

Expert Opinion

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Dental stem cells for tooth regeneration and repair

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Mesenchymal stem cells (MSCs) resident in bone marrow are one of the most studied and clinically important populations of adult stem cells. Cells with similar properties to these MSCs have been described in several different tooth tissues and the potential ease with which these dental MSCs could be obtained from patients has prompted great interest in these cells as a source of MSCs for cell-based therapeutics. In this review we address the current state of knowledge regarding these cells, their properties, origins, locations, functions and potential uses in tooth tissue engineering and repair. We discuss some of the key controversies and outstanding issues, not least of which whether dental stem cells actually exist.

Keywords: dental stem cells, tooth development, tooth engineering, tooth repair

Expert Opin. Biol. Ther. (2009) 9(9):1143-1154

1. Introduction

Teeth are unique structures that contain the hardest biological substance known, the enamel, a unique material that is highly preserved in the fossil record. Tooth origins have been traced back to the earliest vertebrates where skin denticles of extinct fishes are thought to have 'moved' into the oral cavity as jaws evolved. Teeth are only found in vertebrates and their evolution is believed to be associated with the appearance of the neural crest [1,2].

Adult humans have four incisors, two canines, four premolars and six molars in each jaw. Mice, the most commonly used experimental animal model, do not develop canine and premolar teeth; instead the incisors (which continuously grow throughout life) and the molars are separated by a region devoid of teeth called the diastema.

Current replacement tooth methods such as tooth implantation use synthetic materials. Other possibilities include autotransplantation and subsequent reshaping. All these techniques have been used in dentistry for many years, but have never been satisfactory for cosmetic or physiological reasons.

A growing number of studies demonstrate the presence of stem cells in different tooth areas. They present a diverse potential for multilineage differentiation but undoubtedly are potential sources of cells for dental tissue repair and tooth tissue engineering. The understanding of their properties and to what extent they can be used remains one of the greatest challenges in the dental field in the coming years.

Herein we present a concise review of the main dental stem cell populations and discuss their current and future perspectives in repairing lost dental tissues or even creating a new biological tooth.

2. Tooth development

Teeth are formed by epithelial–mesenchymal interactions [3]. The epithelium originates from stomodeum or pharynx and the mesenchyme originates from

neural crest cells. During early development, cranial neural crest cells migrate laterally and anteriorly to form the ectomesenchyme of the first brachial arch and the frontonasal process [4]. Interactions between cells and tissues constitute a central mechanism regulating the development of all multicellular organisms and therefore, the developmental of a tooth is a very similar process to that for other skin derivatives such as hairs, feathers and scales [2].

The first morphological sign of tooth development is a thickening of the oral epithelium. This thickened epithelium yields the dental lamina on the lingual side and the vestibular lamina on the vestibular side. The dental lamina gives rise to the teeth and the vestibular lamina forms a sulcus between the cheek and the teeth. The proliferation of the dental lamina is greatest in a specific location corresponding to the future teeth and the process gives rise to round or oval structures known as placodes that invade the mesenchyme and will form the tooth buds. The buds will penetrate into the underlying mesenchyme which in turn, will start to condense around this bud. The following stages (cap and bell) are marked by cell growth and morphogenesis in a very intricate and self-controlled mechanism that results in the establishment of the form of the tooth crown in the exact location. This mechanism, as in the case of other developing tissues in the body, must be tightly co-ordinated and linked to position. The mesenchymal cells will form the dental papilla, formation of which will culminate with the differentiation of the pulp tissue and the odontoblasts. The dentine-forming odontoblasts and the enamel-forming ameloblasts are unique to teeth and they differentiate terminally during the bell stage of tooth development. This takes place at the interface of the epithelium and mesenchyme and is regulated by interactions between the two tissues [5-7]. The odontoblasts secrete a collagenous extracellular matrix which subsequently mineralizes into dentin, a bone-like hard tissue. The ameloblasts deposit the enamel matrix which directs the mineralization of the enamel into the hardest tissue in the body. The outer cells of the condensed mesenchyme around the epithelium (now called enamel organ) will form the dental follicle which will give rise to the periodontal ligament including cementoblasts. After crown morphogenesis, the roots of the teeth develop and subsequently the teeth erupt into the oral cavity [8].

3. Dental stem cells: different populations with diverse potential

3.1 Stem cells from dental pulp

The dental pulp develops from the dental papilla, occupies the central portion of the teeth, called the pulp cavity, and is a connective tissue permeated by blood vessels and nerves. As the pre-dentin is continuously deposited during crown formation and tooth eruption, the dental pulp becomes progressively smaller in volume [8].

