem Cells from Apical Papilla (SCAP) can undergo odontogenic differentiation in vitro. SCAP also demonstrate the capacity to undergo adipogenic differentiation following induction in vitro and show positive staining for several neural markers even without neurogenic stimulation [90, 103] and they are able to differentiate into functional dentinogenic cells. Periodontal Ligament Stem Cells (PDLSCs) seem to be the most promising dental derived stem cells for periodontal regeneration purposes as they can differentiate into either cementum-forming cells (cementoblasts) or bone-forming cells (osteoblasts) [104, 105]. They still demonstrate osteogenic, adipogenic, and chondrogenic characteristics under defined culture conditions [72, 73, 89, 106] but their capacity to form all of the PDL-related structures in vivo [82] makes of them the ideal candidates in periodontal regeneration [107]. In recent studies evaluating the osteogenic activity of different stem cell populations, the expression of RUNX2—a gene playing a crucial role in osteoblast differentiation [108]—peaked in BMSCs at day 14, while DPSCs and GSCs peaked at day 21 and PDLSCs peaked at day 28. The authors concluded that a possible explanation for

the early RUNX2 expression in BMSCs may be that the embryological niche in the mesoderm area is more conductive for mineralization as compared to the ectodermal origin of PDLSC, GSC and DPSC [109, 110]. Moreover it has been shown that hPDLSC produce negative regulators of osteogenesis such as chordin and PDL-associated protein-1 or aspirin. In particular chondrin specifically binds to BMP-2 suppressing its osteoinductive capacity [111–115]. Despite this piece of knowledge may seem conflicting with the PDLSC task of regenerating a damaged periodontium, a physiological explanation may be hypothesized. Should a damaged PDL come in contact predominantly with bone MSCs in an osteoinductive environment ankylosis and root resorption have been documented [116]. The PDLSC ability to suppress osteoinduction may be needed to maintain normal tooth tissues homeostasis and prevent remodeling of the root.

From the information provided above it seems clear that despite their plasticity and flexibility not every stem cell is the same but one particular stem cell population may be more suitable to a particular task than another. Other cells showing capacity to form periodontal tissues are the Dental Follicle Precursor Cells (DFPCs): progenitor cells that form the periodontium, i.e., cementum, PDL, and alveolar bone which have been isolated from human dental follicles of impacted third molars. Kémoun et al. demonstrated expression of cementum attachment protein and cementum protein-23 (CP-23), two putative cementoblast markers, in EMD-stimulated whole dental follicle and in cultured DFPCs stimulated with EMD or BMP-2 and BMP-7 [117].

Even within a single cell source differences may be greater than one would think. PDLSCs have been isolated from the alveolar socket following extraction, from the root surface after extraction [95, 96], from deciduous [118–120] or permanent teeth [118, 119], from supernumerary teeth [120] and from periodontal granulation tissue [121, 122] each source showed different characteristics and regenerative potential [123], therefore strict identification criteria should be used when reporting and comparing results from each particular cell type.

Other important consideration are related to the culture medium, the oxygen concentration during cell culturing or the combined use of growth factors, which may enhance or reduce their regenerative potential and modify their characteristics [123].

The use of dental derived stem cells in periodontal therapy has important advantages. First of all that, because of their origin, they may be more adequate in the regeneration of periodontal tissues as opposed to other MSCs as dental stem cells appear to be more committed to odontogenic rather than osteogenic development [90]. Secondly, when autologous therapy is considered, the same dentist providing treatment to the patient could also be harvesting the cells beforehand. The problem in periodontal therapy is that not always the patient requiring periodontal regeneration will also need tooth extraction so not all cell types may be available. Periodontitis defects need a large number of cells (about 10^7 cells for one defect), which we may not be able to obtain from a single subject when using autologous stem cells from oral tissues [124]. Also at the time of treatment most periodontal patients are not young and the potential of their stem cells may be reduced [125]. Luckily because of immunoregulatory potential of DPSCs [126] SCAP and PDLSCs [90] allogenic

dental stem cell therapy may be feasible as demonstrated in animal models [127, 128] therefore stem cells collected from deciduous teeth or teeth extracted for orthodontic reasons in younger individuals may be used in older patients. This immunoregulatory capacity is interesting as it has been suggested that it may play a role in reducing the inflammatory reaction to biomaterials used in tissue regeneration thus stimulating faster bone formation [129]. Other solutions to improve stem cell availability could be the cryopreservation of one's stem cells for future autologous use. Cryopreservation of extracted third molars was found to be an adequate and cost-effective method of preservation of MSC cells. After recovery, these cells maintained the characteristics of mesenchymal stem cells (ability to differentiate into osteogenic, adipogenic, and chondrogenic pathways) showing the possibility of banking MSC with minimal processing [130]. Finally, the immortalization of adult cells via gene therapy has been suggested [131] although raising questions on the costs and safety of these therapies.

On this path, adult adipocytes are known to be able to de-differentiated back to levels with higher generative capabilities and because of the large amounts of human lipoaspirates readily available, and the fact that their procurement induces only low morbidity, ASCs may be useful in future clinical cell-based therapy for periodontal disease. Adipose tissue is the richest source of MSCs, 100 times more than bone marrow [132]. The use of adipose-derived stem cells (ASCs) for periodontal tissue regeneration in vivo has been tested in animal models. These studies demonstrated the ability to promote tissue regeneration with this cell construct [133–136].

iPSCs may also represent an alternative source of stem cells. These are induced pluripotent stem (iPS) cells were first created reprogramming mice somatic cells through viral introduction of four transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) from a group from Kyoto University [137]. iPS cells demonstrated to be completely similar to the most widely used Stem Cells and can be created reprogramming adult human dermal fibroblasts into a pluripotent state or practically any human somatic cell source [138]. IPSC lines were derived from human somatic cells reprogram somatic cell nuclei to an undifferentiated state [139, 140]. This technology may allow the implantation of Stem Cells without the drawbacks of the surgery required for their harvesting from the patient. Moreover, in alternative to reprogramming of patients' somatic cells, encouraging results have been conducted towards the establishment of an iPS cell bank consisting of various human leukocyte antigen (HLA) types. Dental Pulp Cells (DPCs) could represent an excellent source for iPS cell banks using retroviral vector for delivery of the exogenous genes [141].

Once proved as safe and effective, the possibility to generate an HLA-type banking of human pluripotent stem cell (hPSC) lines would greatly increase cost-effectiveness and practicality with the medical and oral health care cell therapy delivery systems, as well as safety and quality control measures of these therapies. Studies should determine whether IPSCs are better than DSCs in terms of safety and efficacy in periodontal regeneration.

An important issue recently emerging is the use of xenogenic components and growth factors in culture media, which may transmit animal pathogens [132].

Luckily researchers have been developing xeno-free culture system of human periodontal ligament stem cells, which after culture showed the morphological features of stem cells, expressed the markers associated with pluripotency, and a normal karyotype encouraging the use of this system in future human research experiments [142].

Applications in Periodontal Regeneration

In 2006 ex vivo expanded bone marrow mesenchymal stem cells (BMMSCs) in a PRP gel were shown to facilitate periodontal tissue regeneration, and suggested as a possible treatment solution for aesthetically sensitive sites, while reducing patient morbidity [114, 115]. Despite being a case report with no control the study draw significant attention on cell-based regenerative periodontal therapy.

Li et al. demonstrated that cryopreservation of bone marrow mesenchymal stem cells (BMSCs) had no negative effect on their growth and differentiation in vitro and that both freshly isolated and cryopreserved BMSC induced significantly better periodontal regeneration compared with collagen sponge alone [143].

Autologous PDLSCs were used to treat periodontal defects in a minipig model showing successful regeneration of the PDL and recovery of the heights of the alveolar bone [91].

Ding et al. evaluated both autologous and allogeneic periodontal ligament stem cells (PDLSCs) in the treatment of periodontal defects in a miniature pig periodontitis model. Both the autologous and the allogeneic PDLSCs demonstrated significant periodontal tissue regeneration with no marked difference between the autologous and allogeneic PDLSCs transplantation groups. The authors also observed lack of immunological rejections in the animals that received the allogeneic PDLSCs transplantation [127].

Cultured mandibular periosteum-derived cell sheets with PRP have also been used in the treatment of chronic periodontitis without [112] or with HA in patients suffering from advanced chronic periodontitis [144].

Recently, human osteoprotegerin (hOPG)-transfected periodontal ligament stem cells (PDLSCs) seeded on beta-tricalcium phosphate (β-TCP) were used to enhance periodontal regeneration in New Zealand rabbits. Unfortunately the adopted model was not adequate to evaluate periodontal regeneration but more for alveolar bone regeneration [145].

Future Directions

Several therapies have been evaluated for periodontal regeneration including guided tissue regeneration, and bone grafting. These techniques have been shown to improve clinical outcomes in relation to their control treatments although some authors have expressed concerns regarding the possible interference of mineralized

grafts with bone repair in periodontal defects and some others have shown the long-lasting persistence of grafted particles in the regenerated area [146] arising questions on the clinical implications that these remaining particles might have. Recently an increased interest has been put on tissue engineering approaches, and in particular on the therapeutic potential of different stem cells populations for periodontal regenerative applications.

In the dental field different animal studies have shown encouraging results in bone regeneration. Marei et al. regenerated bone by implanting an engineered porous scaffold seeded with bone marrow mesenchymal stem cells (BMSCs) in an extraction socket in rabbits [129]. De Kok et al. in canine extraction sockets found that MSCs remained in the specified site contributing to bone formation as differentiated osteoblastic cells and that MSC-mediated bone repair was greater than bone repair in controls [147]. Some studies utilized fresh bone marrow aspirates [148] although, when using an autologous bone marrow grafting procedure, it should be kept in mind that while some of the cells populating bone marrow have strong osteogenic capacity, others have more of a regulatory effect and inhibit bone formation. Culturing protocols must therefore be adopted, which should be aimed at expanding those cells with osteogenic capacity while reducing inhibitor cells. Bone Repair Cells (BRCs) are an example of a culturing method of bone marrow aspirates successful in highly enriching Thy1 cells (CD90+) and CD105+ cells [58, 149]. BRC technology has been shown to be able not only to provide mesenchymal cells at an early stage of differentiation but also to produce significant concentrations of cytokines and were shown to maintain their ability to differentiate into endothelial cells and produce angiogenic factors providing supportive osteogenic cells that do not directly create bone but that facilitate bone depositions creating structures that are needed for the regeneration to take place (i.e. vascular network) [76, 77]. In the dental field, this cell construct has been already successfully tested in humans in a socket grafting procedure [150] and maxillary sinus grafting [151] while awaiting for even more advanced applications.

In periodontology mesenchymal stem cells (MSCs) isolated from a patient's iliac crest marrow aspirates in combination with platelet-rich plasma (PRP) were successful in the regeneration of periodontal tissue [114, 115].

Clinicians at times tend to underestimate the importance of a more rapid tissue formation. In periodontology the importance of boosting tissue regeneration may dictate the difference between succeeding or not in the preservation of teeth therefore novel tissue regeneration approaches must be evaluated in order for the periodontal field to advance.

On the other side, though case reports and clinical trials are promising, their results should be interpreted with caution [124]: while the potential of stem cells to induce tissue regeneration is more and more consolidated, the mechanisms involved in these processes are not clearly understood [152, 153]. Also determining which patient would greatly benefit from this advanced therapy has to be properly determined as several factors influence the disease and the clinician's ability to treat it [154]. The type of periodontal defects (number of walls), the type of periodontitis, the type of cell source and culturing method, the scaffold used, adjunctive growth

factors, specific culture medium, or the enhancements of cultured cells via gene therapy, all these factors have to be properly evaluated before stem cell therapy can be applied in clinical practice.

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Dental Stem Cells for Tooth Regeneration

Thimios A. Mitsiadis, Giovanna Orsini, and Lucia Jimenez-Rojo

Tooth Formation

Odontogenesis involves a precisely orchestrated series of cellular, morphogenetic, and molecular events [1]. Generation of teeth relies upon sequential and reciprocal interactions between the oral epithelium and mesenchymal cells that are derived from the cranial neural crest (CNC) [2]. CNC-derived cells migrate from the dorsal part of the neural tube and subsequently generate craniofacial structures of unique morphology and function such as teeth (Fig. 1a) [2–4]. Genetic markers, cell labelling, and lineage tracer techniques have shown that CNC-derived cells are responsible for tooth mesenchyme formation [5–7]. Classical tissue recombination experiments between oral epithelium and CNC-derived mesenchyme have identified the oral epithelium as providing the instructive information for tooth initiation [8, 9]. Tooth formation starts at precise locations of the oral epithelium that thickens and gradually invaginates the underlying mesenchyme (Fig. 1b). The invaginating oral epithelium gives rise to the dental epithelium that progressively acquires characteristic shapes such as the bud, cap, and bell configurations. After tooth initiation the odontogenic potential shifts to the mesenchyme that can instruct any kind of epithelium to form tooth-specific structures [9, 10]. These and other tissue recombination experiments have provided evidence of an essential role of the mesenchyme in establishing tooth shape.

At the bell stage, the mesenchyme gives rise to the dental pulp. Pulp cells adjacent to the dental epithelium start to differentiate into odontoblasts, while epithelial

T.A. Mitsiadis (✉) • L. Jimenez-Rojo
Orofacial Development and Regeneration, Institute of Oral Biology, Centre for Dental Medicine, Medical Faculty, University of Zurich, Plattenstrasse 11, 8032 Zurich, Switzerland
e-mail: thimios.mitsiadis@zzm.uzh.ch

G. Orsini
Department of Clinical Sciences and Stomatology, Polytechnic University of Marche, Ancona, Italy

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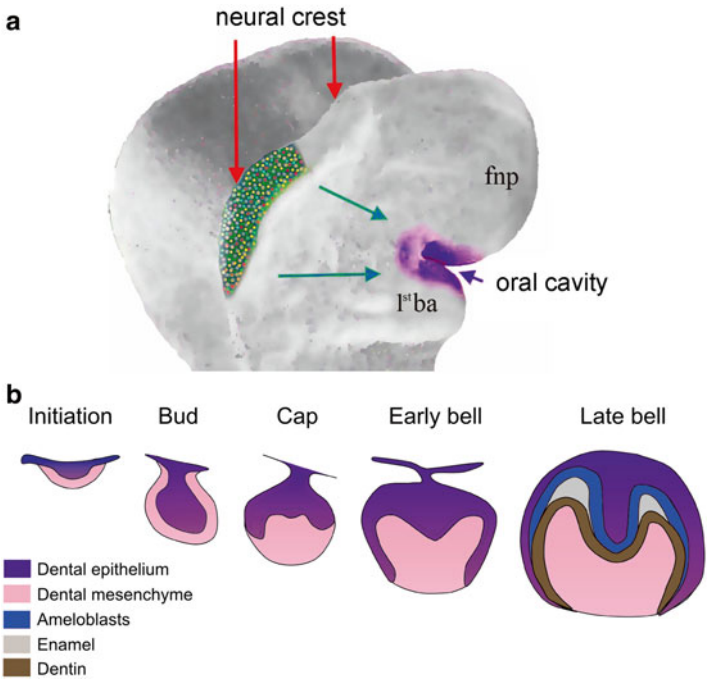

Fig. 1 Schematic representation of (**a**) cranial neural crest cell populations (*red arrows*) represented by the various colors and their migratory paths towards the oral cavity territory (*blue–green arrows*), and (**b**) the various stages of embryonic tooth development. *fnp* frontonasal process, *1st ba* first brachial arch

cells facing the dental pulp differentiate into ameloblasts [11, 12]. Odontoblasts are polarized columnar cells with processes that participate in the secretion of dentin matrix and minerals. Dentin matrix is composed mainly of collagen (90 %) and various non-collagenous proteins such as dentin sialophosphoprotein (DSPP) and dentin matrix protein 1 (DMP1) that represent the 10 % of the matrix. Apatite minerals are deposited on the matrix forming the mature calcified dentin. Following initial dentin deposition, the ameloblasts polarize and start to secrete the enamel matrix along the dentin-enamel junction. Enamel matrix is essentially composed of tissue specific hydrophobic proteins such as amelogenin, ameloblastin, enamelin, amelotin, tuftelin and ODAM (odontogenic ameloblast associated proteins) [12, 13].

Molecular Control of Odontogenesis

Over the last years a big effort has been made to understand the molecular mechanisms that dictate tooth development, pathology, and repair [1, 14]. To this contributed the constantly increasing knowledge on genetics, stem cells, and tissue engineering.

Signaling molecules control all steps of odontogenesis by coordinating cell proliferation, differentiation, apoptosis, extracellular matrix synthesis, and mineral deposition. The same molecules are repetitively used during the different stages of odontogenesis and are regulated according to a precise timing mechanism [1, 15–17]. Signals produced at a wrong time lead to inappropriate cell proliferation, differentiation, and apoptosis, thus affecting the overall tooth development and morphology [1].

The territory where teeth will form is defined early, before any obvious sign of tooth development. The earliest marker of the odontogenic territory within the oral epithelium is the transcription factor Pitx2 [18, 19]. Dental fields are also established by morphogenic signals that determine the display and fate of the CNC cells, thus leading to the generation of distinct tooth shapes [20, 21]. For example, ectodysplasin (Eda) molecules are involved in the determination of dental fields size that controls teeth number [22]. In fact, increased Eda signaling leads to supernumerary teeth formation, while Eda deletion often leads to tooth number reduction [1, 22].

Bone morphogenic proteins (BMPs), Wnts, sonic hedgehog (shh), and fibroblast growth factors (FGFs) regulate the epithelial-mesenchymal interactions during tooth initiation [1, 17]. BMP4 and FGF8 molecules constitute essential early oral epithelial signals that activate tooth specific genes (e.g. *Msx1*, *Msx2*, *Barx1*, *Dlx1*, *Dlx2*, *Pax9*) in the underlying mesenchyme [1, 18, 19, 23], thus controlling tooth patterning (i.e., BMP4 directs incisors shape while FGF8 molars shape) [2, 21].

Signals Specifying Dental Cell Fates

The specification of the various dental cell-types involves genes with restricted expression patterns during odontogenesis. It has been proposed that the determination of cell fates in teeth occurs via inhibitory interactions between adjacent cells through the Notch signaling pathway [17, 24]. Indeed, previous studies have shown that molecules of the Notch signaling pathway are involved in tooth development and dental cell specification [25–29]. In order to influence developmental decisions, molecules of the Notch signaling pathway must obviously interact with other signaling pathways. Notch-dependent cell fate acquisition between non-equivalent dental precursor cells is influenced by extrinsic signals such as BMP and FGF molecules. BMP and FGF molecules have opposite effects on the expression of Notch receptors and ligands in dental tissues [25, 26, 29], indicating that cell fate choices during odontogenesis are under the concomitant control of the Notch and BMP/FGF signaling pathways. Furthermore, it has been shown that Notch-mediated lateral inhibition has a pivotal role in the establishment of the tooth morphology, as shown in *Jagged2* mutant mice where the overall development and structure of their teeth is severely affected [28, 29]. Several genetic findings have shown that Tbx1 plays a significant role for the early determination of epithelial cells to adopt the ameloblast fate. Indeed, hypoplastic incisors that lack enamel are observed in mice lacking the *Tbx1* gene [30, 31]. Mesenchyme-derived FGF molecules activate the expression of *Tbx1* in dental epithelium [32, 33].

Dental Pathologies

Traumatic injuries and external harmful agents such as bacteria and acids jeopardize tooth integrity. Periodontal disease and caries are mainly responsible for pathologies affecting the dental and alveolar bone tissues. Dental pathologies, combined with age, constitute important factors that can trigger tooth loss [34].

In most cases, the reparative mechanisms following dental and periodontal injuries involve a series of highly conserved processes that share genetic programs that occur throughout embryogenesis [14]. For example, after severe injury or inflammation of the dental pulp (i.e., pulpitis), the dying odontoblasts are replaced by progenitor cells, which differentiate into a new generation of odontoblasts and produce the reparative dentin [14, 35]. Signaling molecules released at the injury sites may attract progenitor cells, thus initiating the healing processes.

The increased knowledge on the reparative events within dental tissues has contributed to the proposal of alternative methods for the treatment of dental pathologies. However, traditional treatments continue to be applied in dental clinics since most of these novel approaches are at the experimental level and not well established yet.

Traditional Dental Treatments and Main Limitations

Restorative dentistry has undergone significant advances in recent years [36, 37]. Contemporary techniques to replace partially lost hard tissues consist of direct or indirect tooth-colored restorations (e.g., resin-based composites, porcelain inlays and onlays). The resin-based composites are bonded to the dental tissues through elaborated adhesive materials. While adhesion to enamel is stable over time, adhesion to dentin is weaker and unstable, mainly because of the higher levels of organic matrix of dentin [38]. Therefore, the goal of the adhesive procedures is the widespread infiltration and encapsulation of the demineralized collagen fibrils of dentin with the monomeric resin [37, 39]. Dentin biomodification strategies have as goal to enhance the properties of dentin matrix, thus inducing remarkable resin resistance against degradation that leads to short durability of restorations [39–41].

Endodontic therapy is a procedure implying the removal of contaminated or necrotic dental tissues within the pulp, thus eliminating future tooth contamination. Clinically, vital pulp therapy can be divided into two main groups: indirect and direct pulp capping. Indirect pulp capping is achieved by applying a protective agent on the thin layer of dentin remaining over a nearly exposed pulp, in order to allow the underlying dental pulp to repair [42]. Direct pulp capping necessitates the use of a protective agent directly on the exposed pulp, thus allowing pulp regeneration [43]. In case of large pulp exposure or infection, the coronal pulp has to be removed before direct pulp capping at the tooth root level (i.e., pulpotomy) [44]. This method preserves the vitality of pulp located at the root canal, thus allowing

the accomplishment of the root growth [45]. Apexogenesis is another procedure of vital pulp treatment that promotes physiological development and formation of the root using mineral trioxide aggregates [46]. When the whole pulp is damaged or infected is replaced with inorganic materials such as guttapercha after thorough root canal treatment. However, root canal treatment results in the loss of dental pulp vitality. Dental pulp is an important element of the tooth since it provides nutrition, sensation, and defense against the various pathogens. Therefore, devitalized teeth are subsequent to various complications causing tooth strength reduction, increased fragility, and predisposition to postoperative fracture [47]. In terms of aesthetics, endodontic treatment often results in tooth crown discoloration due to either the filling material or the unsuccessful pulp chamber cleaning [48]. Thus, it becomes obvious that the maintaining of dental pulp vitality is of prime importance and this is highlighted by the emergence of new cell-based techniques focusing on partial or total pulp regeneration.

