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# Dental Pulp Stem Cells and Tissue Engineering Strategies for Clinical Application on Odontoiatric Field

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## 1. Introduction

Recent advances in tissue engineering have drawn scientists to test the possibility of tooth engineering and regeneration. Tooth regeneration is normally referred to as the regeneration of the entire tooth or root that can be integrated into the jaw bone. This technology is still at its infancy and when it matures, it may be used to restore missing teeth and replace artificial dental implants when the tooth is damaged but still in a reparable condition, regeneration of parts of the tooth structure can prevent or delay the loss of the whole tooth. To engineer and regenerate a whole tooth, the cell source, tissue engineering strategies and specific scaffolds needed to be correct choose.

Indeed, for example, to repair partly lost tooth tissues such as PDL, dentin, and pulp, one or two particular types of dental stem cells may be sufficient to fulfill the need. In light of such considerations, aim of the present chapter is to define the main strategies to isolate dental pulp stem cells, their characterisation and differentiation, tissue engineering strategies and clinical applications for the creation of artificial tissue useful in odontoiatric field.

## 2. Dental pulp stem cells

Dental pulp is a well known tissue enrich of adult mesenchymal stem cells: Dental Pulp Stem cells (DPSc). These adult stem cells play an important role in regenerative medicine both for oral and non oral pathoses thanks to their biological properties such as multipotency, high proliferation rates and accessibility (Yamada et al., 2010).

Beyond natural capacity of response to injury, dental pulp stem cells are attractive for their potential to differentiate, in vitro, into several cell types including odontoblasts, neural progenitors, chondrocytes, endothelocytes, adipocytes, smooth muscle cells and osteoblasts. The potential application of dental pulp stem cells and tissue engineering in dentistry are discussed in the present chapter (Sloan & Waddington, 2009).

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## 2.1 Tooth anatomy

Each tooth consists of three main parts: the crown, the neck and the root, that we can define with anatomic or clinical criteria. Here follow a brief review of the tooth anatomy (Fig.1) involved on stem cells-tissue engineering field.

### 2.1.1 Dentin

Dentin is a mesenchymal derived tissue lying between enamel or cementum and dental pulp (pulp chamber and root canal). It is a mineralized connective tissue with an organic matrix. It is made up of 70% inorganic materials (especially hydroxyapatite crystals), 20% organic materials and 10% water by weight. The bulk of organic matrix (85-90%) consists of type I collagen, there is also a minor amount of type V and VI collagen. Noncollagenous molecules of dentin are dentin phosphorines, Gla proteins, acidic glycoproteins, growth-related factors, serum derived proteins, lipids and proteoglycans. Dentin has microscopic channels (0,5-3  $\mu\text{m}$ ), called dentinal tubules, radiating outward from pulp cavity to dentino-enamel or dentino-cementum junction. These tubules contain projections of cells secreting dental matrix, known as odontoblasts. The most peripheral aspect of the pulp is lined by the body of these odontoblasts (Yoshida et al., 2002; Fonzi, 2000).

### 2.1.2 Pulp

Pulp consists of a loose connective tissue enclosed by rigid predentin and dentin. Along the border between the dentin and the pulp are odontoblasts. The thickness of dentinal layer increases with age due to the deposition of secondary and tertiary dentin, reducing the volume of the pulp chamber and the root canals. The most peripheral aspect of the pulp contains four layers of cells: the odontoblastic layer (the most external one), the cell-free zone, the cell-rich zone and the parietal plexus of nerves. Deep inside is the pulp proper, composed of a great amount of fibroblasts and ECM. Blood vessels and nerves enter the tooth mostly through the apical foramen. Other cells in the pulp include undifferentiated mesenchymal cells, deriving from dental papilla, fibrocytes, macrophages and lymphocytes (Fonzi, 2000; Ferguson, 2002).

### 2.1.3 Alveolar bone

The bone that supports the teeth is called alveolar bone. It is composed of compact bone and trabecular or spongy bone. The outside wall of the bone is compact bone, such as the thin layer that lines the socket known as lamina dura.

The spongy 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.