The capacity of the human dental pulp to react after injury to form tertiary dentin has been recognized for decades. Odontoblast replacement involves multiple DNA replications and migration of pulpal cells to the exposure site from the deeper pulp. These replacement cells appear to be derived from a deeper population of cells that can migrate after induction, differentiating as odontoblasts [9,10].

The mechanism underlying the process of odontoblast replacement is not well-understood. However, in 2000 a population of odontogenic progenitor cells with high clonogenic and proliferative capacity was identified and isolated from the dental pulp of humans permanent third molars, dental pulp stem cells (DPSCs). When those cells were compared with bone marrow stromal stem cells (BMSCs), both cell lines shared the same immunophenotype [11] and similar levels of gene expression for more than 4000 known human genes [12]. Functional studies showed that DPSCs produced only sporadic, but densely calcified nodules, and did not form adipocytes, whereas BMSCs routinely calcified throughout the adherent cell layer with clusters of lipid-laden adipocytes resembling bone marrow [11]. Moreover, DPSCs have been shown to maintain an average of 30% higher proliferation rate and growth potential due to the elevated amounts of specific cell cycling molecules such as cyclin-dependent kinase 6 (CDK6) and IGF in the pulp tissue [12]. Both dental pulp and bone marrow stem cell populations express similar perivascular markers also described as putative stem cell surface markers, including CD44, CD106, CD146, 3G5, and STRO-1 [13]. Perivascular cells have been postulated as the source of cells that differentiate into odontoblasts [14].

To further characterise this population as mesenchymal stem cells, previously transplanted DPSCs were re-isolated from immunocompromised mice and re-transplanted into other animals, showing a capacity to form a dentin-pulp-like complex of human origin without any induction. Cells were also capable of differentiating into adipocytes and neural-like cells [15].

Side populations of cells can be isolated on the basis of exclusion of the dye Hoechst 33342. This technique has been used in the hematopoietic system and cells isolated from bone marrow express stem cell markers and can reconstitute the bone marrow of lethally irradiated mice [16,17]. Using the same technique of dye exclusion, side cell populations with stem cell properties were isolated from permanent porcine [18] and human dental pulps [19]. The porcine cells were self-renewable expressing B lymphoma Moloney-murine leukaemia virus insertion region 1 (Bmi1) continuously, showed diverse differentiation potential into chondrogenic, adipogenic and neurogenic tissues and were able to express dentin sialoproteins and enamelysin in a 3D pellet culture after induction with bone morphogenetic protein 2 (BMP-2) [18]. The human side population cells accounted for about 0.79% of the total pulp cells, grew more rapidly than the other cell populations of the pulp and had the capacity to form odontoblast-like cells [19]. Although this approach is very interesting, the side populations are not homogeneous and further investigations are required.

The presence of stem cells was investigated in deciduous teeth. Stem cells were isolated from human exfoliated deciduous teeth (SHED). After *in vivo* transplantation, SHEDs were able to induce bone formation, generate dentin, and survive in mouse brain expressing neural markers. In addition, they were highly proliferative and clonogenic, but compared with DPSCs, SHED cells showed higher proliferation rates, increased cell-population doublings, osteoinductive capacity *in vivo* and failure to reconstitute a dentin-like cell cluster formation. It is been suggested that they represent a population of multipotent stem cells more immature than the previously examined postnatal dental stromal stem cell populations. SHED cells also express neuronal and glial markers, which may be related to the neural crest origin of the dental pulp [20,21].

Dental pulp cells are not only diverse considering their origin in adult or deciduous pulp tissue. They can also behave differently depending on the technique used for their isolation and on the culture conditions. Consequently, populations with different characteristics and potency have been described and often results are difficult to replicate.

A population of c-kit⁺/CD34⁺/CD45⁺ dental pulp cells isolated by FACS has been shown to be self-renewable and able to produce living autologous bone (LAB) *in vivo*. This is a fibrous bone tissue that resembles human bone during mineralization, with an external layer formed by osteoblasts markedly positive for osteocalcin, and after transplantation into immunocompromised rats, LAB formed lamellar bone containing osteocytes [22].

A population of human immature dental pulp stem cells (hIDPSC), which express embryonic stem cell markers octamer-binding transcription factor 4 (Oct-4), Nanog, stage-specific embryonic antigen (SSEA)-3, SSEA-4, TRA-1-60 and TRA-1-81 was isolated from dental pulp of deciduous teeth. Under chemically defined culture conditions, these cells could undergo uniform differentiation into smooth and skeletal muscle, neurons, cartilage and bone. Following intraperitoneal injection into BALB/c nude mice, these IDPSC cells showed engraftment in various organs [23]. Further studies revealed that this immature population from dental pulp could contribute to the formation of mouse embryos, showing biological compatibility with the mouse host environment. The cells could survive, proliferate and contribute to the inner cell mass and the trophoblast cell layer after introduction into early mouse embryos. When transferred to foster mice (n = 5), these blastocysts with human pulp immature stem cells (n = 57) yielded embryos (n = 3) and fetuses (n = 6); demonstrating presence of human cells in various organs, such as brain, liver, intestine and hearts, of the human/mouse chimaeras [24].