Missing teeth have traditionally been replaced with removable dentures, fixed bridges, and dental implants [49]. Currently, dental implants offer the most valuable option for tooth replacement, but a prerequisite for successful implant treatment is the quantity and quality of the remaining alveolar bone. Indeed, dental implant retention requires the close contact between the alveolar bone and the surface of the implant, a process called osseointegration. Dental implants are made of biocompatible titanium and they are inserted into the bone after surgical intervention. Survival rates of osseointegrated dental implants have been reported to be of over 95 % [50]. Apart alveolar bone quality and dimensions, the clinical success of implants also depends on primary implant stability, surgical methods, time of loading, infections, and implant surface characteristics [51]. When the dental implant is not well anchored to the bone, soft tissue encapsulation can occur, thus leading to unsuccessful treatment. Nanotechnology offer the opportunity to develop new implant surfaces that increased implant osseointegration [52], thus allowing the shortening of healing period without jeopardizing implant success [49]. However, the risk of infection of the tissues surrounding the implant (i.e., peri-implantitis) is still high and has to be taken seriously into account [53].

Recently, dental implants have been benefited from outcomes from stem cell biology and tissue engineering in order to maximize their survival rate in cases of poor bone quality. New regenerative technologies using scaffolds, stem cells, drug and growth factor delivery, and gene therapy are expected to enhance host tissue response and implant osseointegration [54]. Indeed, numerous pre-clinical studies have been performed already in various large animal models for guided bone regeneration around implants using growth factors and protein delivery [55, 56]. More recently, clinical studies have been realized using growth factor delivery for osseous and periodontal regeneration [57–59]. Pre-clinical applications for oral tissue regeneration using stem cell and gene therapies have been realized in rat models [60, 61]. Presently, safe and effective cell-based regenerative therapies have started to be applied in the dental clinics [54]. Clinical trials have documented that stem cells seeded in specific scaffolds are capable of generating sufficient amounts of bone in order to achieve primary implant stability [62–66]. However, all these new approaches should be further studied using controlled randomized clinical trials.

Alternative Dental Treatments

Novel cell-based therapeutic approaches have been proposed the last two decades [34, 67–69]. These treatments are promising, challenging, and complement traditional restorative or surgical techniques that are currently used in dentistry. The administration to patients of stem cells in conjunction with scaffolds and bioactive molecules might accelerate and increase the repair process of the impaired dental tissues. Although most of the studies focus on partial dental tissue regeneration, few attempts have been also made for the regeneration of entire teeth.

Here we report on selected dental stem cell populations that are currently used for experimental regenerative purposes in dentistry.

Stem Cells in Tooth Homeostasis and Regeneration

Stem cells play a critical role in tissue homeostasis and repair. Their fate is regulated by cell intrinsic determinants and signals from a specialized microenvironment [70–73]. Molecules of the Wnt, Notch, and BMP pathways have been shown to control stem cell fate specification [71, 74, 75]. The growing interest in the molecular regulation of dental stem cells arises from the potential to influence their fate and consequently their functions during tooth repair.

A considerable effort has been produced the last years to isolate epithelial and mesenchymal stem cell populations from deciduous and adult human teeth. Dental mesenchymal stem cells (DMSCs) were found in the dental pulp of permanent and exfoliated deciduous human teeth (Fig. 2) [11, 34, 69]. DMSCs were also identified in the apical part of dental papilla, the dental follicle, and the periodontal ligament. Because dental epithelial stem cells (DESCs) are very rare in adult human teeth, the current knowledge on DESCs has been obtained from studies in rodent incisors, which are continuously growing teeth [24, 76].

Stem Cell Populations Within the Dental Pulp

Activation of Notch signaling in various dental pulp cell populations during the dynamic processes triggered by dental injury may reflect the existence of specific stem cell pools [71, 77, 78]. Indeed, Notch expression is activated in endothelial cells, in cells located close to injury sites, as well as in cells located at the apex of the roots [71, 77]. Currently there are available protocols for the isolation and in vitro culture adult DMSCs [79–81]. These protocols are continuously being improved in order to further enrich the DMSC populations. Mesenchymal stem cell markers such as STRO-1, CD146 and CD44 are commonly used for the isolation of dental pulp stem cells (DPSCs) [11, 34, 79]. These cells are capable to differentiate

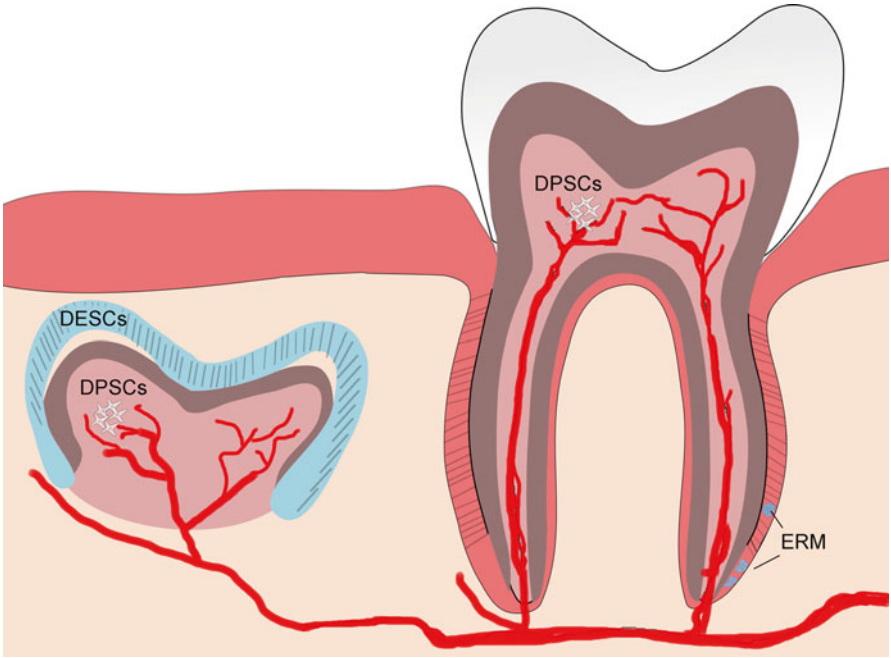

Fig. 2 Schematic representation of the locations of epithelial stem cells (i.e., hDESCs and ERM) and dental pulp stem cells that can be found in unerupted and erupted teeth of adult humans. *ERM* epithelial rests of Malassez, *hDESCs* human dental epithelial stem cells, *DPSCs* dental pulp stem cells

into odontogenic, osteogenic, chondrogenic, adipogenic, myogenic, and neurogenic cells in vitro and in vivo [11, 34]. Pulp-dentin tissues have been generated after ectopic transplantation of DPSCs mixed with hydroxyapatite/tricalcium phosphate [79]. Furthermore, clinical trials using autologous human DPSCs combined with collagen scaffolds for alveolar bone reconstruction have been successfully performed several years ago [82].

Dental Epithelial Stem Cells

In mice, cells responsible for the continual growth of the incisor are located in the cervical loop area (posterior part of the incisor), which is a putative stem cell niche [76]. Cells originated from the cervical loop region are clonogenic and express Notch receptors and stem cell markers such as Sox2 and $p75^{NTFR}$ [26, 28, 76, 83]. This expression pattern indicates that Notch signaling plays a role in the maintenance of the dental epithelial stem cells. FGF3 and FGF10 are important mesenchymal signals involved in the maintenance and creation of the adult stem cell compartment within the cervical [84]. In the FGF10 null mice, the incisors are smaller due to the lack of the stem cell compartment in the cervical loop [84].

Therefore, FGF and Notch signaling pathways appear to interact for the maintenance of dental epithelial stem cells [76, 83].

In humans, dental epithelial stem cells (hDESCs) can be isolated from the third molars that develop after birth (Fig. 2) [11, 68]. Another source of hDESCs is the epithelial root sheath that disintegrates and forms the epithelial rests of Malassez (ERM) (Fig. 2) [11, 68]. ERM cells express epithelial stem cell markers such as Bmi-1, E-CAM, and p75, as well as embryonic stem cell markers such as Oct-4 and Nanog [24, 69].

Induced Pluripotent Stem Cells

Somatic cells can be reprogrammed and turned into pluripotent cells by the expression of a four transcription factors cocktail (i.e., Oct4, Sox2, c-Myc and Klf4) [85]. Similar to embryonic stem cells (ESCs), induced pluripotent stem cells (iPS) can generate cells from all germ layers [85]. This discovery opened new perspectives for the field of regenerative medicine, thus allowing the conception of new treatment scenarios for the replacement of damaged or pathological tissues and organs. Therefore, a plethora of efforts have been produced the last years in order to design and adapt protocols that redirect iPS to specific cell types that could be used for the regeneration of a precise organ. In fact, the first clinical trial using iPS technology has been recently initiated: a sheet of iPS-derived retinal cells has been transplanted in a patient for the successful treatment of the age-related macular degeneration (AMD) pathology [86].

For dental-linked purposes, iPS have been generated from different dental cell types. Furthermore, iPS derived from skin fibroblasts have been used to produce various dental mesenchymal and epithelial cell populations [87].

Experimental Approaches for Tooth Regeneration

Regeneration of Dentin-Pulp Complex

Dental pulp is composed by a highly innervated and vascularized connective tissue containing a variety of cell types such as fibroblasts, immune cells, odontoblasts, and stem cells [88]. DPSCs show a limited potential to regenerate dentin, which becomes insufficient after tooth damage such as to caries or trauma. Several attempts have already been made in order to use DMSCs for the regeneration of the dentin-pulp complex in a variety of animal models. The first ever experiment realized using DMSCs isolated from human teeth has demonstrated that these cells are able to give rise to odontoblast-like cells, which form a dentin-like structure when transplanted together with HA/TCP ceramic powder into the dorsal surface of immunocompromised mice (Fig. 3a) [79]. Infiltration of mouse-derived blood vessels was observed in the dentin-pulp complex generated under these conditions.

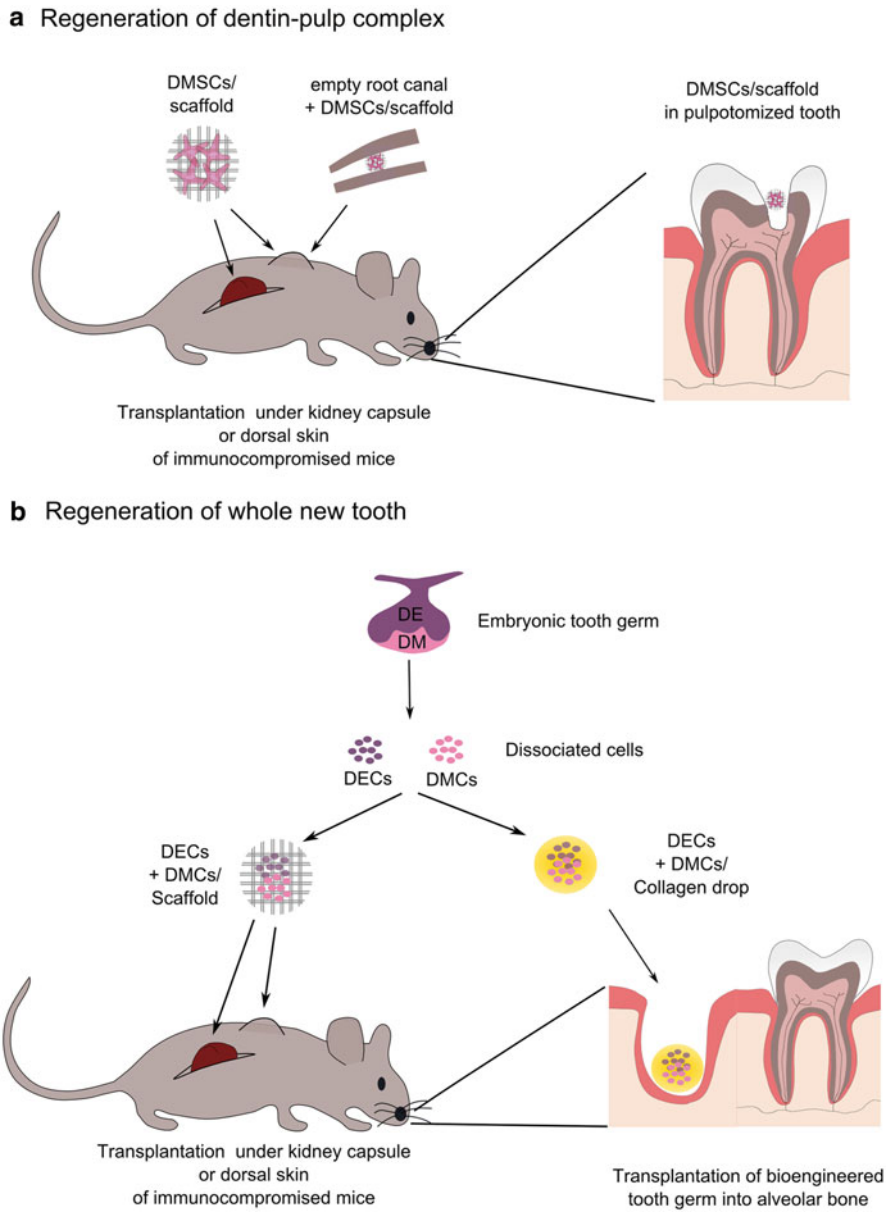

Fig. 3 Summary of different strategies for the regeneration of (**a**) dentin-pulp complex, and (**b**) an entire tooth. *DE* dental epithelium, *DM* dental mesenchyme, *DECs* dental epithelial cells, *DMCs* dental mesenchymal cells, *DMSCs* dental mesenchymal stem cells

More recent studies, using human DPSCs and SCAPs seeded on a poly-D,L-lactide/glycole scaffold and transplanted into the empty root canal space of mouse teeth, have demonstrated the ability of DMSCs to regenerate vascularized dentin-pulp complexes (Fig. 3a) [89–91].

Although being a useful in vivo model to assess the potency of DMSCs to form all or part of the various pulp tissues in vivo, the these experimental strategies were based on the transplantation of DMSCs in ectopic locations (Fig. 3a). Thus, those methods cannot be directly translated into a potential stem cell-based therapy in order to regenerate human dental tissues.

More recent studies have adopted more physiological strategies. For example, an experimental model consists of either the partial or total dental pulp removal (i.e., pulpotomy and pulpectomy, respectively), which is followed by fulfilling the empty space of the pulp chamber with DMSCs either alone or in combination with scaffold and bioactive molecules (Fig. 3a) [92–94]. For instance, autologous DPSCs transplanted together with granulocyte-colony stimulating factor (G-CSF) in pulpectomized teeth of dogs, gave rise to new dentin formation and vascularized and innervated pulp tissues [94]. Interestingly, partial pulp regeneration was also observed in pulpectomized teeth without DPSCs transplantation that were used as control, indicating that an endogenous population of DMSCs may be also participating in the regenerative process.

Regeneration of a Whole New Tooth

Severe dental pathologies can lead to tooth loss. In such cases, whole tooth regeneration could be the considered the right therapeutic approach. Functional teeth have been experimentally bioengineered by the re-association of disaggregated epithelial and mesenchymal cells from mouse embryonic tooth germs [67, 68, 95]. The bioengineered tooth germs can be subsequently transplanted into the alveolar bone and erupt into the oral cavity, thus replacing the missing teeth (Fig. 3b). Bioengineered erupted teeth possess enamel and dentin, are innervated, and can achieve normal occlusion indicating that are fully functional teeth [95]. However, it is quite difficult to control tooth size and shape during the in vitro development of these regenerated teeth. Indeed, the teeth formed using this experimental protocol were smaller when compared to the natural teeth. Thus, a big challenge is the generation of bioengineering functional teeth with the appropriate crown morphology. Therefore, the use of specifically designed scaffolds that will respect the crown anatomy of the various human teeth will greatly help to overcome that limitation [96–99]. Several attempts have been made towards this direction using dental epithelial and mesenchymal stem cells combined with tailor made scaffolds. Nevertheless, the result was the generation of teeth with irregular dental structures [96]. It is obvious that this method needs to be revisited and optimized before any application in dental clinics.

The previously mentioned approaches use embryonic dental stem cells in order to generate bioengineered teeth in the various small animal models. However, for the generation of teeth in humans the use of embryonic stem cells is not possible yet because of the various ethical and regulatory problems [34]. Therefore, the is an absolute need to find alternative sources of human stem cells that could serve for tooth regeneration purposes in the dental clinics. Ideally, those stem cells would be isolated from adult tissues. Although a sufficient number of DPSCs can be collected from the pulp of adult human teeth, harvesting DESCs from adult teeth for the generation of epithelial structures in the bioengineered teeth is almost impossible. In this sense, iPS technology could be of great help for the formation of dental epithelial tissues. Indeed, studies in mice have shown that mouse iPS can form ameloblast-like cells when co-cultured with dental epithelium [100]. In addition, a similar effect has been observed when mouse iPS were cultured in presence of ERM-conditioned medium [101] or recombined with dental embryonic mesenchyme [87]. However, it is still required to identify the precise molecules responsible for the redirection of iPS cells towards a dental epithelial fate. A better understanding of the regulation of iPS reprogramming towards dental cell types is essential in order to develop reproducible and safety strategies for new iPS-based therapies for regenerative purposes in dentistry.

Conclusion

The control of tooth regeneration necessitates a thorough understanding at the cellular and molecular level. The identification and characterization of dental epithelial and mesenchymal stem cell populations is a considerable accomplishment that can offer new perspectives in dentistry. Understanding when and how signaling molecules control the fate, migration, proliferation, and differentiation of dental stem cells will open new unexplored horizons for all dental disciplines. The acquired scientific knowledge together with novel approaches based on tissue engineering and nanotechnology are likely to instruct development of innovative dental therapies and materials.

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Dental Stem Cells for Bone Regeneration

Evangelia Diamanti, Xenos Petridis, Amalia Kaparou, and Efthymia Kitraki

Introduction

Bone fractures related to trauma, neoplasia or metabolic disorders such as osteoporosis, are among the most common human injuries. Unfavorable conditions, such as extended bone fractures (accompanied with soft tissue damage and poor blood supply) or inappropriate fracture stabilization, may lead to delayed union, nonunions or permanent bone loss. These unfavorable outcomes are estimated at a rate of 5–10 %, but the number is expected to increase along with the increase of the aging population. Successful bone repair is thus of immense clinical importance. Notably, bone repair ends up with an osseous part identical to the intact bone. This is a unique feature of the skeleton, compared to soft tissues' repair that results in fibrous scar formation.

Bone regeneration is a highly coordinated physiological process with close similarity to that of skeletal formation during organogenesis. During fetal life, osteogenesis occurs through either endochondral or intramembranous ossification. Endochondral ossification gives rise to the majority of long bones, while intramembranous ossification produces the flat bones of skull, maxilla, mandible and clavicle. Similarly to fetal bone formation, both endochondral and intramembranous ossification contribute to bone restoration. Adult mesenchymal stem cells are driven by

E. Diamanti, DDS, MSc, PhD • A. Kaparou, DDS, MSc • E. Kitraki, PhD (✉)
Faculty of Dentistry, National and Kapodistrian University of Athens,
2 Thivon str., Athens 11527, Greece
e-mail: ekitraki@dent.uoa.gr

X. Petridis, DDS, MSc, PhD student
Faculty of Dentistry, National and Kapodistrian University of Athens,
2 Thivon str., Athens 11527, Greece

Center for Dentistry and Oral Hygiene, University Medical Center Groningen,
1 Antonius Deusinglaan str., Groningen 9713 AV, The Netherlands

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the same molecular signals that regulate fetal osteogenesis. However the amount and potential of adult stem cells is limited compared to the population of embryonic pluripotent and osteoprogenitor cells [1]. Moreover, adult bone repair further depends on inflammation signals and mechanical forces in load bearing skeletal parts. At the beginning of the healing procedure, inflammatory cells, recruited at the fracture cite by pro-inflammatory cytokines, stimulate angiogenesis to support the ossification process [2]. The exerted mechanical load is also critical for the healing of long bones in particular. Complete stabilization of the fracture site results in direct (intramebranous) bone formation, whereas motility is associated with the formation of a cartilage callus intermediate.

The current standard of care for complicated bone fractures is bone grafting. Bone progenitor cells stemming from the grafts can differentiate into osteogenic cells to promote bone regeneration. Due to limitations of the bone transplants, alternative therapeutic strategies have been looked for. To this direction, bone marrow-derived mesenchymal stem cells (BMSCs), first isolated by Friedenstein et al. [3, 4], have shown promising results when applied at the fracture site either alone or in combination with osteo-conductive matrices and growth signals. This cell-based therapy provides an alternative to bone grafting by preventing bone loss and reducing graft-related morbidity [5]. BMSCs-mediated bone repair has been tested in clinical trials to restore extended craniomaxillofacial defects or problematic long bone fractures with established results [6]. However, the use of BMSCs in regenerative medicine is accompanied by several drawbacks the most important being the remaining invasiveness and cost of their collection as well as their limited quantity. Consequently, the ongoing research for other postnatal (adult) stem cells has led to the discovery of a variety of mesenchymal stem cells (MSCs) from different tissues and organs that exhibit osteogenic potential. Among them, MSCs from tissues of dental origin, initially isolated from the adult dental pulp [7] represent a promising player in field of bone regeneration. Due to their simpler accessibility and the non-invasive and low-cost way of harvesting, the dental stem cells could possibly substitute BMSCs in the cell-based approaches for bone healing.

This chapter aims to provide a concise presentation of the different types and properties of stem cells that derive from dental tissues with emphasis on their osteogenic characteristics. Subsequently, evidence for the in vivo applications of dental stem cells in bone repair will be described separately for the craniofacial and long bone defects.

Stem Cells of Dental Tissue Origin

Dental tissues comprised of the tooth pulp, the periodontium, the gingiva and also developmental tissues such as the apical papilla and the dental follicle are identified sources of stem cells. These stem cells originate embryologically from the neural crest, a cell population that comes from the dorsal margins of the closing neural folds and migrates under the induction of several signals to various locations in the embryo. Several distinct populations of dental tissue-derived stem cells have been

isolated and characterized. All of them share common properties with adult MSCs from other tissues. They exhibit self-renewal capacity, multilineage differentiation potential and immunomodulatory properties [8]. According to the guidelines of the International Society for Cellular Therapy, the minimal criteria for human MSCs are the ability to attach and grow on plastic under specific culture conditions, differentiate in vitro into osteoblasts, chondroblasts and adipocytes and express specific surface markers which are >95 % (positive) CD105, CD73 and CD90, and <2 % (negative) CD45, CD34, CD14/CD11b, CD79α/CD19 and HLA-DR [9].