Alveolar bone proper is the part just around the tooth and it gives attachment to the PDL fibres (bundle bone). The alveolar bone proper is also called cribiform plate, due to the presence of perforation for the entry of vessels and nerves.

Bone is made of 65% inorganic material (mainly hydroxyapatite) by weight, 15% water, 20% organic material. The organic matrix is composed of collagen type I (90-95%), Gla proteins, glycoproteins, phosphorines, proteoglycans, growth factors and bone morphogenetic proteins (e.g. osteogenin) (Ferguson, 2002; Fonzi 2000).

## 2.2 Odontogenesis

During the sixth week of embryogenesis, after the migration of neural crest cells into head and neck mesenchyme, the ectoderm of the first brachial arch begins to proliferate giving rise to the vestibular lamina and the dental lamina (Langman, 2008).

Dental lamina is a band of ectodermic cells growing from the epithelium of the stomodeum into the underlying mesenchyme and giving rise to the enamel organs of teeth, along the horse shoe shaped dental arches.

Several transcription factors are implicated in odontogenesis, including Pax9, Pitx2, Runx2, Msx1, Msx2, Bmp2, Bmp4, Fgf8 and Fgf9 (Zhang et al., 2009; Bei & Maas,1998). The development is commonly divided into the following stages: the bud stage, the cap stage, the bell stage.

The early bell stage of odontogenesis is characterized by epithelial expansion and differentiation into the inner and outer enamel epithelium, stratum intermedium and stellate reticulum.

During the late bell stage, two tooth specific cell types are formed: ameloblasts, which derive from the inner enamel epithelium and produce enamel, and odontoblasts, which differentiate from dental papilla and synthesize dentin.

Dentinogenesis starts before enamel formation with the secretion of an organic matrix in the area directly adjacent to the inner enamel epithelium. Dentin is formed by the production of organic matrix (predentin) and the simultaneous mineralization of this matrix (Hao et al.2009). After crown formation, root development begins. The cells of the inner and of the outer enamel epithelium become in contact and give rise to the cervical loop at the base of enamel organ (Fonzi, 2000).

The cells of the cervical loop continue to grow away from the crown and become Hertwig's epithelial root sheath. It induce the adjacent cells of dental papilla to differentiate into odontoblasts and produce dentin. Once this structure fragments, the dentin of the root comes in contact with the dental follicle and stimulates the cementoblasts to begin cementum secretion.

The dental follicle also gives rise to the other supporting structure of the tooth: the periodontal ligament and the alveolar bone proper (Luan et al. 2006).

### **2.3 Dental pulp stem cells**

A stem cell is defined as a cell that has the ability to continuously divide to either replicate itself (self-renewing), or produce specialized cells than can differentiate into various other types of cells or tissues (multilineage differentiation). The microenvironment in which stem cells reside is called a stem cell niche and is composed of heterogeneous cell types, extracellular matrix (ECM) and soluble factors to support the maintenance and self-renewal of the stem cells (Yen & Sharp, 2008).

Stem cells can be generally classified in embryonic stem cells (ESCs) and adult stem cells (ASCs). ESCs derive from the early mammalian embryo at the blastocyst stage and have the capability to give rise to all kinds of cells. Thus, ESCs are considered pluripotent. On the contrary, ASCs are just multipotent because their differentiation potential is restricted to certain cell lineages. ASCs reside in several and perhaps most organs and tissues that have already developed. For this reason, ASCs are also referred to as postnatal stem cells.

Mesenchymal stem cells (MSCs) are ASCs with mesodermal and neuroectodermal origin. They are available from many tissues such as bone marrow, adipose tissue, umbilical cord and dental pulp. MSCs are able to differentiate into cells of mesodermal origin like adypocytes, chondrocytes or osteocytes, but they can also give rise to representative lineages of the three embryonic layers. For instance, it is well known that MSCs posses an extended degree of plasticity compared to other ASCs populations, including the ability to differentiate in vitro into non-mesodermal cell types such as neurons and astrocytes. MSCs,

in addition to their multipotency, are easy to isolate and culture *in vitro* and they do not apparently represent an ethical issue based on their source of origin.