Dental Pulp Stem Cells (DPSCs)

Stem cells from the pulp of permanent teeth are obtained mainly from impacted third molars after enzymatic digestion of the pulp tissue or an explant culture of tissue fragments [7, 10]. As typical MSCs, DPSCs express several surface antigens such as CD73, CD90, CD105, STRO-1, CD29, CD44, CD166, CD271 and CD146 but not CD14, CD34 or CD45 [11]. These cells are highly proliferative, exhibit a faster population doubling time than BMSCs and possess immunosuppressive properties [12, 13].

DPSCs can develop in vitro into odontoblasts, osteoblasts, adipocytes, neural cells, chondrocytes, myocytes, cardiomyocytes, melanocytes, endotheliocytes and hepatocyte-like cells [10, 14–22]. Under specific inductive conditions DPSCs express nestin, a specific marker of functional odontoblasts, and can form mineralized structures that resemble dentin or osteodentin and express alkaline phosphatase (ALP), dentin sialophosphoprotein (DSPP), osteonectin (OCN) and bone sialoprotein (BSP) [7, 23–27].

The osteogenic differentiation capacity of DPSCs seems to be superior compared to BMSCs [12] and has been well demonstrated in vitro in conjunction with several materials [16, 28]. For example, DPSCs that were cultured on different titanium surfaces were differentiated into osteoblasts and produced bone [29]. Immobilization of DPSCs into alginate microspheres resulted in osteo-differentiation and enhanced mineralization as shown by the upregulated osteo-related genomic profile of these cells [30]. DPSCs that were pre-differentiated into osteoblasts and seeded onto collagen-hydroxyapatite-poly (L-lactide-co-ε-caprolactone), provided osteoblast-like cells capable for extracellular matrix mineralization [31].

When DPSCs are transplanted ectopically in experimental animals, they can form dentin-pulp-like complexes [7, 10, 32]. DPSCs in combination with carriers like hydroxyapatite/tricalcium phosphate (HA/TCP) or dentin surfaces, led to the formation of vascularized pulp-like tissue and mineralized nodules resembling dentin [33].

Regarding the osteogenic properties of DPSCs upon ectopic transplantation, numerous studies in animal models have shown bone-like tissue formation after subcutaneous implantation of DPSCs loaded on various carriers [16, 34–40]. More specifically, a subpopulation of DPSCs (c-kit /CD34 positive and CD45 negative,

SBP-DPSCs), able to osteo-differentiate in vitro, generated a fibrous lamellar bone, when transplanted into immunocompromised rats [19]. This bone that contained osteocytes was named LAB (living autologous fibrous bone tissue). Under a similar experimental set-up, the formed bone had its own vasculature, suggesting differentiation of DPSCs into endothelial cells and a tendency to enhance angiogenesis in vivo [16]. In further studies, well-mineralized hard tissue with distinct concentric lamellae and partially developed bone marrow-like haematopoietic tissue was formed after subcutaneous implantation of rat STRO-1-selected DPSCs with a 3D porous HA/TCP carrier [39]. Constructs prepared from DPSCs and an absorbable self-assembling peptide nanofibre hydrogel cultured in an osteogenic medium for 2 weeks and subsequently implanted subcutaneously into nude mice resulted into vascularized mineralized tissue pieces after 4 weeks [34]. The ability to produce ectopic bone-like tissue seems to be enhanced by the addition of the bone morphogenetic protein-2 (BMP-2) [41–43]. Collectively, most studies on bone formation following ectopic transplantation of DPSCs in animal models have provided positive results with very few exceptions [40].

Aspects of DPSCs isolation and handling before implantation can exert a significant influence in bone tissue formation [44]. For example, specific markers have been proposed as indicators of DPSCs' enhanced osteogenic potential such as STRO-1, flk-1, CD34 and very recently a combination of low CD271 with high CD90 and thus have been used for the isolation of specific cell subgroups [19, 45–47]. The number of passages required to generate sufficient cells for clinical application may affect the potency of the cultured cells, while different culture conditions may affect osteogenic lineage differentiation [36]. It is well known that DPSCs are able to differentiate into osteoblasts when cultured for 3–4 weeks in a medium supplemented with dexamethasone, β-glycerophosphate and ascorbic acid [36, 48]. However, the use of standard medium supplemented with 20% FBS has been proposed as equally effective [19], whereas human serum instead of bovine serum is considered more clinically relevant [49, 50]. Other components or additives of the culture medium can also affect the equilibrium between stemness maintenance and cellular differentiation. Indicatively, human DPSCs exposed in vitro to optimal concentrations of small molecules (pluripotin/SC-1/6-bromoindirubin-3-oxime, rapamysin) showed an increased expression of STRO-1, NANOG, OCT4 and SOX2, but a diminished differentiation into odontogenic/osteogenic, adipogenic and neurogenic lineages [51].

Critical for clinical use is the ability of DPSCs to maintain their characteristics after cryopreservation for years. Several studies reported no changes in cell viability and stem cell marker expression as a result of cryopreservation using controlled cooling rates but no cryopreservant [52–54]. Under such conditions, osteoblasts differentiated from DPSCs, as well as DPSCs themselves were still capable of restarting proliferation, differentiating and producing mineralized matrix [55, 56]. The choice of the culture medium and the pre-treatment of DPSCs also depend on the type of the defect. Ectopic bone formation in subcutaneous pockets particularly might require osteoinductive supplementation since this model lacks pre-osteogenic signaling cascades that develop due to injury and exerted mechanical forces [44].

Stem Cells from Human Exfoliated Deciduous Teeth (SHEDs)

SHEDs represent a unique and easily accessible source of MSCs. A significant advantage of these cells is that they are taken from deciduous teeth, a temporary tissue that normally is rejected. They are distinct from DPSCs due to their higher proliferation rate, increased population doublings and an ability to form sphere-like clusters in culture. SHEDs express the typical MSCs markers (CD105, CD146, STRO-1, and CD29; are negative for CD31, CD34) but also the embryonic stem cell markers Oct4 and Nanog, the neural stem cells marker nestin and the stage-specific embryonic antigens SSEA-3 and SSEA-4 [57]. SHEDs exhibit higher plasticity than DPSCs and are able to differentiate in vitro into odontoblasts, osteoblasts, adipocytes, myoblasts, chondroblasts, endothelial cells, neural cells and hepatocytes [58–62]. The addition of dexamethasone, L-ascorbate-2-phosphate and inorganic phosphate in the culture medium led to the production of mineralized tissue within 4 weeks, while further addition of human recombinant BMP-4 promoted their osteogenic induction [57].

In numerous studies, SHEDs seeded onto several carriers (tooth slices, HA/TCP or a polyethylene glycol(PEG)ylated fibrin carrier in combination with dentin discs) and subsequently implanted subcutaneously into immunocompromised mice, were able to differentiate in odontoblast-like cells. These cells were associated with a dentin-like structure and produced a tissue very similar to human dental pulp [57, 58, 63–66]. However, they are not able to reconstitute a dentin-pulp-like complex like the one formed by DPSCs. SHEDs in combination with HA/TCP scaffolds are also capable of inducing new bone formation not by differentiating directly into osteoblasts but probably by recruiting host osteogenic cells around the ectopic transplant [57].

Stem Cells from the Apical Papilla (SCAP)

SCAP are a population of MSCs isolated from the apical papilla, a soft tissue located at the root apex of developing teeth [67, 68]. These cells appear to be the source of the primary odontoblasts responsible for the formation of root dentin. They are located mainly around vessels, show higher proliferation rate and mineralization potential than DPSCs, express typical MSC markers (CD73, CD44, CD105, CD146, CD166, CD24, STRO-1, CD34) and exhibit a distinct gene expression profile from that of DPSCs [25, 67]. Like DPSCs and SHEDs, SCAP can differentiate in vitro into odontoblasts and form 3D osteodentin structures. SCAP represent a population of cells from a developing tissue and might thus exhibit greater plasticity than other stem cells of dental origin. In culture, following induction they can differentiate into osteoblasts, adipocytes and hepatocyte-like cells [20, 67]. Additionally, ex vivo expanded SCAP, without any stimulation, express several neural markers that are further increased after neurogenic stimulation [68].

SCAP transplanted subcutaneously in immunocompromised mice lead to the formation of a typical dentin-pulp-like complex [67, 69], while a vascularized pulp-like tissue is formed when these cells are transplanted into root canals [70]. Regarding their in vivo osteogenic potential, SCAP could generate a cement/woven bone-like tissue with embedded cementocyte/osteocyte-like cells when transplanted subcutaneously in combination with porous ceramic discs into immunocompromised mice for 8 weeks, but the authors could not identify if the tissue was bone, dentin or cementum [71].

Dental Follicle Stem Cells (DFSCs)

This population of dental tissue-derived stem cells is isolated from the dental follicle, an ectomesenschymal tissue that surrounds the developing tooth germ prior to eruption. DFSCs are considered the progenitor cells for the formation of all periodontium tissues including cementum, periodontal ligament and alveolar bone [72]. Similar to SCAP, the fact that DFSCs are isolated from developing tissues could provide them with an advantage compared to other stem cells of dental tissue origin [73]. DFSCs exhibit a considerable proliferative ability and express similar surface antigen markers with the other dental stem cells (positive for CD105, CD44, CD29, negative for CD34, CD117), as well as the putative stem cell markers Notch-1 and nestin [74]. Under the appropriate inductive conditions, DFSCs can differentiate in vitro into osteoblasts, cementoblasts, chondrocytes, hepatocytes, adipocytes and neural cells [20, 75–78]. Long-term cultures of DFSCs with dexamethasone produce membrane-like structures containing compact calcified nodules [79, 80]. Ex vivo expanded mouse or bovine DFSCs transplanted into immunosuppressed mice are capable of forming cementum- or PDL-like structures [81, 82].

Hard tissue formation by DFSCs is still a matter of debate. In studies conducted in immunocompromised mice, the subcutaneous transplantation of DFSCs was reported to had no effect in the formation of any type of hard tissue (cementum or bone) [72, 79, 83] or to lead in bone tissue formation when DFCSs were combined with porous ceramic discs [71].

Stem Cells from the Periodontal Ligament (PDLSCs)

Stem cells from the periodontal ligament are isolated from the roots of extracted teeth, mostly impacted third molars, after enzymatic digestion or explant culture of the PDL. They express the typical MSC markers (STRO-1, CD44, CD90, CD105, CD146) and also high levels of the tendon-specific marker scleraxis and several markers related to osteoblasts and cementoblasts [84, 85]. Under the appropriate inductive conditions, PDLSCs have the ability to differentiate into odontoblasts/osteoblasts forming calcified nodules, cementoblast-like cells,

adipocytes, chondrocytes, neurons, hepatocytes and also form connective tissue rich in collagen type I [67, 69, 84, 86–90]. They have a similar proliferative potential with DPSCs but an apparent higher ability for differentiation into cementoblasts than in osteoblasts [91]. Formation of a typical cementum/PDL-like complex was reported after subcutaneous transplantation of PDLSCs into the dorsal surfaces of immunocompromised mice [84]. Bone formation has been observed in cultures of human PDLSCs with different 3D-scaffolds like fibrin sponges and bovine-derived substrates [92]. Additionally, PDLSCs isolated from dogs developed well on polymer scaffolds and the constructs generated new bone after subcutaneous transplantation in animals of the same species [93].

Gingival Mesenchymal Stem Cells (GMSCs)

This population of MSCs recently isolated from the human gingiva possesses all the stem cell-like properties such as clonogenicity, self-renewal ability, multipotent differentiation capacity and immunomodulatory properties. These cells display a higher proliferation rate than BMSCs, express typical CD markers of MSCs and also Oct4, Sox2, Nanog, nestin, SSEA-4, STRO-1. They can differentiate in vitro into osteoblasts, chondroblasts, adipocytes and neural cells [94–96]. Under typical osteogenic culture conditions, GMSCs show similar osteogenic ability with the BMSCs [97], while, when encapsulated in a RGD (arginine-glycine-aspartic acid tripeptide)-coupled alginate microencapsulation system, their osteogenic differentiation capability becomes inferior to both BMSCs and PDLSCs [98]. GMSCs have been seeded onto HA/TCP scaffolds and were subsequently incubated in osteogenic medium and transplanted subcutaneously in the dorsal surface of immunocompromised mice. This has led to an increase in the expression levels of osteocalcin, osteopontin and collagen I, but not to the formation of any hard tissue [96, 97].

Alveoral-Bone Derived Mesenchymal Stem Cells (ABMSCs)

Mesenchymal stem cells have been isolated from the alveoral bone and they exhibit a fibroblast-like morphology, plastic adherence and colony formation capacity [99]. They express relevant MSC markers and display osteogenic, chondrogenic and adipogenic differentiation potential [99–101]. Expanded ABMSCs demonstrate in vitro high ALP expression. Several treatments can enhance osteogenesis in these cells [99]. They have also been used to test a new fabric (CS (chitosan)/HA (hydroxyapatite)) as a scaffold for bone tissue engineering and the results were excellent, with the cells showing improved differentiation capacity, increased ALP expression and calcium accumulation in vitro [102]. Following subcutaneous transplantation into immunodeficient mice, human ABMSCs induced new bone formation with a significant activity of cuboidal osteoblasts and osteocytes [99].

Tooth Germ Progenitor Cells (TGPCs)

TGPCs refer to a distinct population of progenitor cells identified in the dental mesenchyme of the third molar tooth germ during the late bell stage [75]. These cells express MSC-associated markers, such as STRO-1 and several CDs and an ability to differentiate in vitro into adipocytes, osteoblasts, odontoblasts, chondrocytes, neurons, hepatocytes, while they also seem to contribute to vascularization [75, 78]. TGPCs/HA subcutaneous implants in immunocompromised rats have shown new bone formation with the presence of osteocytes in the newly formed bone matrix and active osteoblasts lining on the matrix surface [75].

The differentiation properties of dental stem cells mentioned in this chapter are summarized in Table 1.

Table 1 Categories and differentiation potential of dental-tissue derived mesenchymal stem cells

	Source	In vitro multipotency	In vivo ectopic tissue formation
DPSCs [7]	Pulp adult teeth	Osteogenic, dentinogenic, adipogenic, chondrogenic, neurogenic, myogenic, cardiomyogenic, hepatocyte-like cell, melanocyte	Dentin-pulp-like complex
			Bone-like tissues
			Adipose
			Muscle
SHEDs [57]	Pulp deciduous teeth	Osteogenic, dentinogenic, adipogenic, chondrogenic, neurogenic, myogenic, bone induction, endothelial cell	Pulp-like tissues Bone-like tissues
SCAP [67]	apical papilla	Osteogenic, dentinogenic, adipogenic, chondrogenic, neurogenic, hepatocyte-like cell	Dentin-pulp like complex
			Bone-like tissues
DFSCs [72, 80]	Dental follicle	Cementogenic, osteogenic, adipogenic, chondrogenic, neurogenic, hepatocyte-like cell	Periodontal tissues (alveolar bone, PDL, cementum)
PDLSCs [84]	Periodontal ligament	Osteogenic, cementogenic, adipogenic, chondrogenic, neurogenic	Periodontal tissues (cementum, PDL)
GMSCs [96]	Gingiva	Adipogenic, chondrogenic, osteogenic, neurogenic, endoderm cell	Cartilage
			Bone
			Muscle
ABMSCs [99]	Alveolar bone	Adipogenic, chondrogenic, osteogenic	Bone
TPSCs [75]	Dental mesenchyme	Adipogenic, chondrogenic, osteogenic, odontogenic, neurogenic, hepatogenic, endothelial cell	Bone

First reference for each category is provided in brackets in the first column

Dental Stem Cells in Craniofacial Bone Healing

The craniofacial structures constitute the epitome of the vertebrate development as they consist of an incongruous medley of tissues derived from the coordinated integration of each germ layer tissue (i.e., ectoderm, mesoderm, and endoderm) [103]. For example, the anterior section of the calvaria originates from neural crest cells and the parietal bones from mesodermal cells. Specifically, the craniofacial mesenchymal tissues originate from the neural crest, paraxial and lateral mesoderm, with the contribution of the cranial neural crest being the most dominant [104]. Indeed, when the cranial neural crest cells migrate superficially, they demonstrate a stounding multilineage differentiation potential towards cells that generate the majority of the components of the craniofacial complex. Whether the neural crest or mesodermal origin of a bone is related to its function and healing potential remains elusive. However there are convincing insights that the two cell populations exhibit molecular differences that are reflected to their osteogenic potential and regenerative capacity [105, 106]. Further investigation is needed to delineate the possible involvement of these embryological differences to the healing capacity of the discrete craniofacial parts.

Calvarial Bone Defects

Experimental Models of Calvarial Bone Defects

Bone tissue reconstruction in the craniofacial area is typically required in cases of trauma, skeletal diseases, congenital malformations and cancer surgery and affects the life quality of millions of patients worldwide [107]. The ‘gold standard’ bone grafts, despite their associated limitations (autogenous, allogenous, xenogenous and synthetic) [108], constitute the basic therapeutic modalities. The recently introduced treatment alternatives are based on regenerative medicine and bone tissue engineering [109, 110]

Irrespective of the treatment choice, designing appropriate experimental models to evaluate the efficacy and safety of any regenerative procedure stands out as an absolute necessity. In vivo animal models could provide a link between in vitro studies and clinical trials. Although a single ideal model is currently unavailable, there are specific criteria that a model used for bone tissue engineering should meet [111]. The calvarial bone defect in rodents is frequently used as anorthotopic model to evaluate bone regeneration, as it fulfills the basic requirements of an adequate experimental design [112]. Specifically, the rodent calvarial defect model constitutes a uniform, reproducible and standardized defect that allows radiographic and histological analysis; calvaria is an easily accessible site providing sufficient area for surgical handling; the dura mater and the overlying skin provide adequate support for the grafts without the need of any kind of fixation; includes

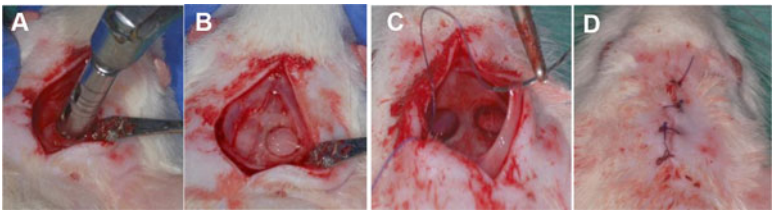

Fig. 1 Surgical procedure in calvaria defects. (**a**) Preparation of the surgical area. (**b**) Creation of the 5-mm critical size defect in the rat calvarium. (**c**) Closure of the periosteum. (**d**) Closure of the overlaying skin [121]. Reproduced upon permission from Elsevier

both cortical and cancellous bone; it is inexpensive and widely used, thus allowing for direct comparisons between the different graft materials; it has a fast healing time [113–116].

An important parameter in the design of the calvarial bone defect is its size. The most widely accepted defect size in assessing the osteogenic capacity of different grafts is the critical-size defect (CSD) [117]. CSDs were originally defined as "the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal" [118] and were later established at the calvaria of rats as the 8 mm [119] or the paired 5 mm cranial defect [120] (see also Fig. 1). Although the value of the CSDs has been keenly criticized [122], it still remains the most encountered osseous defect in the literature and constitutes the 'gold standard' experimental model in appraising craniofacial regeneration.

Dental Stem Cells and Bone Repair in the Calvarial Experimental Model

The rodent calvarial bone defect model has been employed extensively as an orthotopic experimental design to assess the bone regeneration capacity of the DPSCs in the craniofacial complex. Following the first isolation of MSCs derived from the dental tissues [7], a huge impetus towards the extensive study of their stem cell properties has been noted [69]. Within the context of craniofacial bone engineering, DPSCs constitute a favorable population of adult MSCs for several reasons: they have common developmental origin from the cranial neural crest with the craniofacial bones [123]; they are easily harvested; they exhibit enhanced osteogenic potential in vitro and in vivo compared to other MSC populations [10, 12, 44, 46] and they retain their 'stemness' after cryopreservation [54, 56].

As mentioned previously, a series of in vitro studies have clearly demonstrated the capacity of the c-kit$^+$/CD34$^+$/CD45$^-$ sub-population from the mixed DPSCs (SBP-DPSCs) to produce two- and three-dimensional structures of mature bone [19, 55, 124]. Further research on the regenerative efficacy of a selected subpopulation of DPSCs (cells positive for c-kit$^+$/CD34$^+$/STRO-1$^+$) seeded on collagen or fibroin scaffolds, has demonstrated exceptional bone healing of critical-size calvaria

defects in immunosuppressed rats. The human cells were detected in the areas of newly-formed bone and the enhanced bone healing was attributed also to the increased angiogenic potential of the selected stem cell populations [50, 125, 126]. Similar results were obtained recently with a selected $CD271^{low+}/CD90^{high+}$ DPSC population. These cells mixed with Matrigel Matrix and implanted into critical-size calvaria defects in immunodeficient mice survived throughout a 4-week period and promoted significantly bone regeneration [47]. These studies have shown that selective DPSC subpopulations, with enriched osteogenic and angiogenic potential, are of utmost importance for an optimal result. The healing properties of the entire population of human dental pulp cells have also been demonstrated in the rat calvaria model [121]. In our study, dental pulp cells isolated from human third molars were transplanted in the 5 mm calvaria defects of immunocompetent rats following a short osteoinduction period in culture. An ECM-mimicking hyaluronic-based scaffold was used as the cell carrier. Enhanced bone regeneration was detected by histomorphometry in the cell-treated defects, compared to the scaffold-treated and untreated ones (Fig. 2). However, in contrast to the complete bone healing observed in the afore-mentioned studies [50, 125, 126], none of the defects exhibited complete bone regeneration in our study. The mechanisms underlying this incomplete bone regeneration may relate to several factors, including the lower osteo/angiogenic potential of the mixed pulp population used and the type and properties of the scaffold employed that was not degraded properly, thus entrapping many of the pulp cells in it. The latter resulted in inflammatory reactions around the non-degraded scaffold islets that contained dead pulp cells (Fig. 2). Though inflammatory signals have a stimulating role in the beginning of the healing process, the host's response that was detected at a later stage due to pulp cells' entrapment might have hampered the repair procedure. Despite the incomplete bone restoration, the significantly higher percent of new bone formation in the cell-treated group of this study, compared to the control groups, support the osteogenic efficacy of the entire dental pulp cell population. At the same time, these results underline the need for further improvement of our knowledge on the scaffolds' in vivo properties and bio-behaviors.

Stem cell-based bone regeneration is critically relayed on the properties of the stem cell populations used. These properties mostly refer to their osteogenic potential. However, additional characteristics may contribute to their efficiency in bone healing, such as their interplay with the host tissue immune response [127] and their paracrine 'trophic activity' to adjacent cells [128]. Although the bone regenerative properties of DPSCs in vivo have been shown in the calvarial defects of immunosuppressed rats [50, 125, 126], still little is known about the sequence of events following implantation of the DPSCs. More specifically, studies investigating the biological cues in the defect area after the implantation of the biocomplexes are lacking. The DPSCs-induced immunological response and the paracrine action of the DPSCs warrants for further investigation.

Another important parameter that needs to be taken into consideration in bone regeneration is the type of scaffold employed. These three-dimensional matrices accommodate the dental stem cell populations by forming biocomplexes. The

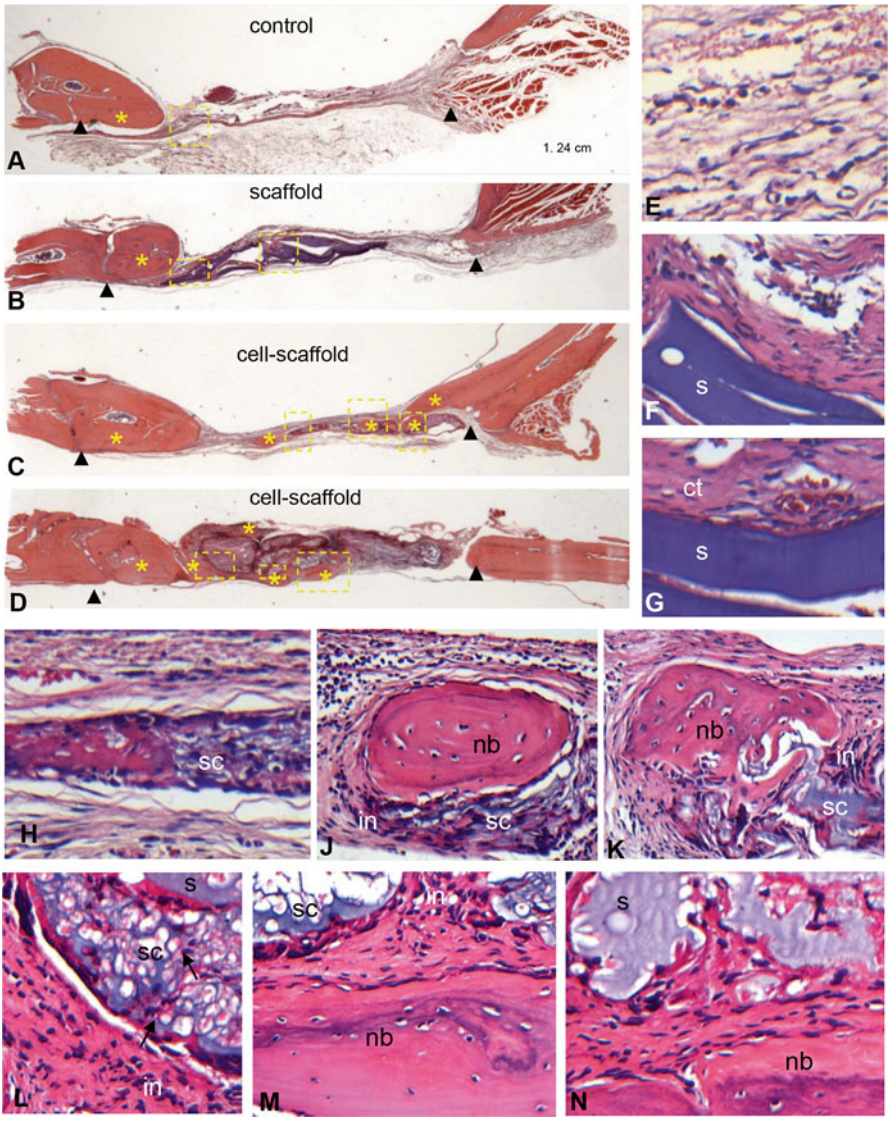

Fig. 2 Histological findings in haematoxylin and eosin stained calvaria sections. Representative low magnification (×1.25) photomicrographs of the defect site in the control (**a**), scaffold (**b**) and cell-scaffold (**c**, **d**) groups. *Arrowheads* indicate defect margins. Areas of new bone formation are marked by *asterisks*. *Rectangles* indicate sites within defect for which higher magnification (×20) images (**e**–**n**) are provided. (**e**): connective tissue (ct) in the framed area of the control group; (**f**, **g**): large areas of scaffold (s) remnants surrounded by dense connective tissue in the framed area of the scaffold group; (**h**–**n**): non-degraded scaffold with entrapped cells (sc), and sites of new bone (nb) formation within the defect in the framed areas of the cell-scaffold group in C (**h**–**k**) and D (**l**–**n**). Note the inflammation (in) around areas of cell-scaffold remnants and dead cells into the scaffold (*arrows* in L) [121]. Reproduced upon permission from Elsevier

constructs are either cultured in vitro leading to the ex vivo production of bone that is subsequently implanted in the osseous defect or they are implanted directly to the site inducing in vivo bone regeneration [129]. In general, the types of scaffolds used to regenerate calvaria bones can be divided into ceramics and polymers (synthetic or natural) and it is noteworthy that the cell-scaffold interactions as well as the interplay between the bioengineered construct and the host tissue can profoundly affect bone regeneration [130]. In a study assessing calvaria bone regeneration after the transplantation of BMSCs-loaded collagen-glycozaminoglycan constructs in rodents, the initial inflammation induced by the biocomplex led to incomplete bone healing [131, 132]. According to the authors, this host response accelerated the degradation of the scaffold, which in turn impaired the bone matrix deposition [132]. In our study, the utterly different host response to the dental pulp cell-biocomplexes, compared to the inflammatory response towards the cell-free scaffolds, was the major cause of the non-optimal repair of the defects [121].

Besides from DPSCs, stem cells from exfoliated deciduous teeth (SHEDs) have also been successfully used to regenerate critical size bone defects in the calvaria rat model [133–135]. Similarly to the DPSCs, the neural crest origin of the SHEDs and their enhanced stemness make them excellent candidates for craniofacial bone tissue engineering.

Bioengineered constructs comprised of stem cells from the periodontal ligament (PDLCs) have been used to regenerate bone in calvarial defects with very encouraging results, thus rendering this treatment modality a viable option in the field of craniofacial bone regeneration [98, 136, 137].

Finally, stem cells from the dental follicle (DFSCs) have shown enhanced bone formation when transferred as scaffoldless transplants in 8 mm critical-size calvarial defects of immunodeficient rats [138].

Overall, the use of dental stem cells in craniofacial bone engineering seems justified. In particular, the results from the transplantation of DPSCs in the calvarial experimental model render this stem cell-based treatment a quite promising future treatment modality for repairing defects in the craniofacial area. Alternatively, stem cell populations from other dental tissues displaying enhanced osteogenic capacity and sharing common developmental origin with the craniofacial bone structures may also prove beneficial in the cranial bone tissue engineering.

Mandibular and Alveolar Bone Defects

Mandibular bone defects due to traumas, neoplastic tumors or other pathological conditions are challenging problems for oral and maxillofacial surgeons. In most cases, repair of such defects requires bone grafts for restoration of continuity, function and aesthetics. In dentistry, bone grafts are mainly applied in order to repair alveolar bone defects caused by periodontal or peri-implant disease and to preserve or reconstruct the alveolar ridge for future implant placement. Dental stem cells and tissue engineering technology in oral and maxillofacial region stand out as new alternatives for repairing mandibular and alveolar defects.

Experimental Models of Mandibular and Alveolar Bone Defects

The majority of in vivo studies evaluating the application of stem cells of dental origin on mandible defects are performed in experimental animals. Various animal models of mandibular bone defects have been used. Dogs, pigs, rabbits and rats are the most frequently used animals in this field, with dogs and pigs possessing anatomical and physiological features more close to the human ones [139]. The rat model, on the other hand, is dissimilar in terms of size and bone structure compared to humans [36], but is easily handled and cost effective [112].

The type of the mandible defect varies from segmental [140] or cylindrical [141] alveolar defects to orofacial defects in the parasymphyseal region of the mandible [142] or in front of the mandibular corner [143]. All of the above defects are considered critical size defects, in which by definition complete bone healing does not spontaneously occur and therapeutical intervention is needed [118]. The efficacy of dental stem cells on bone regeneration has also been tested in a mandibular distraction osteogenesis model in rabbits [144]. In the clinical setting, alveolar defects of the mandible, produced after extraction of impacted third molars, have been treated with autologous DPSCs seeded onto collagen sponge scaffolds [145].

Alveolar defects of the mandible often linked with periodontal degeneration may result in loss of cementum, periodontal ligament and alveolar bone. Animal models simulating these conditions have been also used for evaluating the regenerative efficacy of dental stem cells. Experimental models of periodontitis are surgically created and can be classified to chronic, acute and acute/chronic ones [146]. Models of chronic defects arise from the reproduction of conditions that induce periodontal tissue loss, such as the placement of orthodontic elastics around the teeth at or slightly apical to the gingival margin [147]. The models of acute defects are created by surgical removal of bone, cementum and periodontal ligament, while the insertion of a foreign body in the surgically created acute defect leads to an acute/chronic defect. The type of acute defect displays heterogeneity; furcation defects [148], intrabony defects in the mesial or distal aspect of the tooth [149] or fenestration defects [150] have been used. Acute defect models have been used for testing bone regenerative properties of dental stem cells, due to their cost-effectiveness and the reduction of experimental time. Regarding the acute/chronic defect, Liu et al. [151] created such defects in the mesial region of mandibular first molars of pigs by surgical removal of bone and subsequent silk ligament suture. Similarly, Park et al. [152] created surgically circumferential defects in the mesial root of mandibular premolars of dogs, which were then filled with a rubber base impression material. The acute/chronic model resembles ‘true’ periodontitis and is the one recommended in periodontal regeneration research. In cases of bone regeneration around dental implants, experimental models in dogs have been used to examine the effect of dental stem cells on peri-implant bone regeneration [153–155].

Unfortunately, there is no ideal experimental model and the selection of appropriate animal and defect model for each clinical application is complicated [146]. The establishment of reliable and validated animal models is necessary for evaluating and comparing the effect of stem cell-based therapies on mandibular bone regeneration.

Dental Stem Cells in the Repair of Mandibular and Alveolar Defects

In addition to the proven in vitro and ectopic in vivo osteogenic properties of DPSCs and SHEDs, these cells exhibit great regenerative capacity when transplanted orthotopically in critical-sized mandibular defects. Dental pulp stem cells from deciduous or permanent teeth, enriched with platelet rich plasma (PRP), enhanced bone formation in cylindrical alveolar defects of the mandible in dogs [140–143]. Importantly, the stem cells of dental origin showed bone regeneration capacity similar to that of BMSCs, which have been considered the gold standard in bone regenerative medicine [141].

Growth factors, scaffolds and stem cells, constitute the three key elements for bone tissue engineering. Growth factors, such as the recombinant human bone morphogenetic protein-2 (rhBMP-2,) promote the osteogenic capacity of the DPSCs. Autologous DPSCs combined with rhBMP-2 induced more new bone formation than even the autologous bone, when engrafted in segmental alveolar defects in rabbits [140]. The osteogenic properties of DPSCs appear to be scaffold-dependent. The resorption rate of the scaffold material is of paramount importance and, thus, mixing DPSCs into biomaterials with suitable resorption rates creates a synergetic effect that enhances bone regeneration [143].

In the only human study so far, autologous DPSCs seeded onto collagen sponge scaffolds successfully repaired an alveolar mandibular defect produced after extraction of an impacted third molar [145]. A three-year follow-up study revealed that the regenerated bone was uniformly vascularized and qualitatively compact, though differed from the physiological cancellous (spongy) bone [45]. The formation of compact bone by the DPSC-grafts is alleged to have positive clinical impact, favoring implant stability and rehabilitation of large bone defects in oral cancer patients [36, 45]. However, this alteration in the regenerated bone type raises some concern on the possible diversion of the transplanted DPSCs' fate towards undesirable cells and tissues [156]. Further studies are required to assure the absolute control on the behavior of the transplanted multipotent cells in the host environment.

For periodontal tissue regeneration, PDLSCs are considered to be the best dental stem cell type. Several animal studies have evaluated the potential of PDLSCs for regeneration of alveolar periodontal defects and have shown that these cells induce new bone, cementum and periodontal ligament formation in the treated defects [148–152]. In a clinical case series, the application of autologous PDLSCs for the treatment of periodontal defects led to improved periodontal parameters in all cases [157]. DFSCs may be an alternative to PDLCs for periodontal regeneration, although not an equally effective one [152]. Although DPSCs exhibit great bone regeneration capacity, their potential for periodontal regeneration is doubtful due to their limited capacity to form cementum [152, 158]. Instead, SCAP have shown to possess the ability to form PDL-like, cementum-like and bone-like tissues in an in vivo ectopic transplantation rat model [158], but there is lack of evidence regarding their potential to repair periodontal defects. Similarly, there is no sufficient data supporting the use of SHEDs for periodontal regeneration.

As mentioned above, there are few studies evaluating the effect of stem cells of dental origin on peri-implant bone regeneration. PDLSCs have been shown to enhance alveolar bone regeneration in surgically created peri-implant saddle-like defects, although to a lesser extent than BMSCs [154]. Notably, the osseointegration of dental implants, assessed by measuring bone/implant contact, was higher in DPSCs/PRP-treated alveolar defects, compared to analogous BMSCs/PRP-, periosteal cells/PRP-treated and untreated defects [153]. Furthermore, Omori et al. [155] suggested that treatment of Ti implants with SHED-conditioned medium promotes bone morphogenesis not only around the implant interface, but also at distant locations from the implant surface, thus improving early osseointegration.

Dental Stem Cells in Long Bone Healing

During osteogenesis, the long bones of the body are formed by endochondral ossification through distinct steps that include: differentiation of mesenchymal stem cells into chondrocytes; proliferation, hypertrophy and cartilage production by the chondrocytes; differentiation of osteoblasts and bone matrix deposition. In adult bone repair, both endochondral and intramembranous ossification (i.e. without prior cartilage formation) participate in the healing process depending on the degree of fracture stabilization.

The need to enhance the healing of long bone fractures is well appreciated, especially in cases of unsuccessful unions or non-unions. The use of autologous or allogenic bone grafts is the current gold standard. However, this application is accompanied with severe drawbacks including invasiveness, limited graft volume and bone loss at the donor site. Bone tissue engineering, by using isolated bone marrow stem cells in combination with osteoinductive or osteoconductive scaffolds, emerges as a promising alternative to bone transplants. Animal studies in rodents, rabbits, or bigger mammals have used BMSCs to successfully treat long bone fractures [159]. In these models of bone regeneration, BMSCs have been mostly isolated from same-species donors and the stem cells have been used in combination with scaffolds of various types, including calcifying agents, synthetic polymers and ECM-mimicking biomaterials. In some occasions, scaffold-free approaches have also been used, where the cells are applied as multilayered cell sheets produced in culture prior to transplantation [160, 161]. In the majority of the published animal data, the mesenchymal stem cells enhanced the healing process [6]. The ongoing clinical trials in the field, using therapeutic approaches based on the use of bone marrow stem cells/osteoinductive scaffolds have not yet provided published results [162].

The osteogenic properties of DPSCs are quite comparable with those of stromal BMSCs, both in vitro and upon ectopic transplantation [7, 10]. During the last decade DPSCs have been used in animal models of craniofacial bone healing with promising results. However, neither DPSCs nor other stem cells of dental origin have been tested in long bone repair. Given the mesenchymal origin and properties

of DPSCs, we were interested to examine the efficacy of dental pulp cells, isolated from human impacted third molars, in the restoration of osteotomized rat tibia. In humans, the dental pulp from impacted immature third molars that are extracted from healthy young subjects is the preferred source of DPSCs. Additionally, dental pulp contains a substantial population of progenitor cells conferring a niche for the 'true' stem cells [163]. We therefore used the entire population of dental pulp cells instead of isolating the DPSCs. The presence of typical mesenchymal stem cell markers and the osteo-differentiation potential of the entire dental pulp population were verified in vitro by flow cytometry. To avoid reduction of the stemness of the pulp cell population following osteo-differentiation and/or repeated subcultures [164, 165], early passage, undifferentiated, pulp cells were transferred into the fracture site. It was also decided to follow a scaffold-less transfer of the cells in order to clearly detect any stem cell-induced effect. Osteotomies were performed in adult male rats and stabilized with intramedullary nails (Fig. 3). The healing process was monitored radiologically for 8 weeks. Quantification of the X-ray films showed a significantly smaller and more homogenous callus in the cell-treated rats compared to the controls, denoting advanced bone healing in the former group. The radiological results were further supported by the histological observations at 8 weeks post surgery [166]. Cell-treated animals exhibited a remodeled bone marrow cavity and higher percentage of lamellar new bone, compared to the vehicle-treated ones that had a higher score of non-bridging and scar tissue (Fig. 4).

To our knowledge, this is a first approach to examine in vivo the effectiveness of dental pulp cells to heal long bone fractures. The results provide evidence that the whole population of dental pulp cells from adult human dentition can promote bone healing in a rat osteotomy model. The developed model was purposely kept simple (i.e. scaffold-less, without specific cell sorting, without immunosuppression of the

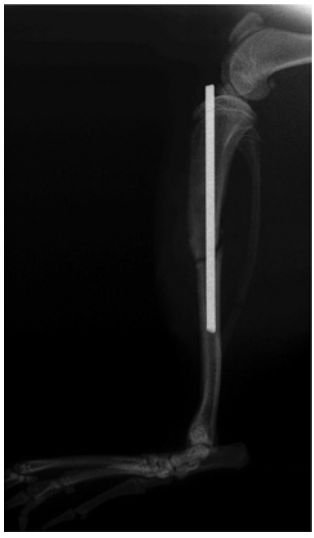

Fig. 3 Representative X-ray of the rat tibia fracture model used in the study of [166]

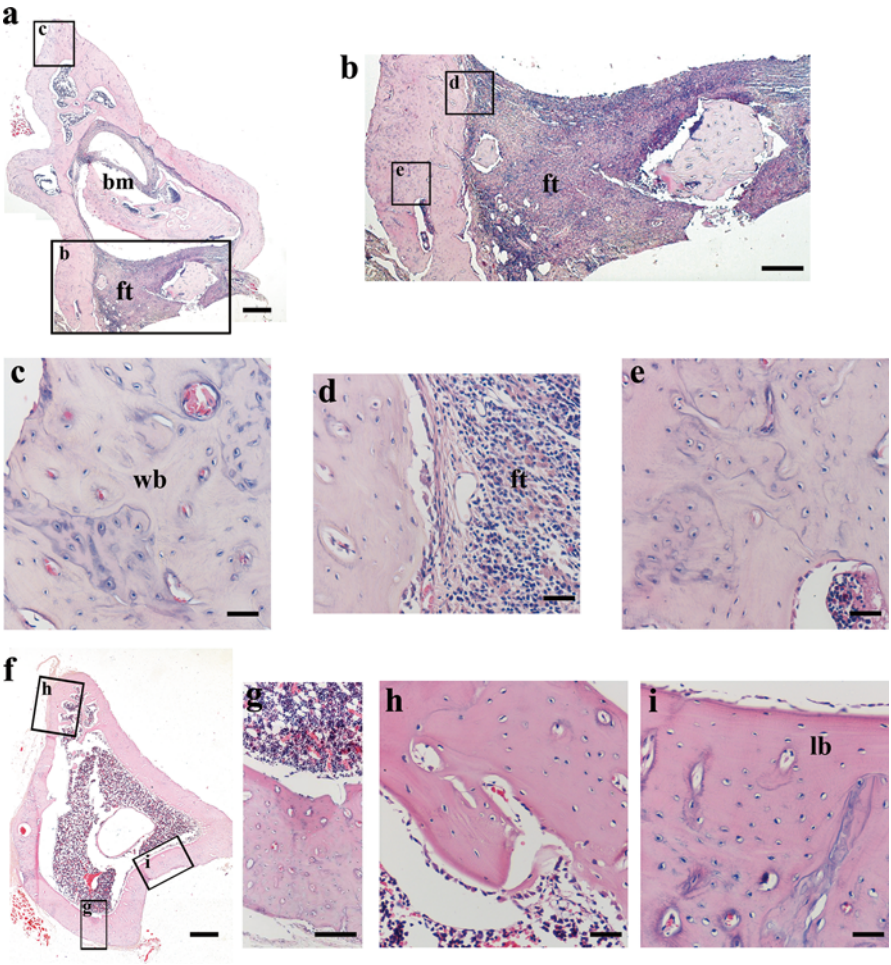

Fig. 4 Representative histological sections stained with haematoxylin and eosin (H&E) from rat tibias at 8 weeks post-fracture of control (**a**–**e**) and dental pulp cell-treated (**f**–**i**) animals [166]. H&E stained sections show smaller bone marrow cavity (**a**), non-bridging (**a**, **b**), extended fibrous tissue (**a**, **b**, **d**) and mostly woven bone (**c**, **e**) in the controls, compared to cell-treated (**f**–**i**). *Framed areas* in **a**, **b** and **f** refer to higher magnification images, according to the letter inside the *upper left side* of the frame. *bm* bone marrow, *ft* fibrous tissue, *lb* lamellar bone, *wb* woven bone. Reproduced upon permission from J Sci Med Central

host that could potentially hinder healing initiation; [2]), in order to investigate a potential cellular effect. Further refinement of the protocol is necessary to delineate the optimal conditions for the best biological outcome. Additionally, there is need for detailed characterization of the cellular interactions (including dental pulp cells' fate in the host tissue) and the molecular responses during the process of bore repair.

Conclusion and Future Perspectives

For successful cell-based bone repair, the candidate stem/progenitor cell population must fulfill the following key criteria: (a) to differentiate into bone forming cells or to stimulate endogenous tissue repair, (b) to impede uncontrolled growth and differentiation, (c) to be tolerated by the host immune system, (d) to support neoangiogenesis and blood supply, (e) to form biocomplexes with scaffolding materials, (f) to be easily harvested in a harmless manner, (g) to retain stemness upon cryopreservation and (h) to be cost effective.

Experimental evidence from mesenchymal stem cells of dental origin (DSCs) suggests that several types of these stem cells can satisfy the above criteria. Indeed, they are responsive to osteogenic signals and exhibit a bone forming potential comparable to that of BMSCs. Their origin (adult mesenchymal stem cells) assures a minimal oncogenic potential compared to pluripotent cells. DSCs are well tolerated in the fracture site and some categories have immunosuppressive properties. Discrete subpopulations of DSCs have been shown to promote new vessel formation at the fracture site. Most types of DSCs can form composite grafts with several types of scaffold materials. Importantly, they can be obtained in a non-invasive and low cost procedure and retain their osteogenic properties upon storage.