MSCs were firstly discovered and characterized in bone marrow (Friedenstein et al., 1970). Bone marrow-derived stem cells (BMMSCs) can be easily obtained, greatly expanded in culture and used in cell-mediated therapies and tissue engineering applications. However, the clinical use of BMMSCs is limited by several problems, including a painful recovery often associated to a low number of harvested cells. For these reasons, many researchers begin to investigate alternative tissues for more abundant and accessible sources of MSCs with least invasive collection procedures.

Dental tissue from human third molar represents an easily accessible and often discarded source for MSCs harvesting. The first type of dental stem cell was isolated from the human pulp tissue and termed dental pulp stem cells (DPSCs) (Gronthos et al., 2000). Subsequently, four more types of dental-MSCLike populations were identified: stem cells from exfoliated deciduous teeth (SHED) (Miura et al., 2003), periodontal ligament stem cells (PDLSCs) (Seo et al., 2004), stem cells from apical papilla (SCAP) (Sonoyama et al., 2006), and dental follicle precursor cells (DFPCs) (Morsczeck et al., 2005). Among them, all except SHED are from permanent teeth. All of these stem cells demonstrate multipotentiality and the capability to regenerate multiple dental/periodontal tissues *in vitro* and *in vivo* (Huang, 2009).

Dental pulp is the soft connective tissue entrapped within the dental crown. It is divided into four layers. The external layer is made up of odontoblasts producing dentin; the second layer, called "cell free zone", is poor in cells and rich in collagen fibers; the third layer, called "cell rich zone", contains progenitor cells and undifferentiated cells, some of which are considered stem 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 is the core of the pulp and comprises the vascular area and nerves (D'Aquino et al., 2009).

Stem cells that reside in dental pulp, called dental pulp stem cells (DPSCs), are considered a population of MSCs, therefore markers that have been used for identifying MSCs are also used for DPSCs. DPSCs result positive to STRO-1, CD13, CD24, CD29, CD44, CD73, CD90, CD105, CD106, CD146, Oct4, Nanog and  $\beta$ 2 integrin, and negative to CD14, CD34, CD45 and HLA-DR. The persistence of negativity for CD45 and positivity for CD34 demonstrates that these cells are not derived from a hematopoietic source, nevertheless they are of mesenchymal origin (D'Aquino et al., 2007). Like all MSCs, DPSCs are also heterogeneous and the various markers listed previously may be expressed by subpopulations of these stem cells (Huang et al., 2009).

DPSCs were firstly identified and isolated by Gronthos and co-workers in 2000, based on their clonogenic abilities and rapid proliferative rates. In addition, they demonstrated that DPSCs can develop into odontoblasts, the cells that form the mineralized matrix of dentin (Gronthos et al., 2000). Moreover, when transplanted in immunocompromised mice, DPSCs mixed with hydroxyapatite/tricalcium phosphate (HA/TCP) form a pulp-dentin-like tissue complex. This complex is composed of a mineralized matrix with tubules lined with odontoblasts, and fibrous tissue containing blood vessels in an arrangement similar to the dentin-pulp complex found in normal human teeth. Interestingly, and in contrast to BMMSCs, DPSCs do not form areas of active hematopoiesis and adipocyte accumulation at the transplantation site. In another study, when DPSCs are seeded onto human dentin surface and implanted into immunocompromised mice, reparative dentin-like structure is deposited on the dentin surface (Batouli et al., 2003). These results together raise the

possibility that a protocol for pulp tissue regeneration and new dentin formation for clinical therapeutic purposes could be established.

Later, Laino and co-workers isolated a selected subpopulation of DPSCs called stromal bone producing dental pulp stem cells (SBP-DPSCs), which roughly represent 10% of dental pulp cells (Laino et al., 2005). These cells display a great capability of self-expanding and differentiating in pre-osteoblasts which are able to self-maintain and renew for long time. SBP-DPSCs differentiate into osteoblasts, producing in vitro a living autologous fibrous bone (LAB) tissue. When transplanted into immunocompromised rats, SBP-DPSCs form a lamellar bone containing osteocytes. In this setting, SBP-DPSCs produce bone but not dentin, as shown by mRNA expression of bone markers including osteocalcin, Runx-2, collagen I, alkaline phosphatase, but not dentin sialo phospho protein (DSPP), which is specific for dentin. Moreover, it has been observed that, during their differentiation, about 30% of SBP-DPSCs becomes endothelial cells. These cells are found lining the vessel walls of the newly formed woven bone. In addition, after in vivo transplantation, a complete integration of vessels within bone chips takes place, leading to the formation of a vascularised bone tissue (D'Aquino et al., 2007).

Further characterization revealed that DPSCs also possess adipogenic and neurogenic differentiation capacities by exhibiting adipocyte- and neuronal-like cell morphologies and expressing respective gene markers. In addition, DPSCs were also found to undergo chondrogenic and myogenic differentiation in vitro. The plasticity and multipotential capability of DPSCs can be explained by the fact that dental pulp is made of both ectodermic and mesenchymal components, containing neural crest-derived cells (D'Aquino et al., 2009). DPSCs can be collected from dental pulp by means of a non-invasive practice that can be performed in the adult during life and in the young after surgical extraction of wisdom teeth. DPSCs can survive for long periods and can be passaged several times. It is possible to obtain more than 80 passages without clear signs of senescence. Furthermore, DPSCs can be cryopreserved and stored for long periods without losing their multipotential differentiation ability (Laino et al., 2005).

### **3. Role of DPSCs in regenerative medicine**

Teeth have a complex structural composition that ensures both hardness and durability. However, this structure is vulnerable to trauma and bacterial infections. When the tooth is damaged but still in a reparable condition, regeneration of parts of the tooth structure can prevent or delay the loss of the whole tooth. This fact is of importance because tooth loss affects not only basic mouth functions but aesthetic appearance and quality of life (Huang, 2009). The regenerative response of teeth to damage and structural degeneration are diverse and compartment-dependent, since teeth, as complex structures, harbour both living (periodontal ligament, cementum, pulp) and acellular (enamel, dentine) tissues. Of all the dental structures, only enamel is incapable of regenerating its original structure, while the remaining tissues possess that capacity in varying degrees, dependent on multiple factors (Inanç & Elçin, 2011).

The dental pulp plays a major role in tooth regeneration after injury, by participating in a process called reparative dentinogenesis. When pulp tissue is exposed as consequence of the loss of the overlying dentin, direct pulp-capping therapy allows the pulp to form new dentin. It has been observed that the use of various cement-based compounds, such as calcium hydroxide and mineral trioxide aggregate (MTA), promotes the activity of

dentinogenesis. Cells that remain in the healthy portion of the pulp migrate to the injured site, proliferate by the growth factors released from surrounding dentin matrix and attach the necrotic layer to form osteodentin. Later, the cells attached to osteodentin differentiate into odontoblasts to produce tubular dentin, thus forming reparative dentine. This early mineralized tissue preserves the pulp integrity and serves as protective barrier upon the injury (Nakashima, 2005).

When the tooth is further damaged, dentin regeneration becomes difficult as it requires a healthy pulp. Thus, bigger traumas or advanced caries are clinically treated with root canal therapy, in which the entire pulp is cleaned out and replaced with a gutta-percha filling. However, living pulp is critical for the maintenance of tooth homeostasis and essential for tooth longevity.

An ideal form of therapy might consist of regenerative approaches in which diseased or necrotic pulp tissues are removed and replaced with regenerated pulp tissues to revitalize the teeth. In particular, the regenerative pulp therapy would reconstitute the normal tissue continuum at the pulp-dentine border by regulating the tissue-specific processes of reparative dentinogenesis. Two types of dental pulp regeneration can be considered based on the clinical situations: *in situ* regeneration of partial pulp or *de novo* synthesis of a total pulp replacement (Sun et al., 2010).