The results from the use of DSCs in animal models and the one human study of bone repair are encouraging, especially for the restoration of craniofacial bone defects. However, the existence of different models of bone repair in combination with the different subpopulations of DSCs and scaffolds used, has not allowed all proper comparisons. More studies are thus required to define the most valuable healing approach in terms of the bone defect, and the cell population/scaffold used. Furthermore, there is need to expand our knowledge on the biobehavior of the transplanted cells in the in vivo situation, regarding their interaction with the carrier material, their cell fate and possible induction properties.

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Neuronal Properties of Dental Stem Cells

Letizia Ferroni, Sara Ricci, and Chiara Gardin

The neurodegenerative disorders are characterized by loss of nerve structure or function. The prevalent disorders are Alzheimer's disease, frontotemporal dementia, amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, and multiple sclerosis. These neurodegenerative diseases are increasing worldwide, not only due to increased incidences of direct or indirect injury to the central nervous system (CNS) but also due to the increase in the percentage of the aging population [1]. Despite great progress in understanding the etiology of these disorders, the underlying mechanisms are still indistinct. Furthermore, no means of treating the underlying cause have been devised. For the past decade, researchers have been interested in stem cells and the prospect of using them for understanding the pathogenesis of disease and for facilitating the development of novel therapeutics [2]. In model organisms, both endogenous and exogenous neural stem cells (NSCs) have been investigated for their capacity to regenerate a damaged nervous system [3]. Due to the low incidence of human adult NSCs and problems with accessibility, the use of exogenous sources of stem cells with neural potential has been suggested as a plausible approach to stem cell therapy. Although embryonic stem cells (ESCs) and bone marrow stem cells (BMSCs) have been assessed as potential candidates for neuronal therapy, adult stem cell populations derived from cranial neural crest cells may possess a greater propensity for neuronal differentiation and repair [4]. Interestingly, the dental pulp tissue, termed "ectomesenchyme", derives from ectodermal cells growing on the periphery of the neural tube during embryonic development, migrating into the oral region, and transdifferentiating into a mesenchymal phenotype [5]. Consequently, Dental Stem Cells (DSCs) have been proposed as a

L. Ferroni • C. Gardin (✉)
Department of Biomedical Sciences, University of Padova, via Ugo Bassi 58/B, 35131 Padova, Italy
e-mail: chiaragardin@gmail.com

S. Ricci
Department of Neurosciences, University of Padova, via Giustiniani 5, 35128 Padova, Italy

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promising source of stem cells to treat nerve regeneration due to their close embryonic origin and easy accessibility. Indeed, DSCs can be obtained from extracted teeth and their surrounding tissue that are usually discarded during dental procedures without invasive methods and additional injury.

Preliminary investigations into the neural potential of human adult DSCs have shown that, under non-neuronal inductive conditions, these cells expressed the neural progenitor marker nestin, and the glial marker glial fibrillary acidic protein (GFAP), at both the gene and protein levels. The DSCs retain their neural crest properties following ex vivo expansion, and express the postmitotic neuron-specific marker neuronal nuclei (NeuN), when cultured under neural inductive conditions [4, 6–8]. Therefore, neural crest-derived adult DSCs exposed to the appropriate environmental cues could differentiate into functionally active neurons and could provide an alternative stem cell source for the treatment of neuronal disorders and injury.

The neural differentiation potential of human adult dental pulp stem cells (DPSCs) was demonstrated by Arthur and colleagues [4]. DPSCs were cultured in neuronal inductive conditions with mitogen factors Epidermal Growth Factor (EGF) and basic Fibroblast Growth Factor (FGF) for 3 weeks. Following 3 weeks of induction, DPSCs acquired a bipolar and stellate morphology consistent with that of sensory and motor neurons, respectively. Assessment of the cell proliferation status of DPSCs over the same time period showed that there was a significant decrease in the proliferation rate of DPSCs cultured in neuronal inductive media compared with non-neuronal inductive condition. Immunocytochemical analysis showed that ex vivo expanded DPSCs constitutively express the proteins nestin and GFAP. While nestin protein expression continued to be detected by the majority of DPSCs following neuronal induction, nestin gene transcription was found to be downregulated at 3 weeks after neuronal induction, correlating to neuronal maturation. Also the expression of βIII-tubulin, a neuronal specific marker expressed during early brain development and downregulated in adult brain [9], was evaluated. Whereas βIII-tubulin protein increased over the 3 week induction period, the mRNA level was found to be downregulated, which also correlated to the observed reduction in nestin gene expression. Moreover, the number of DPSCs expressing either neurofilament (NF) medium (NFM) or NF heavy (NFH) gene or protein were found to be significantly higher following neuronal induction compared with non-neuronal inductive condition [4].

Also human exfoliated deciduous teeth (SHED) showed neural differentiation potential. SHED differ from DPSCs with respect to their higher proliferation rate, increased cell-population doublings, sphere-like cell-cluster formation, osteoinductive capacity in vivo, and failure to reconstitute a dentin-pulp-like complex [8]. Therefore, SHED apparently represent a population of multipotent stem cells that are perhaps more immature than previously examined postnatal stromal stem-cell populations. SHED under non-neuronal inductive conditions express a variety of neural cell markers including nestin, βIII-tubulin, glutamic acid decarboxylase (GAD), NeuN, GFAP, NFM, and 2′,3′-cyclic nucleotide-3′-phosphodiesterase (CNPase). After 4 weeks of neural inductive culture, SHED lost their fibroblastic

morphology developing multicytoplasmic processes and increased the expression levels of neuronal markers including βIII-tubulin, GAD, and NeuN, whereas the levels of nestin, GFAP, NFM, and CNPase remained unchanged [8]. These findings were confirmed by Govindasamy and co-workers that compared the neural differentiation potential of SHED and DPSCs [10]. They observed that, under non-neuronal inductive conditions, the proliferation rate of SHED was higher than that of DPSCs. The fold expression of several pluripotent markers such as OCT4, SOX2, NANOG, and REX1 were higher in SHED as compared with DPSCs. Conversely, DPSCs showed higher expression of neuro-ectodermal markers PAX6, GBX2, and nestin. These data supported the notion that SHED are more primitive or pluripotent cells than DPSCs. Indeed, the overexpression of the transcription factors OCT4, SOX2, and NANOG are responsible for the maintenance of pluripotency in early embryos and ESCs [11]. However, when SHED and DPSCs were cultured in non-coated dishes containing the neuronal induction factors FGF and EGF for 15 days, both cells were capable of forming distinct neurospheres, in which growing cells aggregate in floating spheres. Once attached in coated dishes, the neurospheres derived from SHED and DPSCs spontaneously showed outgrowth and dendrite-like structure and expressed neuronal markers, such as βIII-tubulin, NF and GFAP. Despite SHED and DPSCs came from the same origin, higher neurosphere formation and neuronal marker expression were found in the differentiated DPSCs into neuron-like cells as compared with SHED. Since nestin is essential for the induction of neurospheres [12], the high level of nestin expressed by undifferentiated DPSCs could potentially enable them to differentiate more efficiently than SHED into neuronal cells. Nevertheless, SHED could also be forced to form neuronal cells under the influence of appropriate microenvironment [10].

In a study of Lee and colleagues the neural differentiation potential of DPSCs was compared to that of stem cells from apical papilla (SCAP), and periodontal ligament stem cells (PDLSCs) [13]. After induction in neurogenic medium for 24 h, the morphology of DPSCs, SCAP, and PDLSCs changed into neurite-like cells, including cell processes. Most of the cells were similar in shape to neuronal cells, with very thin and long cytoplasmic processes, resembling axons and dendrites. The neurogenic potential of DPSCs, SCAP, and PDLSCs was analyzed assessing the transcription levels of βIII-tubulin, microtubule-associated protein 2 (MAP2), and GFAP. In each cell line, mRNA level of the mature neuronal marker MAP2 tended to increase after neurogenic induction. The RNA level of βIII-tubulin significantly increased within SCAP and PDLSCs in neurogenic differentiation medium but not in DPSCs. DPSCs showed a significantly increased GFAP mRNA expression under differentiation conditions, whereas SCAP expressed very little GFAP mRNA in both control and differentiation groups. Interestingly, MAP2 and βIII-tubulin mRNAs were also found in undifferentiated conditions. These results suggest that SCAP might have a neural origin and thus have a capacity for neurogenic differentiation. In terms of protein level, MAP2 was strongly expressed in SCAP, DPSCs, and PDLSCs after neurogenic induction. The protein expression of βIII-tubulin increased in DPSCs under differentiation conditions, whereas GFAP was not detected. However, the expressions of neuronal markers in DPSCs were lower than

those in SCAP and PDLSCs. These results indicate that PDLSCs and SCAP, as well as DPSCs, may be appropriate cell sources for neuronal regeneration [13].

Altogether these in vitro studies support the finding that DSCs are capable of differentiating into neuronal cells when cultured under appropriate inductive conditions basing on analyses of cellular morphology and expression of early neuronal markers. However, functional neurons express voltage-gated potassium, sodium, and calcium channels, which are required for the generation and propagation of action potentials [14]. The protocol of Arthur and co-workers demonstrated that DPSCs can be differentiated into neuronal-like cells by means of an inductive medium supplemented with FGF and EGF. Their protocol, however, resulted in an incomplete neuronal differentiation, since only voltage gated sodium channels could be detected without the presence of voltage gated potassium channels which are also regarded as a basic criterion for functional neuronal cell identification [4]. In a later work of Király and colleagues, DPSCs were differentiated into neuronal cells that not only express neuronal markers, but also display simultaneous voltage dependent sodium and potassium channels [15]. Their protocol consists of three steps in 10 days: pretreatment, induction, and maturation. First, DPSCs were seeded onto poly-L-lysine coated surfaces and pretreated with a medium consisting of basic FGF and 5-azacytidine. The 5-azacytidine causes hemi-demethylation of DNA leading to dedifferentiation of partly committed cells to a multipotent state. It was also reported to be a potent maturation inducing factor for neurogenesis [16]. Then, neural induction was performed by basic FGF, nerve growth factor (NGF), and neurotrophin-3 (NT-3), and the simultaneous activation of protein kinase C (PKC) and cyclic adenosine monophosphate (cAMP) pathways. In cell lines of diverse origins, it was demonstrated that neuron and glia differentiation can be promoted by activation of PKC and cAMP pathways [17]. Finally, maturation of the induced cells was achieved by continuous treatment with NT-3, and other neuroprotective factors [15]. The DPSC-derived neuronal cells generated following the protocol of Király et al. gave good results even after the engraftment into rat brain. The DPSC-derived neuronal cells were integrated into the host brain, even into the injured cortex, and showed neuronal properties not only by expressing neuron-specific markers but also by exhibiting voltage dependent sodium and potassium channels [18]. However, Király and co-workers did not observe action potential firing by the differentiated cells, addressing the incomplete differentiation of DPSCs to neuronal cells. Recently, Gervois and colleagues have implemented a two-step protocol to improve the differentiation outcome [19]. First, they adopted the neuronal induction step of Arthur's protocol based on FGF and EGF signaling [4]. These signaling molecules are essential to induce the formation of the three-dimensional neurosphere structures, in which neural progenitor cells are close in a favorable microenvironment for the creation of close physical contacts essential for neuronal commitment [20, 21]. The second step concerning the neuronal maturation is based on activation of cAMP and NT-3 signaling pathways, as previously reported by Király [15]. Elevated intracellular cAMP is essential in sustaining neurogenic differentiation of early neuronal committed cells [22], whereas NT-3 signaling is essential for neurogenic maturation [23]. After neuronal differentiation, patch-clamp analysis demonstrated the

functional activity of differentiated DPSCs by the presence of voltage-gated sodium and potassium channels selectively blocked by tetrodotoxin and tetraethylammonium, respectively. Moreover, a subset of neuronal-differentiated DPSCs was able to fire a single action potential. However, a train of repeated action potential firing after stimulation was not observed, which would be the ultimate proof of functional neurons. The failure to fire repeated action potentials might be attributed to the gating kinetics of the delayed rectifier potassium channels, that is resulting in an incomplete repolarization. The incomplete repolarization failed to deactivate sodium channels, which would be necessary for repetitive firing [19].

The above studies have demonstrated the capability of human DSCs to differentiate in neuronal cells when they are cultured in vitro in presence of neuronal inductive conditions. However, interesting results were also achieved after in vivo transplantation of not manipulated DSCs in rodent and avian brain. In particular, neural developmental potential of SHED was studied by injecting them into the dentate gyrus of the hippocampus of immunocompromised mice. Histological examination showed that SHED survived for 10 days inside the mouse brain microenvironment and continued to express neural markers such as NFM [8]. Human DPSCs were found to express neuronal-associated markers (NFM and βIII-tubulin) within 48 h after injection into 4-day-old chicken embryos, indicating a rapid response to endogenous environmental cues [4]. They survived and differentiated into neuronal derivatives, and potentially integrated into host neuronal networks within 7 days. The location of DPSCs into the embryo was essential for their specific neural morphology and differentiation capacity. DPSCs located near sensory trigeminal ganglion neurons displayed a bipolar morphology characteristic of sensory neurons, whereas in the CNS, DPSCs exhibited multidendritic processes associated with motor neurons. These observations suggest that not manipulated DPSCs are able to respond directly to surrounding environment and differentiate into specific neurons when they are transplanted in host brain [4].

The interesting studies of Nosrat and colleagues have demonstrated the capability of DPSCs to promote the survival of sensory neurons of the trigeminal ganglion, and motoneurons of the spinal cord by providing neurotrophic support [24, 25]. It was shown that DPSCs have the ability to produce neurotrophic factors, such as NGF, brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) both in vivo and in vitro. Neurotrophic factors are general modulators of neuronal survival, plasticity, and target innervation and play important roles in axon growth and synaptogenesis [26]. In vivo experiments have demonstrated that NGF, BDNF, and GDNF transcripts are present in the dental pulp at the time of the onset of dental pulp innervation. These neurotrophic factors are crucial in supporting trigeminal nerves as they grow into the developing dental pulp and as the innervation of teeth is established [27]. Primary cultures of DPSCs have also shown the expression of NGF, BDNF, and GDNF mRNA transcripts. Interestingly, the expression levels of these transcripts in DPSCs were upregulated after sub-culturing, suggesting their long-term neurotrophic competence in culture [24]. Generally, the maintenance in vitro of central and peripheral nervous tissues requires the presence of exogenous neurotrophic factors to regulate neuronal

survival and differentiation as well as axonal growth and path-finding [28]. However, in vitro experiments proved that neurotrophic factors produced by DPSCs promote survival of trigeminal neurons and neurite outgrowth [24]. Moreover, an in vitro study demonstrated that the neurotrophic factors produced by DPSCs promote the survival of dopaminergic neurons and provide neuroprotection for dopaminergic neurons against 6-hydroxy-dopamine [25]. DPSCs not only produce neurotrophic factors for supporting the survival of neuronal cells, but also support the homing of endogenous neural stem cells to the injury site during transplantation. In an avian embryonic model system, Arthur and coworkers demonstrated that DPSCs are able to coordinate axon guidance within a receptive host nervous system. In particular, they observed that DPSCs chemoattracted avian trigeminal ganglion axons toward them by the release of the chemokine CXCL12 [29]. The neurotrophic support of DPSCs was also confirmed by ectopically transplantation into the anterior chamber of eye in rats. Dental pulp grafts induced collateral sprouting from existing iris nerve fibers and promoted innervation of the ectopically transplanted dental pulp tissue. Additionally, dental pulp grafts increased the density of catecholaminergic nerve fibers of the irises [24]. Interestingly, dental pulp grafting also promoted the survival of injured motoneurons in rat model of spinal cord injury (SCI), indicating a functional bioactivity of the dental pulp-derived neurotrophic factors in vivo by rescuing motoneurons [24].

Similarly, SHED exhibited therapeutic benefits for recovery after SCI [30]. It was shown that SHED inhibited SCI-induced apoptosis of neurons, astrocytes, and oligodendrocytes, which promoted the preservation of neural fibers and myelin sheaths. SHED also regenerated transected axon through the direct inhibition of multiple axon growth inhibitors signals by paracrine mechanisms. Finally, SHED replaced lost or damaged oligodendrocytes after SCI through specific differentiation into mature oligodendrocytes under the extreme conditions of SCI [30].

SHED have also been proposed as a source of stem cells for the treatment of Parkinson's disease, as it has been demonstrated their differentiation into dopaminergic neurons [31]. Wang and colleagues have first induced SHED into neurospheres in serum-free culture medium supplemented with EGF and basic FGF, and then have induced SHED-derived spheres into dopaminergic neurons with a cocktail of sonic hedgehog (SHH), FGF8, GDNF, and forskolin. Under this condition, SHED-derived spheres generated a number of βIII-tubulin- and MAP2-positive neurons, and some of them were positive to tyrosine hydroxylase (TH), the enzyme responsible for synthesis of dopamine. In order to increase the cell survival capacity, Wang and coworkers transplanted into the striatum of 6-hydroxy-dopamine-treated rats the SHED-derived spheres instead of the quite matured differentiation neurons, because the latter has been reported poor survival rate [32]. Intrastriatal transplantation of SHED-derived spheres ameliorated behavioral deficits in parkinsonian rats, and enhanced the dopamine content in the striatum. Probably, this in vitro induction system activated in SHED-derived spheres some transcriptional factors for the regulation of TH expression. Once these cells were transplanted to striatum, they responded to the microenvironmental signals to undergo the committed differentiation [31].

The exposure to a cocktail miming embryonic midbrain cues was also proposed by Kanafi and colleagues to differentiate DPSCs into functional dopaminergic neuron [33]. The cocktail consisted of basic FGF, SHH and FGF8. The last two factors are secreted by the ventral neural tube and at the mid/hindbrain boundary, respectively. The induced cells were characterized by an increase in mature neuronal markers (βIII-tubulin, MAP2) and dopamine-specific markers, such as Engrailed 1 (En1), Nuclear Receptor related protein 1 (Nurr1), paired-like homeodomain transcription factor 3 (Pitx3), and TH. The induced cells presented a simultaneous decrease in early neuronal markers nestin, musashi12, neural crest marker HNK1 and mesenchymal markers CD73, CD90, and CD105. Functional studies indicated that the induced DPSCs secreted dopamine constitutively and upon stimulation with potassium chloride (KCl) or adenosine triphosphate (ATP). Additionally, the induced DPSCs showed intracellular calcium influx in the presence of KCl, and after ATP stimulation. These in vitro findings suggest that DPSCs in the presence of embryonic midbrain cues have efficient propensity towards functional dopaminergic cell-type [33].

In conclusion, in the development of a successful autologous transplantation therapy for repairing and regenerating injured nervous system, accessibility of the cells intended for grafting is a very important issue. This type of therapy should preferably use easily accessible tissues from the patient to be treated. Dental tissue constitutes an interesting source of cells that could be explored for possible autologous transplantation therapy. DSCs are easily available from adult teeth such as wisdom teeth that are sometimes extracted due to mal positioning. This procedure of preparation of DSCs is devoid of any relevant ethical concerns. In addition, DSCs can be easily handled in the laboratory. They are expandable, cryopreservable, and robust in culture. The achieved results support the use of DSCs as promising candidates for cell-based therapies for neuron disorders. DSCs could produce neurotrophic and neuroprotective factors for supporting the survival of neuronal cells, but also could support the homing of endogenous neural stem cells to the injury site during transplantation. Moreover, DSCs exposed to the appropriate environmental cues could differentiate into functionally active neurons and may provide an alternative stem cell source for therapy-based treatments of neuronal disorders and injury.

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MSCs and Biomaterials

Adriano Piattelli and Giovanna Iezzi

Introduction

Osseointegration was first observed, although not recognized as such, by Bothe and coworkers in 1940. These researchers found that titanium dental implants placed in animal bone were found to be in close and tight contact with bone tissue [1]. They, moreover, reported that titanium, for its strength and hardness, could have a potential as a prosthesis material. Another researcher, Leventhal, in 1951 placed titanium screws in rat femurs and observed that after 16 weeks, the screws were so strongly apposed to bone that the bone fractured when it was tryied to remove the screws [2]. The peri-implant bone did not show untoward processes and no reaction to the presence of the titanium implants. The bone trabeculae appeared to be absolutely normal. In 1952, Per-Ingvar (PI) Brånemark, a young researcher in anatomy in Lund, Sweden, did a study with a titanium implant chamber used to evaluate the blood flow of bone in rabbits. At the end of the experiment, when trying to remove these chambers from the bone, he found that the bone had so completely and fully integrated with the implant that the titanium chamber could not be removed. Brånemark called this process "osseointegration," from the Latin words os—meaning bone, and integrate—meaning to make whole, in the sense of a combined and close presence between the metal biomaterial and the living bone, and, like the beforementioned authors before him, saw the possibilities for human use [3].

Osseointegration was defined by Branemark as "the formation of a direct interface between the implant and bone, without the presence of an intervening soft tissue". In dental implants, this meant that bone tended to grow right up to the implant metal surface without an interposing layer of soft tissue. This direct contact between bone and the implant metal surface had to be verified histologically [4].

A. Piattelli (✉) • G. Iezzi
Department of Medical, Oral, and Biotechnological Sciences,
University of Chieti-Pescara, Chieti, Italy
e-mail: a.piattelli@unich.it

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The concept of osseointegration was applied for the first time in dentistry in the mid-1960s. In 1965 Brånemark, who had become Professor of Anatomy at the University of Gothenburg, inserted dental implants into the first human patient, Gösta Larsson. Mr. Larsson had a cleft palate and he needed dental implants to support a palatal obturator. He died in 2005, and the original implants were still in place, working successfully, after 40 years of function. There are many pictures of him with PI Branemark, and Mr. Larsson became a minor celebrity and he was interviewed many times by television.

In the mid-1970s PI Brånemark started a commercial partnership with a Swedish defense manufacturer called Bofors, to produce dental implants and the metal instruments needed for their insertion. Osseointegrated implantology was criticized by the official dental academia at that time, but, many years and many fights later, the careful documentation of the efficacy and safety of the dental implants originated the widespread and enthusiastic acceptance of implantology as a viable, and in some cases, preferred treatment by the worldwide dental community. Brånemark spent almost three decades trying to get the acceptance of osseointegration from the dental community. In Gothenburg the University stopped the funds for his research, and he was forced to transfer to a private clinic to continue the implant treatment of patients. Toronto's George Zarb, a Canadian prosthodontist, who, by the way speaks Italian fluently, played a pivotal role in presenting the concept of osseointegration to the wider world. The 1983 Toronto Conference was the turning point, and, at long last, the dental scientific community accepted Brånemark's concepts. Today osseointegration is considered by all clinicians and practitioners to be a highly predictable and commonplace treatment modality, with a more than 99.00 % success percentages. The osseointegration concepts have been also transferred in other fields, e.g. orthopedics, where an intramedullary prosthesis was inserted into the residual bone of amputees and then connected to a limb prosthesis. This fact allowed amputees to move in a more comfortable way, and with less energy consumption. Osseointegrated prosthesis can also been combined with replaced joints, allowing, in such a way, below knee amputees with arthritis of the knee or a small residual bone to move without the necessity of a socket prosthesis.