Engineering and regeneration of dental pulp tissue still remain a difficult task. A regenerated pulp tissue should be functionally competent: it should be vascularised, contain similar cell density and architecture of ECM to those of natural pulp, be capable of giving rise to new odontoblasts lining against the existing dentin surface, produce new dentin and be innervated. The first step to engineer tissues is to isolate cells with the right phenotype and propagate them in suitable culturing environments. DPSCs can be isolated by two methods: the enzyme-digestion method and the explants outgrowth method. The first method involves the collection of the pulp tissue under sterile conditions, the digestion with appropriate enzymes (collagenase, dispase, trypsin), the seeding in culture dishes containing a special medium supplemented with necessary additives, and then the incubation at 37°C. The second method implies that the extruded pulp tissue is cut into 2 mm<sup>3</sup>, and directly incubated in culture dishes containing the essential medium with supplements. A period of two weeks is generally needed to allow a sufficient number of cells to migrate out of the tissue. It has been demonstrated that cells isolated by enzyme-digestion have a higher proliferation rate than those collected by outgrowth (Huang et al., 2006).

Once these cells are grown on a two-dimensional surface, it is possible to transfer them to a three-dimensional scaffold construct. The scaffold provides a 3D environment for cells to attach and grow, therefore mimicking the *in vivo* condition (Fig.2). An ideal scaffold should be biocompatible, biodegradable, and have adequate physical and mechanical strength. Then, it should be porous to allow placement of cells and effective transport of nutrients, oxygen, waste as well as growth factors. Finally, it should be replaced by regenerative tissue while retaining the shape and form of the final tissue structure (Saber, 2009).

Scaffolds can be fabricated from natural polymers or synthetic materials. The natural polymers have advantages of good biocompatibility and bioactivity. On the contrary, synthetic matrices enable precise control over the physiochemical properties such as degradation rate, porosity, microstructure, and mechanical strength (Sharma & Elisseeff, 2004).

Examples of natural polymers are collagen, gelatin, dextran and fibronectin. Although collagen is a commonly used matrix in which to grow cells in three-dimensions, several cell types are known to cause the contraction of collagen. It has been demonstrated that pulp

cells markedly cause the contraction of collagen with a reduction down to ~30%, which might affect pulp tissue regeneration (Huang et al., 2006).

Examples of synthetic polymers are polylactic acid (PLA), polyglycolic acid (PGA) or their co-polymers, poly lactic-co-glycolic acid (PLGA). Recent experiments demonstrate that DPSCs seeded onto PLGA scaffolds regenerate a pulp/dentin-like tissue (Huang, 2009). Other artificial scaffolds are hydrogels, like polyethylene glycol (PEG)-based polymers, or inorganic compounds such as hydroxyapatite (HA), tricalcium phosphate (TCP) and calcium polyphosphate (CPP). These are used to enhance bone conductivity and have proved to be very effective for tissue engineering of DPSCs (Wang et al., 2006).

Apart from DPSCs and an appropriate scaffold, dental pulp regeneration also requires the use of growth factors and ECM molecules that induce specific differentiation pathways and maintain the odontoblast phenotype. It is known that several factors, such as transforming growth factor  $\beta$  (TGF $\beta$ ), bone morphogenic proteins (BMPs), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF), are secreted by odontoblasts and incorporated within the dentine matrix during dentinogenesis. When these molecules are released from the dentin, they are bioactive and fully capable of inducing cellular responses, as for example those that lead to the generation of reparative dentin and to dental pulp repair (Casagrande et al., 2011).

Dental pulp tissue engineering is an emerging field that can potentially have a major impact on oral health. However, the source of morphogens required for stem cell differentiation into odontoblasts and the scaffold characteristics that are more conducive to odontoblastic differentiation are still unclear. (Demarco 2010) investigated the effect of dentin and scaffold porogen on the differentiation of human dental pulp stem cells (DPSCs) into odontoblasts. Poly-L-lactic acid (PLLA) scaffolds were prepared in pulp chambers of extracted human third molars using salt crystals or gelatin spheres as porogen. DPSCs seeded in tooth slice/scaffolds or control scaffolds (without tooth slice) were either cultured *in vitro* or implanted subcutaneously in immunodeficient mice.