Osseointegration is a dynamic process where the implant features (i.e. macrogeometry, surface properties, mechanical properties, structure of the metal, etc.) play an important and pivotal role in the behavior of molecules and cells. Osseointegration has been observed using different materials (e.g. tantalum, niobium), but most studies concerned the bone reactions to titanium. Titanium implants were found to have mineralized bone tissue at the interface, either through direct contact between calcium and titanium atoms, or by a chemico-physical bonding due to a cement line-like layer located at the implant/bone interface. The healing processes during osseointegration seemed to mimic the mechanisms observed during the healing of bone fractures [5].

For an implant to be osseointegrated, the bone to implant contact (BIC) does not need to be 100 %, and the concept of osseointegration is more related to the stability of the fixation than to the degree of bone to implant contact in histologic terms; we must also consider that about 20–25 % of the bone tissue is composed by marrow

spaces, needed to bring oxygen and nutrients to the bone cells. In short, osseointegration is deemed to be successful when the fixation of titanium implants in bone is achieved and, especially, maintained during functional loading in the chewing cycles. After implant insertion, the healing period lasts several weeks or months before the implant is fully integrated into the surrounding bone, and could then be loaded [6]. The first appearance of metal-bone integration occurs usually after a few weeks, while a more strong connection is progressively observed over the next months or years [7]. Although the osseointegrated interface can be resistant to external shocks over time, it may be damaged by prolonged adverse stimuli and overloading forces, which may determine a complete failure with loss of the implant and prosthetic superstructure. Furthermore, clinicians must bear in mind that there is a critical threshold of micromotion above which there is the formation of fibrous tissues at the interface, rather than bone, with subsequent mobility and possible loss of the implant [8].

In the last decade, implants with a porous metal structure have been introduced in dentistry and orthopedics. Porous metals may allow the formation of vascular systems within the porous area and they show high tensile strength and corrosion resistance with excellent biocompatibility. Moreover, the porous structure of the metal has been shown to allow an extensive bone infiltration, allowing osteoblasts to move inside the metal and to form bone there [9–11]. These porous materials are nowadays used in hip replacement, knee replacement and dental implantology.

Other applications of the osseointegration concept have been in:

- Construction of different types of epitheses, used in retention of a craniofacial prosthesis such as an artificial ear (ear prosthesis), maxillofacial reconstruction, eye (orbital prosthesis), or nose (nose prosthesis) [12–14];
- An hearing conduction amplification anchored to the bone (bone anchored hearing aid) [15, 16];
- Replacement of knee and joints.

PI Brånemark has been awarded many prizes for his work, including the Swedish Soederberg Prize, and the Swedish Engineering Academy medal for technical innovations.

Once the dental implants become osseointegrated, a prosthetic suprastructure needs connected to them and loading forces, during the chewing cycles, are transmitted via the implants to the surrounding bone tissue [17, 18]. This peri-implant bone tends to remodel to arrive to a steady state around the implants. When osseointegrated, the implants not only become a part of the body, but also of the mind. Professor Brånemark has called this special type of mental acceptance “osseoperception”. Osseoperception seems to be of very important, for example, when bone anchored prosthetic replacements communicate with the mind to restore function, i.e. picking an object using an osseointegrated finger prosthesis [4, 19].

Osseointegration, then, was born and came of age in Dentistry, but has been extremely useful in other medical fields such as orthopedics and maxillofacial reconstruction.

Tissue Engineering

Tissue engineering (TE) is a quite new and very promising approach to obtain the repair and regeneration of tissues and organs lost, damaged or compromised due to trauma, injury, disease or aging [20]. A key component of TE approach to bone regeneration is represented by natural or man-made scaffold that are used as template for the interactions between different types of cells, and the formation of bone extracellular matrix providing structural support to the newly formed tissue. An ideal scaffold should have the following features (1) a three-dimensional (3-D) and highly porous structure with interconnected pores to allow cells migration, flow transport of nutrients, and removal of metabolic waste; (2) biocompatibility and resorbability, with a rate of resorption similar to that of the forming new bone tissues; (3) a surface chemistry that favors cells attachment, proliferation and differentiation; (4) mechanical properties comparable to bone and soft tissues at the implantation site; (5) a possibility to be commercially produced and safely sterilized without any alteration of its properties [21–25]. Several approaches have been used in bone regeneration procedures, and calcium phosphate ceramics are, probably, extremely effective as scaffolds. There is a necessity of further studies of the best ways in which materials, cells and biologically active molecules could interact. Different types of cells and growth factors are two pivotal elements in bone biology/healing, and their interaction is extremely important towards an effective regeneration process. The best combination of materials, cells and growth factors seems to be a must for a very effective bone TE strategy [26]. A system to be used for bone repair and regeneration would ideally require osteoconductive and osteoinductive properties, so that new bone formation can be improved through an adequately shaped three-dimensional (3D) scaffold (osteoconduction) and by a biological stimulus (osteoinduction) [27]. Ceramic materials, e.g. hydroxyapatite, tricalcium phosphate and coralline-derived calcium phosphate, due to their inorganic nature and ionic composition, are extremely useful in several applications. These materials are known for their ability to bond to bone and stimulate new bone formation. 3D systems have been produced with the use of particulates or blocks having a porous interconnected structure [28]. The formation of 3D scaffolds in particulate or block shape creates a potential for their use either without cells (placing of the scaffold in the tissues, and its colonization by surrounding cells) or combining them in vitro with cells, creating a hybrid cell–material construct. These 3D scaffolds can be used also as a delivery system, releasing bioactive agents and enhancing the regenerative potentialities of the system [29]. The ability of micro-CT to evaluate 3-D structures in a non-destructive way has made its use and application extremely wide in several fields, such as physics, materials science, medicine, mineral processing and powder technology. Furthermore, the possibility to use synchrotron radiation X-ray sources has further improved the application of micro-CT due to its numerous advantages compared to conventional X-ray sources, including a higher beam intensity, a higher spatial coherence and the monochromaticity [30]. The monochromaticity property of synchrotron radiation reduces significantly the beam hardening effects, thus

allowing to simplify the segmentation process of the whole image analysis. Synchrotron radiation X-ray micro-CT has been used to evaluate the 3-D porous architecture and microstructure of several different calcium phosphate scaffolds after a long-term healing period in humans [31]. In the last decade, bone substitute biomaterials have been used in combination with cells for the fabrication of artificial bone grafts. The use of multipotent mesenchymal stem cells (MSCs) has opened up new therapeutic perspectives for the in situ or in vitro TE of bone. The success of tissue regeneration is related to the structure of the scaffold and its ability to allow invasion by cells and tissues. This construct can then be placed in living tissues to act as replacement tissue after the in-vitro colonization of MSC. Blood vessels [32] begin to grow around and into the construct, and as the scaffold undergoes resorption, the newly formed bone tissue starts blending with the surrounding tissues and finally replaces the scaffold. The scaffolds can be reproducibly manufactured with a specific, desired structure obtained according to stochastic, fractal, or periodic principles. In recent years, the efforts in TE have been focused mainly on the characterization of the regenerative properties of different sources of stem cells (dental pulp, periodontal ligament, amniotic fluid) [33–36]. Amniotic derived stem cells (ADSCs) are an intermediate stage between embryonic stem cells and lineage-restricted adult progenitor cells. Their high proliferation rate together with their differentiation potential into cells of all three embryonic germ layers (ectoderm, endoderm and mesoderm) are important advantages over most of the known adult stem cell sources. In vitro studies and tests are needed to evaluate the attitude of the constructs to support cellular events such as adhesion, proliferation and osteogenic cells differentiation [37]. All the results obtained from in vitro and animal experimentations will give essential informations to try to transfer and apply these novel therapeutic strategies to the field of tissue regeneration.

Approaches to tooth regeneration. The approaches to tooth regeneration are still in their infancy and face many obstacles. The different types of approaches that have been tried include: (a) remineralization of carious dentin by inorganic polyphosphates; (b) calcium phosphate coatings; (c) engineering of bone and tooth root using bioactive materials; (d) regeneration of different dental tissues with the use of different substances, i.e. amelogenins for the regeneration of the periodontal tissues, and calcium-phosphate ceramics and collagen for the reconstruction of the bone [38]. The deep and complete understanding of the principles that support the formation of teeth and periodontium represents the basic foundation to design innovative biomaterials to be used in the possible regeneration of these structures. When enamel undergoes demineralization, the residual mineral crystals can serve as templates for the new formation of apatite crystals [39]. The same process can be used in demineralized dentin, as for example can occur in a carious tooth, where dentin apatite crystals tend to remain and can be used as templates. It is also possible to attempt to remineralize dentin with the use of agents likes polyacrylic or polyaspartic acid [40]. These acids bind to collagen and serve to bind calcium and promoting apatite nucleation. The biomineralization processes, such as the formation of tooth enamel, is under the influence of various proteins such as the amelogenins. Mimicking nature, it has been tried to restore enamel by inducing remineralization

of hydroxyapatite on the surface of the tooth. The regeneration of different parts of a tooth by implementation of biomimetic mineralization processes will represent a significant step for the future development of scaffolds for dental regeneration [41]. This will lead to the possible formation of teeth, and this fact could have an enormous benefit to human health with a huge socio-economic impact. Different hybrid composites will be evaluated to mimic the different regions of the tooth and of the periodontal tissues. These composites could have a structure as follows:

1. an unmineralized, collagen central portion, the dental pulp;
2. an unmineralized, layer, the pre-dentin;
3. a highly mineralized layer, the dentin.

The different structure characteristics of the different tooth tissues could be obtaining by different degree of cross-linking. To regenerate the periodontal tissues could be used constructs composed of highly mineralized portions, i.e periodontal bone and cementum, connected by fibrous layers, mimicking the unmineralized periodontal ligament. Tooth regeneration could be a difficult but very important part of regenerative medicine in the future and could have a relevant importance in healthcare.

Graphene

Graphene is a relatively new allotrope of carbon composed of a single layer of monocrystalline graphite with hybridized carbon atoms. Due to its structure, a one-atom-thick two-dimensional (2D) carbon material, graphene has attracted increasing attention in the past several years due to its high surface area, remarkable thermal conductivity, excellent charge mobility, and mechanical properties [42]. The unique structure and outstanding properties render graphene highly promising for a wide range of applications in the fields of electronics, sensor, and energy storage/conversion. Some of the other interesting aspects of graphene include high transparency toward visible light, high values of elasticity, unusual magnetic properties, and charge transfer interaction with molecules, that allowed it to gain a lot of interest in the biomedical field as a new component for biosensors, tissue engineering, and drug delivery. Graphene can be obtained following different approaches [43]. Interestingly, the majority of studies on chemistry of graphene do not involve "pristine" graphene, but rather carbon materials produced upon reduction of graphene oxide (GO). Graphene-based materials show unique interactions with DNA and RNA, which make them attractive in DNA or RNA sensing and delivery. GO shows preferential adsorption of single stranded DNA over double stranded DNA and protects the adsorbed nucleotides from attack by nuclease enzymes opening up a wide range of application opportunities [44]. As opposed to interaction with DNA and RNA, there are only a few data on the interaction of graphene with proteins and lipids. It will be, hoewer, very important to understand interaction of graphene with lipid bilayer in the cell membrane. Protein adsorption on nanomaterials surface has

received increasing attention for the past several years. This phenomenon affects in a significant way the behaviour of these materials in biological systems (e.g. cellular uptake and toxic responses). Nanomaterial surfaces are immediately covered by proteins, lipids, enzymes when put in a biological medium. These coated surfaces give new features to the nanosystem, i.e. hydrophilicity/hydrophobicity, surface charges and energy, topography [45, 46]. These new characteristics will produce the responses at the cell/tissue level. Due of their high specific surface area, the carbon family of nanomaterials including graphene has a potentially larger protein adsorption capacity than other nanostructures. After interaction with cells, tissues, or organs, graphene sheets surfaces change and have completely different biological properties. Hydrocarbons, organic molecules, and elements was reported to change the surface composition and surface energy, which affected the protein absorption and cell attachment, proliferation, differentiation, and final integration in the tissues [47–51]. The high active surface area of graphene, over other nanomaterials, is one of the main advantages of graphene based materials, which allows a high-density drug loading. Due to the specific geometry of graphene (2D structure), both sides of a single layer graphene sheet can be used as a substrate for the controlled adsorption of molecules and functional groups for surface modification [52]. For instance, it has been shown that covalent attachment of chitosan, folic acid, and polyethylene glycol (PEG) to GO produces a potential platform for the delivery of anti-inflammatory and water insoluble anticancer drugs such as doxorubicin (Dox) and SN38, a camptothecin analogue [53]. The idea is to study drug-graphene interaction as to be able to deliver, in a controlled manner and exploiting graphene-drug interactions, drug in proper quantity directly to the desired target site. It is important to understand such interactions from two points of view, one for biomedical applications and other for their toxicity and biocompatibility. Like other employed materials in nanomedicine, toxicity of the graphene is strongly dependent to its physicochemical properties (e.g., size and its distribution, surface charge, particulate state, number of layers, surface functional groups and particularly shape). One of the most important issues for biomedical applications of graphene is its short- and long-term toxicity [54–56]. Carbon-based materials (carbon nanotubes or nanocrystalline diamonds) have been widely tested for both their potential toxicological risks and their possible use in biomedical applications. Moreover, the antibacterial activity of graphene-based materials can be used in wound healing applications to prevent infections or to potentiate and protect the integration process of diffeent types of biomaterials biomaterials. Graphene, when used as a delivery vehicle, could, probably, potentiate the effects of antibacterial drugs. Gene therapy to treat genetic disorders and cancer is another area where graphene could be usefully employed. Successful gene therapy requires efficient and safe gene vectors that protect DNA from nuclease degradation as well as facilitate DNA uptake with high transfection efficiency. Graphene has been explored for applications in gene delivery, gene–drug co-delivery and protein delivery [43, 55, 57]. Osteogenic differentiation of Mesenchymal Stem Cells was enhanced on titanium surfaces coated with GO carrying BMP-2, compared to titanium surfaces coated only with BMP-2 [58]. In vivo studies in mouse also showed a higher new bone formation when using titanium–GO–BMP2 implants

compared to only titanium or titanium–GO or titanium–BMP2 implants; these new composites could be very effective carriers for delivery of drugs. Several studies have emphasized the potential of graphene-based materials as drug and gene delivery vehicles in vitro; however, there is a need to demonstrate their potential in vivo with particular focus on safety, biodistribution and efficacy [59]. Therefore, graphene is an ideal model material for experiments with adherent (anchorage-dependent) cells (e.g. osteoblasts, mesenchymal stromal cells (MSCs), etc.). Adhesion of osteoblasts is a crucial prerequisite to subsequent cell functions, such as proliferation, synthesis of proteins (e.g. proteins of extracellular matrix (ECM), morphogenic factors and osteoinductive molecules) and formation of mineral deposits [43, 60, 61]. Adhesion is generally dependent on time, adhesive forces at the cell/material interface, and surface topography. Cell adhesion is primarily mediated by integrins, a widely expressed family of transmembrane adhesion receptors. Upon ligand binding, integrins rapidly associate with the actin cytoskeleton and cluster together to form focal adhesions (FAs), which are discrete complexes that contain structural (e.g. vinculin) and signalling molecules (e.g., focal adhesion kinase). FAs are central elements in the adhesion process because they function as structural links between the cytoskeleton and ECM to mediate stable adhesion and migration. Furthermore, in combination with growth factor receptors, FAs activate signalling pathways that regulate transcription factor activity and direct cell growth and differentiation [62]. Human MSCs are mononuclear cell population adherent to tissue culture plastic and have been isolated from adult bone marrow. They are capable of further proliferation as well as differentiation into multiple lineages involved with connective tissue (osteoblasts, adipocytes and chondrocytes) when exposed to various growth factor combinations or substrates with different topography and rigidity). Thus, these cells may serve as a good model for testing the possible increased/accelerated differentiation induced by adhesion onto graphene surfaces. Recently, the utilization of graphene foam, a 3D porous structure, showed to be succesfully used as a novel scaffold for Neural Stem Cells (NSCs) in vitro. It was found that three-dimensional graphene foams (3D-GFs) can not only support NSC growth, but also keep cell at an active proliferation state. These findings show that 3D-GFs could offer a powerful platform for NSC research and neural tissue engineering [63].

Digital Dentistry

It has been said that by the end of the decade there will be as many digital bits as there are stars in the universe, some 44 trillion gigabytes. With that degree of digitization it is no wonder that dentistry has been highly influenced by the presence of digital media and digital Technologies [63]. The challenge for the dental community is to try to understand and use at best what the digital world has to offer. In order to do so we need to understand the nuances and complexities of the digital world and with the high speed of its advances, this could be really a major

challenge. Probably digital technologies can and will be used in all dental field, e.g. digital data capture systems such as X-ray, CT and MRI [64]. More recently, there have been really interesting and quite exciting new developments in the production and use of intra-oral cameras. The latter will change in a profound way the daily practice of restorative dentistry as it provides us with digital data of the oral cavity that can then be processed by a digital dental laboratory. This will provide an even greater incentive to develop new design software that will allow us to manipulate all the data that we are able to obtain from our patients. Another aspect of the digital world is the transition from traditional manufacturing routes to the use of CAD-CAM and more recently additive manufacturing. In the immediate future there will be a need to explore the extent to which new manufacturing technologies and new materials could be extremely useful to treat our patients.

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DSC for Ocular Regeneration

Fatima N. Syed-Picard

Introduction

The eye is a complex organ that converts light into visual signal accomplishing one of our five senses, sight. Multiple ocular tissues must function in chorus to properly orchestrate the overarching goal of vision. As light travels through the eye, it first enters through the transparent cornea that focuses the light through the pupil. It is then further focused by the lens onto the retina. The retina converts the light into a decipherable visual signal that is transported to the brain via the optic nerve (Fig. 1). This simplistic description of the pathway of light through the eye that doesn't convey the multiple intricate components involved in ensuring proper function of the eye. For instance, the cornea itself comprises three distinct cellular layers serving multiple functions such as controlling corneal hydration and matrix structure, and preventing vascular infiltration; the disruption of any of these cellular processes results in corneal opacity obstructing the passage of light into the eye. The retina contains multiple types of neurons involved the converting light into visual signal and transporting the signal to the brain, diseases causing neuronal death or axon damage prevent this final necessary step for acquiring visual signal. Cellular therapies could provide mechanisms to repair or replace cells in these types of ocular pathologies.

This chapter reviews ongoing research involved in assessing the potential of dental stem cells for regenerative ocular therapy. Several populations of adult stem cells have be identified in dental tissues, and due to their neural crest developmental origins, maybe be advantageous for use in ocular cellular therapy. These populations of stem cells include those isolated from the dental pulp of adult of exfoliated deciduous teeth, or periodontal ligament. Portions of the cornea are derived from the cranial neural crest; therefore due to their similarity in developmental origins, dental

F.N. Syed-Picard (✉)
Department of Bioengineering, University of Pittsburgh,
302 Benedum Hall, 3700 O'Hara St., Pittsburgh, PA 15260, USA
e-mail: fns4@pitt.edu

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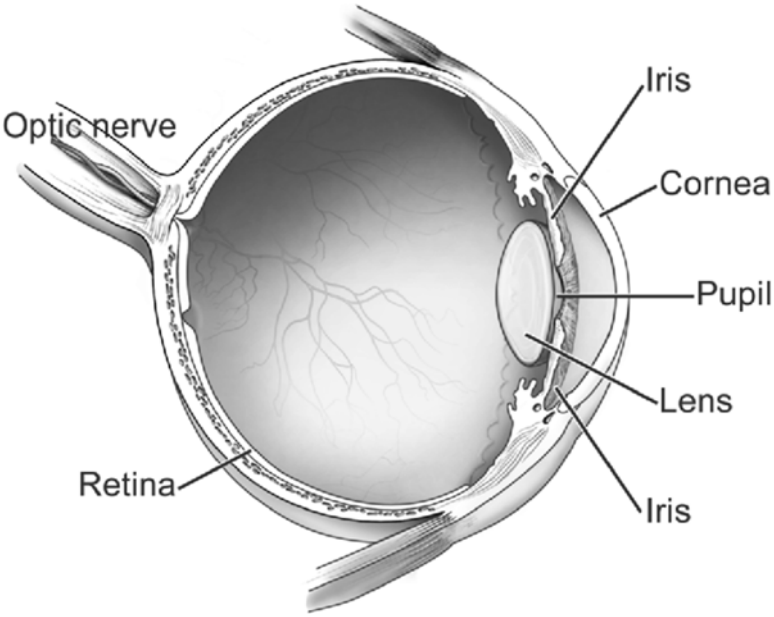

Fig. 1 Drawing of a section of the human eye. Image courtesy of the National Eye Institute, National Institutes of Health

stem cells may potentially possess a stronger affinity to differentiate towards corneal cell lineages. Additionally, the neural crest gives rise to the neurons of the peripheral nervous system, so dental stem cells may have the capacity to differentiate into neurons for replacing damaged cells of the retina. An additional benefit of dental stem cells is their accessibility. These cells can be easily collected from patients for autologous use. These attributes make dental stem cells a potential viable option for developing cellular ocular therapies.

Dental Stem Cells to Treat Corneal Blindness

The cornea is the anterior most tissue of the eye that serves multiple functions such as allowing the passage of light with minimal scatter, acting as a barrier to prevent penetration or infiltration into ocular tissues, and providing two-thirds of the refractive power of the eye to focus light onto the retina [1]. The complex structure of the cornea allows it to facilitate these multiple functions. The cornea comprises three cellular layers: the epithelium is the anterior-most, the stroma is central, and the posterior-most layer is the endothelium. Millions of individuals worldwide suffer from bilateral corneal blindness due to diseases associated with these cellular layers or trauma to the cornea [2]. Stem cell research shows promising results for developing cellular therapies to restore vision to patients suffering from corneal disorders.

Corneal Epithelium

The anterior surface of the cornea comprises a stratified squamous epithelium that acts as a barrier to the eye. The corneal epithelium is a self-renewing structure where the superficial terminal cells are naturally shed and repopulated by a population of stem cells found in the limbus, the transition zone between the cornea and the sclera, termed limbal epithelial stem cells [3]. In addition to functioning in the natural process of epithelial self-renewal, these stem cells are capable of regenerating the entire corneal epithelium following injury.

Disease or injury leading to total limbal stem cell deficiency (LSCD) results in the inability of the corneal epithelium to renew and regenerate itself and therefore leads to corneal opacity due to corneal inflammation and vascular infiltration. Unilateral LSCD is currently treated by transplanting limbal epithelial cells from the contralateral eye [4]. However, treating bilaterial LSCD requires allogeneic tissue transplantation, which is subject to failure due to immunological rejection [5]. Therefore the development of autologous stem cell therapies utilizing cells from an alternative tissue source to repopulate the limbal epithelial stem cells would bypass limitations of current treatments.

Stem cells from exfoliated deciduous teeth (SHED) are a highly investigated population of stem cells since they can be easily isolated from autologous tissues and have the capacity to differentiate into multiple cell types [6]. Recent studies have shown promising results on the ability of SHED to regenerate corneal epithelial cells. Epithelial and stem cells gene expression profiles of SHED were shown to be more similar to that of limbal epithelial stem cells than differentiated corneal epithelial cells for genes such as ABCG2, K12, and K3 [7]. SHED were able to restore corneal epithelial tissue in an LCSD animal model in vivo. LSCD was induced in New Zealand white rabbits through chemical burning. One month following injury, keratectomy was performed to remove pannus formed from the chemical burn, and cell sheets comprising SHED were transplanted onto the exposed rabbit stroma. To prevent damage to the SHED sheet, acellular human amniotic membrane was placed over the cell sheet and sutured to the episclera. Without intervention, the chemical assault to rabbit corneas resulted in corneal vascularization, conjunctivalization, and opacification. Histological analysis of untreated eyes lacked the presence of a stratified epithelium and confirmed the presence of a disorganized, neovascularized conjunctival tissue [7, 8]. Alternatively, the introduction of sheets of SHED to chemically injured rabbit eyes led to clearer corneas with less neovascularization than the control eyes. Furthermore, histological analysis showed that injured eyes receiving SHED had a regenerated corneal epithelium containing cuboidal basal cells, intermediate flattened cells, and polygonal surface cells, similar to nature corneal epithelial tissue [3, 7, 8]. These studies provide promising results for the potential use of SHED in cellular or tissue engineering therapies to regenerate corneal epithelial tissue.

Corneal Stroma

The corneal stroma is the central-most tissue of the cornea and makes up the bulk of the cornea comprising about 90 % of its structure [9]. The stroma has a complex, lamellar structure that facilitates its mechanical integrity and transparency. Each stromal lamella contains long fibrils of collagen that are organized in parallel, have approximately uniform diameter, and are uniformly spaced. The direction of collagen fibril orientation rotates orthogonally between adjacent lamellae [10]. The lattice structure formed by these tightly packed collagen fibrils minimizes light scatter facilitating the transparency of the cornea [11]. Keratocytes are the cells that maintain the stroma. These are quiescent cells serve multiple functions including generating specific molecules that regulate collagen fiber spacing to maintain corneal transparency.

Trauma or disease can cause disruptions to the collagen organization within the corneal stroma diminishing its transparency. Keratocytes respond to stromal trauma by differentiating into fibroblasts and depositing an unorganized scar tissue. This scar tissue does not have the highly organized collagen structure characteristic of healthy stromal tissue and is consequently opaque [12]. Millions of individuals suffer from bilateral corneal blindness resulting from the formation of corneal scars [2]. The current method of treatment is a keratoplasty, which involves using cadaveric grafts to replace the damaged tissues. However, there is a worldwide shortage of donor tissue and this treatment has a failure rate of 38 % after 10 years due in part to immune rejection [13].

The development of a cellular therapy or engineered tissues using autologous stem cells to treat stromal scarring could bypass the limitations associated with current allogeneic grafting treatment. Researchers have started investigating adult stem cell populations such as those derived from adipose tissue or corneal stromal tissue [14–16]. Dental pulp isolated from adult teeth contains a population of multipotent stem cells that are easily accessible and may have a higher affinity for corneal stromal differentiation since, similar to the corneal stroma, they are neural crest-derived.

Human dental pulp stem cells (DPSCs) isolated from adult third molar cells were shown to be able to differentiate into keratocytes in vitro and maintain this phenotype in vivo [17]. The DPSCs were cultured as pellets in a keratocyte differentiation medium, in a similar manner as the culture treatment used to induce keratocyte differentiation from corneal stromal stem cells [18]. After in vitro differentiation, DPSCs had increased expression of molecules characteristic of keratocytes at both the gene and protein levels [17]. Of particular significance was the upregulation in the expression of the proteoglycan keratocan. This molecule is critical in maintaining proper collagen fibril spacing required for corneal transparency. Importantly, keratocan is only found in the corneal stroma and is therefore considered a keratocyte specific marker [19]. The expression of keratocan at the gene and protein levels verifies that DPSCs differentiated into keratocytes in vitro.

DPSCs differentiated into keratocytes in vitro were injected into mouse corneal stroma to assess the behavior of the cells in the in vivo environment. The DPSCs remained in the mouse stroma for up to 5 weeks and produced corneal stromal matrix molecules such as type I collagen and keratocan in vivo. Furthermore, the mouse stroma remained transparent with the injected DPSCs indicating that the DPSCs were not inducing any adverse reaction vivo [17].

The ability of DPSCs to generate tissue engineered corneal stromal constructs with similar structure as native tissues was assessed. DPSCs were cultured on aligned nanofibers to direct cell and matrix organization. In these engineered tissues, DPSCs aligned in parallel and produced a parallel-aligned collagenous matrix, similar to native corneal stromal tissue. Furthermore, the direction of collagen fibrils rotated at different depths within engineered tissue indicating the formation of multiple lamellae. The collagen fibrils were approximately uniform in diameter similar to engineered tissues generated by CSSC [17]. This study showed that DPSCs can differentiation in keratocytes in vitro, maintain this phenotype in vivo, and be used to generate engineered corneal stromal tissue. This shows promise for the development of DPSC-based cellular or tissue engineering therapies to treat corneal stromal scarring.

Corneal Endothelium

The corneal endothelium is the posterior most cellular layer of the cornea. It is a single layer of neural crest derived cells that function to regulate hydration of the stroma through barrier and pump functions [9]. Correct water content is critical in controlling the stromal collagen fibril spacing needed to maintain corneal transparency. Therefore, hydration defects such as edema cause increased light scatter resulting in corneal haze [1]. Corneal endothelial cells have limited proliferative capacity and severe decreases in cellularity due to trauma or diseases such as Fuch's dystrophy results in an edematous cornea with reduced transparency [20]. More than half of corneal transplants occur due to damage or disease to the corneal endothelium [21, 22] indicating a need for the development of cellular therapies. A recent study showed that neural crest derived stem cells isolated from the corneal stroma maintain the capacity to differentiate into corneal endothelial cells [23]. However, few, if any, additional studies have identified alternative adult stem cell populations with this ability. Currently, there are no studies reporting the differentiation competence of dental stem cells into corneal endothelial cells. The capacity of dental stem cells to differentiate into corneal endothelial cells seems plausible since both tissues share similar developmental origins. Potentially, the methods developed to induce corneal endothelial cells from corneal stromal stem cells could be adapted to dental cells. This is still an open scientific question that needs to be answered since, if possible, the use of dental stem cells to treat disorders of the corneal endothelium would greatly impact the field of corneal regeneration.

Dental Stem Cells to Treat Retinal Degeneration

The retina is a complex tissue in the posterior region of the eye involved in converting light into visual signal and transmitting it to the brain. This process is orchestrated by multiple, specialized neuronal cells organized into nuclear layers. The photoreceptor cells, the rods and cones, are located in the outer nuclear layer and are the light-sensitive cells that first detect light and initiate the process of translating it into visual signal. The signal is then processed by neurons in the inner nuclear layer, including horizontal cells, bipolar cells, and amacrine cells. Finally, the signal is received by the retinal ganglion cells (RGCs), which transmit the signal to the brain. The RGC somas are positioned in the ganglion cell layer and their axons stretch towards to posterior of the eye, along the surface of the retina in the retinal nerve fiber layer. The RGC axons collect at the optic nerve head, and assemble to form the optic nerve [24, 25].

Retinal degeneration due to genetic disease or age-related disorders can lead to retinal cell damage or death resulting in vision impairment or loss. Common genetic disorders affecting retinal function result from mutations of the gene for rhodopsin, the light-sensitive protein expressed by the photoreceptor cells [24]. Additional types of retinopathies include glaucoma, a group of diseases resulting from optic nerve degeneration due to RGC loss or damage. Over 60 million individuals worldwide suffer from glaucoma, and this disease has caused bilateral blindness in approximately eight million of these people [26]. Glaucoma is the second leading cause of blindness [27], and currently a cure for this disease is not available. The development of therapies to enhance cell viability and restore cell function in these types of retinopathies could be used to treat these blinding conditions.

Dental Stem Cells for Retinal Cell Regeneration

Multiple groups have shown that dental pulp stem cells have an affinity to differentiate into mature neuronal cells both in vitro and in vivo [28–30]. This characteristic is attributed to the neural crest origins of these cells, since the neural crest gives rise to the nerves of the peripheral nervous system [28]. Under appropriate in vitro conditions, DPSC develop the characteristic stellate neuronal morphology, express neuronal markers at both the gene and protein levels, and produce voltage-dependent current similar to mature neurons [28, 30]. Furthermore, in vivo, neuronally- differentiated DPSC maintained their neuronal phenotype and integrate into brain tissue after transplantation [28, 29]. Because of these capabilities, researchers are investigating the capacity of DPSC to differentiate into retinal neurons.

A recent study has shown that human DPSC cultured with conditioned media from rat retinal explants differentiated into retinal neurons [31]. Conditioned medium was collected from cultures of normal rat retinal explants or explants treated with *N*-methyl-*N*-nitrosourea (MNU), which chemically damages the retina

by inducing apoptosis of the photoreceptor cells. Researchers found that DPSC adopt a neuronal cell morphology when cultured with either normal or damaged retinal explant conditioned medium [31]. However, DPSC only expressed rhodopsin after being cultured with conditioned medium from damaged retinal explants [31]. This shows promising data that DPSC have the capacity to differentiate into retinal neuron-like cells, and damaged retinal cells secrete signals to induce this differentiation. Elucidating the specific signals necessary to induce retinal cell differentiation from DPSCs would be additionally powerful and allow for the development of an efficient mechanism to develop a cellular therapy to treat retinal disorders.

Similar to DPSC, periodontal ligament stem cells (PDLSC) are able to differentiate down neuronal lineages [32], and this also is attributed to the neural crest origins of this tissue. Research is now also emerging on the use of PDLSC for retinal cell differentiation [33]. To induce differentiation, PDLSCs were first cultured as neurospheres and then plated on Matrigel-coated dishes in differentiation medium containing Noggin and Dkk-1, inhibitors of the bone morphogenic protein and Wnt/β-catenin pathways, respectively [33]. Antagonizing these pathways mimicked developmental processes necessary for anterior neural plate formation and retinogenesis. Differentiated PDLSCs expressed genes and proteins characteristic of retinal fate induction such as Pax6 and Rx, and markers of photoreceptors such as rhodopsin and Nrl [33]. This study shows promising data on the potential use of PDLSCs for retinal cell regeneration. Additional animal studies showing the in vivo behavior of these differentiated PDLSCs such as cell integration into retinal structures and cell functionality would provide further support on the potential use of PDLSCs for regeneration therapies to treat retinopathies.

Dental Stem Cells to Facilitate Neuroprotection

Neurotrophic factors (NTFs) are a family of proteins that promote neuronal cell survival and function, and the regeneration of damaged axons. This class of proteins includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3). During development, the expression of NTFs by dental pulp cells is critical for pulp innervation [34–37]. Cultured dental pulp cells maintain the ability to express these factors in vitro and through this production of NTFs promote neuron survival [38]. These studies give promise to the development of cellular therapies utilizing dental pulp cell to treat disorders involving the neuron degeneration.

The introduction of neurotrophic factors is being considered as a strategy to repair damaged RGCs and prevent RGC death in glaucoma [26]. Intraocular injections of recombinant neurotrophic factors results in significant increases in RGC survival following optic nerve injuries in animals [39–43], however, this type of therapy would require repeated injections in patients. Intravitreal injections of viral-mediated NTF genes or cells genetically modified to express NTFs has shown promising results as a method of NTF delivery for optic nerve repair [44, 45]. Alternatively, cellular therapies involving the delivery of adult stem cell populations

that naturally express NTFs are being investigated. The intravitreal injection of NTF-secreting bone marrow stromal cells facilitated a neuroprotective response as seen by a significant increase in RGC survival following optic nerve transection [46]. Recently, a study comparing the NTF secretion among adult stem cell populations isolated from different tissues showed that DPSCs produce significantly greater amounts of NTFs when compared to stem cells derived from adipose or bone morrow [47] suggesting that DPSC may be a favorable cell choice to provide NTFs for retinal cell repair.

Research investigating the ability of DPSC to provide neuroprotection and axon regeneration to RGCs after optic nerve injury showed promising results. In vitro, retinal cells cultured with DPSC-conditioned medium had increased neurite number and neurite length when compared to retinal cells cultured with untreated medium or BMSC conditioned medium [48]. Furthermore, this affect was lost by blocking tropomyosin receptor kinase receptors, which bind NTFs, confirming that the increase in neuritogenesis was due to NTF production by the DPSCs [48].

The ability of DPSC to restore retinal cell function in vivo was evaluated by injecting suspensions of DPSC or BMSC intravitreally into rats after optic nerve crush. One observable effect of optic nerve crush is decreased thickness of the RNFL due to RGC atrophy, which can be quantified from optical coherence topography images. Unlike control eyes, DPSC treated eyes did not have reduced RNFL thickness after optic nerve crush [48]. Furthermore, injured eyes with transplanted DPSC had significantly greater RGC survival, determined by positive Brn3a staining, than control or BMSC injected injured eyes, indicating a neuroprotective effect of DPSCs [48]. Additionally, DPSCs promoted a significant increase in RGC axon repair in the optic nerve at distances of up to 1200 μm distal to the crush site as compared to BMSC injected eyes or control eyes [48]. This data shows promising results that through the expression of neurotrophic signals, DPSC could provide a cellular therapy to promote RGC survival and repair to treat glaucoma.

Conclusions and Future Prospects

Populations of multipotent stem cells have been identified in several dental tissues. These stem cells have been shown to have the competence to differentiate down multiple lineages and are therefore being considered for numerous regenerative therapies. This chapter highlighted the exciting current work on investigating the potential of dental stem cells for ocular regeneration. Due to their neural crest origins, researchers postulated that dental stem cells may have a strong affinity to differentiation down ocular lineages, and these recent studies provide strong data to support this. Future studies could also reveal alternative dental stem cell populations for treating the ocular disorders described here or for use in additional ocular diseases not yet investigated. Dental stem cells can be easily isolated from autologous sources and therefore present a viable cell source for the development of new dental stem cell based cellular or tissue engineering therapies to treat blinding ocular disorders.

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DSC-Differentiated Hepatocytes for Treatment of Liver Diseases

Francesco Paduano, Massimo Marrelli, Akhilesh K. Gaharwar, and Marco Tatullo

Abbreviations

ADSCs	Adipose stem cells
AHF	Acute hepatic failure
ALB	Albumin
ALF	Acute liver failure
ALT	Alanine aminotransferase
AST	Aminotransferase
BM-MSCs	Bone marrow stem cells
CHC	Carboxymethyl-hexanol chitosan
CHC/PU-miR122	miR122 complexed with PU-PEI in nanostructured CHC
CK-18	Cytokeratin 18
CPS-1	Carbamoyl phosphate synthetase
Dex	Dexamethasone
DP-iPSC-Heps	Hepatocytes-like cells from dental pulp derived-iPSCs
DP-iPSCs	Dental pulp-derived iPSC
DPSCs	Dental pulp stem cells

F. Paduano • M. Tatullo (✉)
Research and Development in Biomedicine – Stem Cells Unit, TECNOLOGICA Research Institute, st. E. Fermi Loc. Passovecchio I. Z., 88900 Crotone (KR), Italy
e-mail: marco.tatullo@tecnologicasrl.com

M. Marrelli
Department of Maxillofacial Surgery, Calabrodental, Crotone, Italy

A.K. Gaharwar
Department of Biomedical Engineering, Texas A&M University, College Station, TX 77843, USA

Department of Materials Science and Engineering, Texas A&M University, College Station, TX 77843, USA

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DSCs	Dental stem cells
DTCPs	Deciduous tooth pulp cells
EGF	Epidermal growth factor
ESC-Heps	Embryonyc stem cell-derived hepatocytes
ESCs	Embryonic stem cells
ETF	Embryotrophic factor
FBS	Fetal bovine serum
FGF-1	Fibroblast growth factor 1
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
hdSHED	Human hepatically differentiated SHED
HGF	Hepatocyte growth factor
HTCP	Tooth-pulp stem cells
IL-6	Interleukin 6
iPSC-Heps	iPSCs-derived hepatocyte-like cells
iPSCs	Induced pluripotent stem cells
ITS	Insulin-transferrin selenium
MAPS	Multipotent adult progenitor cells
miR122	MicroRNA122
OSM	Oncostatin M
PU-PEI	Polyurethane-graft-short-branch polyethylenimine copolymer
SHED	Exfoliated deciduous teeth stem cells
STAT3	Signal transducer and activator of transcription 3
TAA	Thioacetamide
TGPCs	Tooth germ progenitor cells
WJ-MSCs	Wharton's jelly-derived MSCs
WTPCs	Wisdom tooth pulp cells
α-FP	Alpha-fetoprotein

Introduction

Chronic liver disease or acute liver failure are caused by several factors including viral infections, genetic disorders, toxic injury and auto-immune defects [1]. Chronic liver injury, such as that triggered by hepatitis C virus (HCV), induces inflammation and fibrosis, often followed by the development of liver cirrhosis, which the most advanced stage of hepatic fibrosis [2]. Current pharmaceutical treatments are able to cure patients with hepatitis virus C thought the administration of direct acting-antiviral agents (DAAs) such as *sofosbuvir* in combination with *simeprevir* [3]. However, the drug therapy alone is not enough to treat the liver fibrosis, especially in patients with decompensated cirrhosis [4]. Consequently, the development of effective treatments for liver fibrosis are necessary for HCV-infected patients. Cirrhosis generally progress to hepatocellular carcinoma (HCC), resulting

in a liver failure without the typical self-regenerative capability of liver. Therefore, the inhibition of hepatic fibrogenesis and liver inflammation could prevent the progression of cirrhosis and reduce the incidence of HCC. Similarly, Acute hepatic failure (AHF) is another severe liver injury that sustained liver damage and deterioration of liver functions, leading complications including hepatic encephalopathy and multiorgan failure. The management of patients with AHF continues to be one of the most interesting obstacles in clinical medicine.

Currently clinical treatment for cirrhosis and AHF include liver transplantation. However, transplantation of liver is hampered due to lack of donors and graft rejection. Therefore, there is an unmet need to develop an alternative treatment for acute and chronic liver diseases. Recently, to alleviate the excessive demand for liver transplants, alternative cellular therapy are investigated [5].

Hepatocyte transplantation has been considered a therapeutic alternative for the treatment of patients having hepatic disorders such as cirrhosis and HCC. However, the source of hepatocytes is limited due to scarcity of suitable liver donors. In addition, it has been shown that the hepatic transplantation of hepatocytes could provoke injury to both recipient and donor. Therefore, the researchers have put great effort into obtaining hepatocyte-like cells from sources other than the human adult liver. Consequently, stem cells have been investigated as an alternative source of hepatocytes to overcome these limitations.

Stem cell therapy may represent a new treatment method for tissue and organ regeneration. In fact, one of the main focuses of regenerative medicine in recent years has been to obtain large population of hepatocyte-like cells derived from stem cells for transplantation. In the beginning, the strategies were focused principally on embryonic stem cells (ESCs) as they can successfully differentiate into three germ layers and have limitless capacity of self-renewal [6]. However, safety conditions and ethical issues are some of the obstacle to clinical translations. The difficulties regarding the use of ESCs can be avoided by using the adult stem cells found in different tissue.

Cellular Therapies for Liver Disease

Recently, cell-based therapies and hepatic tissue engineering have been explored as an alternative for the clinical treatment of liver-related diseases [7]. Mesenchymal stem cells (MSCs) have recently attracted considerable attention as promising a promising cell source for the treatment of liver disease because they can be easily isolated, expanded and cryopreserved. MSCs obtained from various tissue sources include human bone marrow mesenchymal stem cells (BM-MSCs) [8], human adipose stem cells (ADSCs) [9], Wharton's Jelly-derived MSCs (WJ-MSCs) [10] and multipotent stem cells from amniotic fluid (AF-MSCs) [11]. These MSCs have been shown to differentiate into endodermal lineages such as hepatocytes. In addition, several clinical phase I, I/II, and II trials have demonstrated that human MSCs transplantation recovers hepatic function in patients with liver cirrhosis [12–14]. This

demonstrates that human MSCs might be strong candidate for the treatment of liver dysfunction.

In comparison with other MSCs, dental stem cells (DSCs) offer important advantages of lower ethical controversies and easily accessibilities. DSCs maintain stemness characteristics such as cell proliferation, multi-lineage differentiation potential, and immunomodulatory functions [15], and can be cryopreservation without losing these functions [16]. Thus, DSCs are considered an attractive alternative cell source for cell-based therapies for the treatment of liver-related diseases. In addition, DSCs are good cell-source to generate hepatocyte-like cells as they possess multipotent characteristics. DSC can be expanded allowing autologous transplantation and, most importantly without the need of genetic manipulation.

Effect of Soluble Signals on Hepatic Differentiation of Stem Cells

Several studies demonstrated that DSCs such as tooth germ progenitor cells (TGPCs) [4], dental pulp stem cells (DPSCs) [17–19], tooth-pulp stem cells (HTCP) CD117+ [19] and exfoliated deciduous teeth (SHED) [20] or SHED-CD117+ [21] can differentiate into functional hepatocytes in vitro (Table 1). DSCs under definite stimulation, are able to generate hepatocyte-like cells that resemble hepatocyte morphology, express liver-specific markers at mRNA and protein levels and perform functions typical of hepatocytes. For the hepatic maturation of DSCs, use of fibroblast growth factor (FGF), hepatocyte growth factor (HGF), Dexamethasone (Dex) and Oncostatin M (OSM) as hepatic inductors (Table 1) are reported. However, the mechanism behind the differentiation of DSCs into hepatocytes has not yet been completely known.