DPSCs seeded in tooth slice/scaffolds but not in control scaffolds expressed putative odontoblastic markers (DMP-1, DSPP, and MEPE) *in vitro* and *in vivo*. DPSCs seeded in tooth/slice scaffolds presented lower proliferation rates than in control scaffolds between 7 and 21 days ( $p < 0.05$ ). DPSCs seeded in tooth slice/scaffolds and transplanted into mice generated a tissue with morphological characteristics similar to those of human dental pulps. Scaffolds generated with gelatin or salt porogen resulted in similar DPSC proliferation. The porogen type had a relatively modest impact on the expression of the markers of odontoblastic differentiation. Collectively, this work shows that dentin-related morphogens are important for the differentiation of DPSC into odontoblasts and for the engineering of dental pulp-like tissues and suggest that environmental cues influence DPSC behavior and differentiation potential.

Yang et al (Yang 2010) investigated, moreover the *in vitro* and *in vivo* behavior of dental pulp stem cells (DPSCs) seeded on electrospun poly( $\epsilon$ -caprolactone) (PCL)/gelatin scaffolds with or without the addition of nano-hydroxyapatite (nHA). For the *in vitro* evaluation, DNA content, alkaline phosphatase (ALP) activity and osteocalcin (OC) measurement showed that the scaffolds supported DPSC adhesion, proliferation, and odontoblastic differentiation. Moreover, the presence of nHA upregulated ALP activity and promoted OC expression. Real-time PCR data confirmed these results. SEM micrographs qualitatively confirmed the proliferation and mineralization characteristics of DPSCs on both scaffolds. Subsequently, both scaffolds seeded with DPSCs were subcutaneously



implanted into immunocompromised nude mice. Scaffolds with nHA but without cells were implanted as control. Histological evaluation revealed that all implants were surrounded by a thin fibrous tissue capsule without any adverse effects. The cell/scaffold composites showed obvious in vivo hard tissue formation, but there was no sign of tissue ingrowth. Further, the combination of nHA in scaffolds did upregulate the expression of specific odontogenic genes. In conclusion, the incorporation of nHA in nanofibers indeed enhanced DPSCs differentiation towards an odontoblast-like phenotype in vitro and in vivo.

Galler et (Galler 2008) developed an approach to develop novel regenerative strategies and engineer dental tissues, two dental stem cell lines were combined with peptide-amphiphile (PA) hydrogel scaffolds. PAs self-assemble into three-dimensional networks of nanofibers, and living cells can be encapsulated. Cell-matrix interactions were tailored by incorporation of the cell adhesion sequence RGD and an enzyme-cleavable site. SHED (stem cells from human exfoliated deciduous teeth) and DPSC (dental pulp stem cells) were cultured in PA hydrogels for 4 weeks using different osteogenic supplements. Both cell lines proliferate and differentiate within the hydrogels. Histologic analysis shows degradation of the gels and extracellular matrix production. However, distinct differences between the two cell lines can be observed. SHED show a spindle-shaped morphology, high proliferation rates, and collagen production, resulting in soft tissue formation. In contrast, DPSC reduce proliferation, but exhibit an osteoblast-like phenotype, express osteoblast marker genes, and deposit mineral. Since the hydrogels are easy to handle and can be introduced into small defects, this novel system might be suitable for engineering both soft and mineralized matrices for dental tissue regeneration.

In the future, the success of regenerative endodontic therapy will depend on the ability to yield a functional pulp tissue within cleaned and shaped root canal systems to revitalize teeth. This may be achieved by an in vivo approach, where pulp tissue is regenerated in situ into root canals, or by an ex vivo approach, which implies a de novo engineered pulp relying on the tissue-engineering triad (DPSCs, scaffold, growth factors).

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These contribution books collect reviews and original articles from eminent experts working in the interdisciplinary arena of biomaterial development and use. From their direct and recent experience, the readers can achieve a wide vision on the new and ongoing potentials of different synthetic and engineered biomaterials. Contributions were not selected based on a direct market or clinical interest, than on results coming from very fundamental studies which have been mainly gathered for this book. This fact will also allow to gain a more general view of what and how the various biomaterials can do and work for, along with the methodologies necessary to design, develop and characterize them, without the restrictions necessarily imposed by industrial or profit concerns. The book collects 22 chapters related to recent researches on new materials, particularly dealing with their potential and different applications in biomedicine and clinics: from tissue engineering to polymeric scaffolds, from bone mimetic products to prostheses, up to strategies to manage their interaction with living cells.

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