Several studies have showed that biochemical factors that have an essential role in liver development are also shown to induce hepatic differentiation of DSCs. Moreover, those biochemical factors have also been useful for maintaining hepatocytes cultures in vitro [26]. For example, the use of HGF and FGF-4 as inductors for hepatic differentiation of multipotent adult progenitor cells (MAPS) [27] and human umbilical cord blood-derived mesenchymal stem cells [28] are reported. HGF is considered a key growth factor for liver growth and function that controls different processes, including morphogenesis, motogenesis and mitogenesis.

HGF initiates multifunctional regulatory roles by interaction with HGF receptor (c-Met) [29], a transmembrane protein-tyrosine kinase expressed in normal hepatocytes, intestinal, ovarian, epithelium and endometrial endothelia [7]. HGF possesses strong antifibrotic activity and contributes to the onset or progress of liver fibrosis/cirrhosis and directly promotes the differentiation of MSCs into hepatic lineages, and transplantation of these cells improves liver injury in rats [30]. Therefore, HGF can potentially be used for the treatment of acute hepatic failure (AHF) [31] and CCl_4 induced liver necrosis [32]. FGF is another growth factor that works as mitotic agent for a variety of cells and tissues [33]. It stimulates differentiation and

Table 1 Studies that assessed the use of human dental stem cells (DSCs) for liver regeneration

Dental stem cells (DSCs)	In vitro hepatic differentiation	Animal model	Remarks	Reference
Human tooth germ progenitor cells (TGPCs)	DMEM low glucose, 2 % FBS, 100 ng/mL a-FGF (5 days); DMEM low glucose, 2 % FBS, 20 ng/mL HGF (5 days); 20 ng/mL DMEM low glucose, 2 % FBS, 20 ng/mL HGF, 10 nM Dexamehtasone, ITS-X, 10 ng/mL oncostatin M (11 days)	Liver fibrosis in CCl_4 treated rats	TGPCs subjected to in vitro hepatic differentiation had therapeutic effect on CCl_4-induced liver injury (portal veins)	Ikeda E. et al. [4]
Human dental pulp stem cells (DPSCs)	20 ng/mL HGF (5 days), 2 % FCS; 10 ng/mL oncostatin M, 10 nM Dex, 1 % ITS-X (15 days)	No in vivo model	Differentiation of DPSCs into hepatocyte like-cells	Ishkitiev et al. [22]
Human dental pulp stem cells (DPSCs)	DMEM low glucose, 1 % FCS, 20 ng/mL HGF, 10 ng/mL oncostatin, 10 mM nicotinammide, 1.25 μg/mL LDL,, 10 ng/mL FGF-4, 4 μg/mL insulin, 1.25 g/L glucose, 180 mg/L linoleic acid (40 day)	No in vivo model	Hepatic function of differentiated DPSCs	Ferro et al. [18]
Human tooth-pulp stem cells (HTCP) $CD117^+$	Serum free-DMEM, 1 % ITS-X, 100 μg/mL embryotrophic factor (ETF) (until 70 % confluence); 20 ng/mL HGF (5 days); 10 ng/mL oncostatin M, 10 nM Dex (15 days)	No in vivo model	H_2S increased the ability of human tooth-pulp cells to undergo hepatogenic differentiation	Ishkitiev et al. [19]
Human dental pulp stem cells (DPSCs) and human exfoliated deciduous teeth (SHED) $CD117^+$	Serum free DMEM, 1 % ITSX, 100 μg/mL embryotrophic factor (ETF) (until 70 % confluence); 20 ng/mL HGF (5 days); 10 ng/mL oncostatin M, 10 nM Dex (15 days)	No in vivo model	Hepatic differentiation of DPSCs and SHED in serum free medium	Ishkitiev et al. 2012 [17]

(continued)

Table 1 (continued)

Dental stem cells (DSCs)	In vitro hepatic differentiation	Animal model	Remarks	Reference
Cryopreserved human dental pulp stem cells (hDPSCs) isolated from vital extracted teeth with disease.	Serum deprived IMDM medium, 20 ng/mL HGF, 10 ng/ml β-FGF, nicodidamine 0.61 g/L (7 days). IMDM, 20 ng/mL oncostatin M, 1 μM Dex, 50 mg/mL ITS (34 days)	No in vivo model	Hepatic differentiation of hDPSCs from cryopreserved dental pulp tissues of vital extracted teeth with disease	Chen et al. [23]
Human exfoliated deciduous teeth (SHED) $CD117^+$	DMEM serum free, 1 % ITS-x 100 μg/mL embryotrophic factor (ETF) (until 70 %); 20 ng/mL HGF (5 days); 10 ng/mL Oncostatin M, 10 nM Dex (15 days)	No in vivo model	The level of hepatic differentiation in SHED compared with BM-MSCs was the same or higher. H_2S increased the level of hepatic differentiation.	Okada et al. [24]
Human exfoliated deciduous teeth (SHED) $CD117^+$	Serum free medium, 20 ng/mL HGF (5 days); 10 ng/mL oncostatin M, 10 nM Dex, HGF 10 ng/mL (15 days)	Acute liver injury (ALI) and secondary biliary cirrhosis in rats	hdSHED engraft morphologically and functionally into the livers of rats (transplantation to the spleen)	Ishkitiev et al. [21]
Human dental pulp stem cells (DPSCs)	DMEM 2 % FCS, 20 ng/mL HGF (5 days); DMEM 2 % FCS, 20 ng/mL HGF, 10 ng/mL oncostatin M, 10 nM Dex (15 days)	CCl_4-induced liver fibrosis in mice	Combined treatment of DPSCs and melatonin for the treatment of liver fibrosis (teil veins)	Cho et al. [25]
Human exfoliated deciduous teeth (SHED)	No in vitro hepatic differentiation	CCl_4-induced liver fibrosis model mice	SHED transplantation recovered liver dysfunction and led anti-fibrotic and anti-inflammatory effects (intrasplenically transplanted into mice)	Yamanaza et al. [20]

proliferation by binding to its receptor epidermal growth factor receptor (EGFR) [34]. Both, HGF and FGF have important roles in liver development, but it is unclear if they can induce definitive endoderm without the contribution of additional factors.

Recently, Lin et al. showed that hepatocytes differentiation of BM-MSCs is induced by cooperation of HGF and FGF-4 through the HGF/c-Met, a transmembrane receptor endowed with tyrosine kinase activity [35]. In some studies, in order to induce the late hepatic differentiation, the medium was supplemented with OSM [36], insulin-transferrin selenium (ITS) [37], Dex [38] and embryotrophic factor (ETF) [39]. OSM is a cytokine belonging to the interleukin 6 (IL-6) family and is recognized as having an essential role in liver development, it is essential for maturation of hepatocytes in combination with Dex [40]. Moreover, OSM plays a crucial role in progression of hepatocyte development through the signaling pathway of activator factor and signal transducer of transcription 3 (STAT 3) [41]. Some studies have shown that Dex is able to preserve the expression of transcription factors essential for the regulation of liver specific genes [38, 42]. Whereas, insulin-transferrin selenium (ITS) have been shown to induce survival and proliferation of primary hepatocytes [43].

In summary, HGF, EGF and OSM have important effects on the maintenance of primary human hepatocytes in vitro [44] and several studies have proposed that there is a need to expose cells to HGF and FGF4 in a sequential approach in order to increase the hepatic differentiation efficacy of BM-MSCs [45].

BM-MSCs and DPSCs have similar function and morphology, and thus several studies speculated that DSCs have the ability to differentiate not only in tissue of ectodermal and mesodermal origin, but also in those of endodermal origin. As a consequence, the combination of HGF, EGF, OSM, Dex and ITX previously established for ESCs and BMSCs was widely used in protocols to differentiate DSCs to hepatocytes [4, 17–19, 21, 22, 25].

Various Tissue Sources of DSCs

DSCs obtained from various sources can be directed towards hepatic differentiation using soluble factors as listed in Table 1. For example, Ikeda et al. demonstrated that stem cells derived from the neural crest-derived dental tissues can differentiate into endoderm cell lineages [4]. In particular, they showed that the tooth germ progenitor cells (TGPCs) were able to differentiate into functional hepatocytes. The differentiated hepatocytes were able to suppress inflammation and liver fibrosis in carbon tetrachloride (CCl_4) treated rats [4]. They further showed that the hepatic differentiated TGPCs can improve to the restoration of liver function as evaluated by the expression of hepatic serum marker such as albumin, bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT). In their protocol, TGPCs were first treated with a-FGF and HGF followed by the addition of the maturation factors OSM and Dex when the TGPCs already were committed to the hepatic fate.

The changes in morphology were evident and the production of albumin was observed starting from day 10.

In a similar study, Ishkitiev et al. showed that the differentiation of DSCs into hepatic-like cells was possible from DSCs obtained from full-grown wisdom or primary tooth pulp [22]. In fact, they showed the differentiation of wisdom tooth pulp cells (WTPCs) and deciduous tooth pulp cells (DTCPs) into hepatocytes-like cells using HGF, Dex, OSM and ITS-x. The differentiated DSCs produced proteins specific to hepatocytes and acquired hepatic specific functions including the storing of glycogen and the production of urea [22]. Subsequently, the same research group showed that hydrogen sulfide (H_2S) at physiological concentrations improved the capacity of human tooth-pulp stem cells (HTCP) to undergo hepatogenic differentiation [19]. The CD117-positive cells ($CD117^+$) were isolated from deciduous HTCP by using a magnetic cell sorting system and then cells were grown in medium supplemented with HGF, ETF, OSM and insulin-transferrin-selenium-x (ITS-x) with or without H_2S. The results demonstrated that the hepatic markers albumin (ALB), α-fetoprotein (αFP), and carbamoyl phosphate synthetase (CPS-1) were expressed more in H_2S treated HTCP with respect to control. This study demonstrate that H_2S at physiological concentrations is able to improve hepatogenic differentiation of HTCP in vitro [19].

More recently, Okada et al. have compared the hepatic differentiation of SHEDs with those of BM-MSCs in vitro with or without H_2S [24]. In their experiments they observed that either SHEDs or BM-MSCs $CD117^+$ without H_2S expressed similar levels of stem cell transcription factors. However, after differentiation, the expression of hepatic markers such as ALB, αFP, CPS-1 was significantly higher in SHEDs compared to BM-MSCs. Unlike the previous results, no significance differences were observed between H_2S-treated SHEDs and BM-MSCs after hepatic maturation in terms of ALB, αFP, CPS-1 expression, glycogen production and urea concentration. These results strongly suggest the possibility to use SHEDs in combination with H_2S for clinical treatment of liver disease.

In another study, Ferro et al. further demonstrated the hepatic-differentiation potential of DSCs [18]. They reported that under hepatic stimuli, DSCs increased their mRNA expression of albumin and hepatocyte growth factor receptor (C-met/HGF-r). Moreover, they showed that DSCs acquired the pattern of cytokeratin expression (CK8, CK18 and CK19) of normal hepatocytes and exhibited some hepatic functions such as the production and secretion of albumin [18].

The previous studies used fetal bovine serum (FBS) complemented medium for hepatic differentiation. The use of animal products in the differentiation medium represents a enormous obstacle for the application of in vitro cell-therapy methods in clinical applications due to the fact that FBS can cause hypersensitivity reaction and can be a potential vector for prion transmission [46]. Therefore, in order to overcome these limitations, Ishkitiev et al. described the differentiation of DPSCs $CD117^+$ and SHEDs $CD117^+$ into a high-purity hepatic lineage using serum-free medium [17].

Recently, Chen et al. have studied the hepatic potential after cryopreservation of DSCs isolated from human dental pulp tissues of teeth affected by disease such as

periodontitis and pericoronitis [23]. In their experiments they observed that hepatically differentiated DSC had a polygonal shape and expressed the specific liver genes including αFP, ALB, HNF1α and CK18.

Moreover, urea production and glycogen storage results denoted that the differentiated human DPSCs were functionally similar to normal hepatocytes-like cells. Although research studies regarding hepatic differentiation of DSCs are at beginning, the use of cryopreserved tissue to generate hepatocytes-like cells represents an encouraging alternative for the treatment of liver diseases.

To highlight the clinical potential of DSCs for the treatment of hepatic diseases, Ishkitiev et al. showed that human hepatically differentiated $CD117^+$ SHED (hdSHED) were able to engraft functionally in the liver of experimental rat models of hepatic diseases [21]. In fact, human specific markers such as albumin and αFP were found in the serum of the rats, confirming that transplanted hdSHED can be incorporated into structural components of the liver of rats having acute injury. This study showed that in vitro differentiation of DSCs in hepatocyte-like cells and the following transplantation could be a useful method to treat human liver diseases.

As described above, the transplantation of human TGPS in the liver of CCl_4 injured mice were able to inhibit fibrosis and inflammation [4]. Using the same animal model, Cho et al. recently reported that the use of melatonin in combination with the transplantation of human DPSCs drastically decrease liver fibrosis demonstrating that the treatment of DPSCs transplantation combined with melatonin administration could be used to improve liver functions [25]. Melatonin promoted hepatic differentiation by regulating the NF-κB pathway, p38, BMP, and ERK. Moreover, the grade of liver fibrosis measured as restoration of ALT, ASP and levels of ammonia was lower in CCl_4-injured mice treated with a combination of DPSCs transplantation and melatonin administration compared to either melatonin administration or DPSCs transplantation alone [25].

In the earlier studies, DSCs were pre-differentiated in vitro before transplantation in animal model [4, 21, 25]. Recently, Yamanaza et al. demonstrated that naïve SHEDs, not previously in vitro differentiated, were able to improve hepatic dysfunction and directly transform into hepatocytes in CCl_4 treated mice [20]. Moreover, these in vivo SHEDs converted hepatocytes participated in the hepatic recovery via tissue replacement and possessed anti-fibrotic and anti-inflammatory effects.

DSCs-Derived Induced Pluripotent Stem Cells (iPSCs) as Potential Cell Source

Recently, human somatic cells have been reprogramming into induced pluripotent stem cells (iPSCs) by addition of four transcription factors [47, 48]. These results offer the possibility of using adult somatic cells to obtain cells that shared many features with ESCs but with the advantage of being obtained from an adult source. As a consequence, in the last years, it became possible to obtain iPSCs from several adult tissues, including the hepatic tissue. iPSCs are typically generated by ectopic

expression of four transcription factors, *OCT3/4, Sox2, Klf4* and *c-Myc* in fibroblasts. Several researchers have demonstrated that iPSCs can be derived from different cell sources including skin fibroblasts [47], keratinocytes [49], blood progenitor cells [50], pancreatic cells [51] and primary human hepatocytes [52].

It has recently been showed that iPSCs can be considered a new cell source for stem cell therapy of liver diseases because they were able to differentiate into functional hepatocytes [53, 54]. Because of its accessibility and differentiation potential, DSCs has gradually drawn attention in the applications of iPSCs in regenerative medicine, in fact, DSC has been widely demonstrated as a source of iPSCs [55, 56]. The knowledge previously acquired in hepatic-differentiation of DSCs was successfully applied in iPSCs and two reports of efficient methods to obtain hepatocytes-like cells from dental pulp derived-iPSCs (DP-iPSC-Heps) have been published [57, 58]. A summary of these reports is shown in Table 2.

Interestingly, Chiang et al. reprogrammed human dental pulp fibroblasts into iPSCs and induced the differentiation of these dental pulp-derived iPSC (DP-iPSCs)

Table 2 Studies that assessed the use of human dental pulp derived iPSCs (DP-iPSCs) for liver regeneration

Dental stem cells (DSCs)	In vitro hepatic differentiation	Animal model	Remarks	Reference
Induced pluripotent stem cells (iPSCs) from dental pulp stromal cells Transduction of pMX vectors encoding the transcription factors: OCT4, Sox-2, Klf4 and c-Myc	RPMI 0.5 mg/ml Albumin fraction V, 100 ng/mL activin A (1 day); 0.15 and 1 % ITS (2 days); 30 ng/mL FGF-4, 20 ng/mL BMP-2 (4 days); 20 ng/mL HGF, 20 ng/mL KGF, oncostatin M, 0.1 μM Dex (5 days); DMEM N2 B27, glutamax, 0.1 mM β-mercaptoethanol (3 days)	Acute hepatic failure (AHF) mouse model induced by thioacetamide (TAA)	Engraftment of iPSC-Heps with injectable hydrogel (CHC) containing HGF reduced the TAA-induce hepatic necrosis and rescued liver function	Chiang et al. [58]
Induced pluripotent stem cells derived from human dental pulp (DP-iPSCs) Transduction of pMX vectors encoding the transcription factors: OCT4, Sox-2, Klf4 and c-Myc	Nanostructured amphiphatic carboxymethyl–hexanoyl chitosan (CHC)/ Polyurethane-graft-short-branch polyethylenimine copolymer/ miR122 mixture to accelerate the hepatic differentiation of iPSCs	Acute hepatic failure (AHF) mouse model induced by thioacetamide (TAA)	miR122-iPSC-Heps improved liver functions and rescued recipient survival	Chien et al. [57]

into functional iPSC-Heps [57]. Microarray analysis indicated that DP-iPSC-Heps, normal liver and ESCs derived hepatocytes (ESC-Heps) possessed a similar gene expression profile. Moreover, similar to mature ESC-Heps, DP-iPSCs-Heps acquired regular functions for glycogen storage and LDL uptake during hepatic differentiation. Moreover, DP-iPSCs-Heps expressed several hepatic markers including αFP, hepatocyte nuclear factor 3β (HBF-3β) and cytochrome c P450 enzyme CYP2E1. In their in vivo studies, Chiang et al. used an injectable carboxymethyl-hexanol chitosan hydrogel (CHC) having a continuous release of HGF (HGF-CHC), as delivery vehicle for transplantation of DP-iPSCs-Hep in immunocompromised acute hepatic failure (AHF) mouse model induced by thioacetamide (TAA) [58]. They observed that intrahepatic delivery of CHC-HGF hydrogel coupled with iPSC-Heps decreased the TAA-induced hepatic necrotic area and recovered liver functions, suggesting that HGF-CHC is an excellent vehicle for iPSCs-Heps engraftment in iPSCs based therapy against AHF [58].

In another study from the same group, Chien et al. investigated an alternative strategy to obtain mature hepatocytes-like cells from DP-iPSCs using a nanotechnology based delivery microRNA (miRNA) system. At this purpose, they used CHC to encapsulated the complex of polyurethane-graft-short-branch polyethylenimine copolymer (PU-PEI) and microRNA122 (miR122), a hepatocyte-specific miRNA [57]. Subsequently, PU-PEI complex was used as vehicle to deliver miR122 into DP-iPSCs. miR122 has been chosen because it is associated with some biological functions of the liver [59]. They observed that combination of miR122 plasmids with PU-PEI in nanostructured CHC (CHC/PU-miR122) were able to enhance miR122 delivery into human DP-iPSCs. As consequence, after the entry of miR122 into DP-iPSCs, these cells were able to successfully differentiate into iPSC-Heps having mature functions of hepatocytes [57]. In this work, using microarray analyses it has been observed that CHC/PU-PEI-miR122 shifted gene expression profile of DP-iPSCs toward that of the liver. Moreover, they observed that the delivery of a construct containing CHC/PU-miR122 and iPSCs-Heps into thioacetamide (TAA)-injured rat liver was able to significantly rescued liver functions. These results demonstrated for the first time that miR122-iPSCs-Heps transplantation could be used as new therapeutic approach to obtain liver regeneration of recipients with acute hepatic failure (AHF).

Conclusions and Future Directions

Several groups reported successful differentiation of DSCs into hepatocyte-like cells expressing defined hepatocellular antigens and functional properties that include the following: (a) phenotypic changes leading the acquisition of polygonal and polarized morphology; (b) expression of the specific proteins like α-FP, ALB, CPS-1, CK-18 (c) the capacity to synthetize urea and glycogen and (d) expression of protein or enzymes, such as AST, ALT, HNF-4. In all studies described above,

in vitro hepatic differentiation was established on a defined scheme of experimental protocol, which usually required first a differentiation step followed then by maturation step. The differentiation step was sustained by the treatment of DSCs with HGF and EGF, whereas in the maturation step DSCs were exposed to medium containing OSM, Dex and ITS-x. Once hepatocytes-like cells were obtained, several laboratories transplanted in vivo these DSCs to analyse their efficiency in animal models of CCl_4 induced liver injury or acute liver injury (ALI). Most of these experimental studies have reported that transplanted DSCs can effectively engraft in injured liver. These studies have provided evidence indicating that DSCs engrafting the liver during the course of experimental model of liver injury have potential to differentiate in hepatic like cells [4, 20, 21, 25]. All studies described in this chapter lead us to believe that DSCs is one of the promising cell-source to generate functional hepatocyte-like cells in appropriate quantities and purity for cell replacement therapy. However, for clinical applications there are yet several studies that need to be done to better understand the capacity of these cells. In vivo methodologies to check the comportment of the hepatocyte-like cells in humans will be essential to verify their potential application in cell therapy.

A new approach for generating hepatocyte-like cells from DSCs has taken advantage of induce pluripotent stem cells (iPSCs), one of the greatest inventions in the field of regenerative medicine. Human dental pulp stromal cells can be reprogrammed into iPSCs (DP-iPSCs) and can be differentiated into functional iPSCs-derived hepatocyte-like cells (iPSC-Heps) by using constructs of HGF-CHC or CHC/PU-miR122 [54, 58]. HGF-CHC and CHC/PU-miR122 are excellent vehicles for iPSCs-Heps engraftment in iPSCs based therapy against AHF.

In conclusion, this chapter shows the differentiation of DSCs into hepatocyte-like cells, indicating that MSCs of dental tissues have a hepatic potential and can be considered a new cell source for liver cell therapy. The described models offer suitable and convenient cell source of non-embryonic origin to obtain functional hepatocyte-like cells. Wisdom teeth are regularly extracted and deciduous teeth fall out naturally. This potentially makes them exceptionally useful as a simply accessible cell source. Furthermore, dental tissues can be easily manipulated and stored in stem-cell banks. The possibility of preserving DSCs, so that they can be later differentiated into hepatocytes and used for transplantation without risk of immunologic reaction could have an extraordinary effect on the cell therapy of liver deceases in the future. In the past, liver diseases were often considered irreversible, but today progress of research in cellular and molecular biology has demonstrated that hepatic cellular recovery is possible. At this purpose, the prospect of using DSCs as cell therapy for treating liver diseases is very encouraging. However, further studies are necessary to demonstrate long-tem safety of DSCs-based transplantation and moreover, clinical trials are necessary to verify their efficacy to recover liver functions in human patients affected by liver diseases.

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