3.2 Stem cells from the periodontal ligament

Periodontal ligament is a soft connective tissue located between the cementum and the inner wall of the alveolar bone socket and its functions include sustaining and helping

to constrain teeth within the jaw maintaining tooth nutrition and homeostasis.

The presence of progenitors/stem cells in the periodontal ligament has been known for many years [25]. Dental stem cells from human periodontal ligament (PDLSCs), proved to be capable of differentiating into multilineages, and when implanted into nude mice the generated cementum/periodontal ligament structures resemble native periodontum ligament with a thin layer of cementum interfaced with dense collagen fibers similar to the Sharpey's fibers. Under defined culture conditions, PDLSCs differentiated into cementoblast-like cells, adipocytes, and collagen-forming cells. Transplanted human PDLSCs were able to form a dense type I collagen-positive PDL-like tissue and the collagen fibres generated *in vivo* could connect with the cementum-like structures forming functional attachment of cementum/PDL [26].

When PDLSCs were cryopreserved they were able to maintain normal periodontal ligament stem cell characteristics, including expression of the mesenchymal stem cell surface molecule STRO-1, single-colony-strain generation, multipotential differentiation, cementum/periodontal-ligament-like tissue regeneration, and a normal diploid karyotype, indicating that a solid human tissue can be preserved for subsequent post-natal stem cell isolation and tissue regeneration [27].

PDLSC populations have been derived from ovine periodontal ligament using immunomagnetic bead selection, based on expression of the mesenchymal stem-cell-associated antigen CD106 (vascular cell adhesion molecule 1). The cells formed adherent clonogenic clusters, exhibited a high proliferation rate *in vitro* and expressed a phenotype (CD44⁺, CD166⁺, core binding factor alpha-1 (CBFA-1)⁺, collagen-I⁺, bone sialoprotein⁺) consistent with human-derived PDLSCs. *Ex vivo*-expanded ovine PDLSCs demonstrated the capacity to regenerate both cementum-like mineral and periodontal ligament when transplanted into NOD/SCID mice [28].

A PDL is similar to a tendon since both tissues are capable of absorbing mechanical forces of stress and tension. Scleraxis, a tendon-specific transcription marker [29-30], is more highly expressed in PDL stem cells than DPSCs or BMSSCs, implying that the periodontal ligament cells represent a unique population of adult mesenchymal stem cells different from pulp tissue or bone marrow [13].

PDL stem cells also seem to be located in the perivascular area since they express similar perivascular markers to those observed in DPSC and SHED cells. In addition, perivascular cells located in the endothelial spaces of the alveolar bone area around the PDL are slow-cycling and their progeny can rapidly migrate out of the compartment [31], both characteristics attributed to stem cells. The periodontal ligament contiguous with the endosteal spaces exhibited five times more migrating cells from the alveolar bone, implying that cells migrate from endosteal spaces into the periodontal ligament and there express the phenotype for osteoblasts or cementoblasts [31].

Interestingly, when PDL cells were transplanted into nude mice using gelfoam (collagen-based gelatine sponge) as a carrier, they produce significant amounts of collagen fibers with no mineralization, improving the facial wrinkles of the mouse [32], but when hydroxyapatite and tricalcium phosphate (HA/TCP) is used as a carrier, both collagen fibres and cementum are formed [26].

3.3 Stem cells from the root apical papilla

The root apical papilla is a small tissue located in the exterior of the root foramen area where it exists while the root apex is still open during the process of root development until complete eruption in the oral cavity. Apical papilla is apical to the epithelial diaphragm, and there is an apical cell-rich zone lying between the apical papilla and the pulp. The tissue is loosely attached to the apex of the developing root and can be easily detached with a pair of tweezers [33].

A population of stem cells from this area (stem cells from apical papilla (SCAP)) at passage 1 expressed many surface markers including STRO-1, CD24, CD29, CD73, CD90, CD105, CD106, CD146, CD166 and alkaline phosphatase (ALP) but were negative for CD34, CD45, CD18 and CD150. Comparing SCAP with previous described stem cell populations from dental pulp (DPSC) and bone marrow (bone marrow mesenchymal stem cell; BM MSC), it was shown that CD24 appears to be a specific marker for SCAP cells. In response to osteogenic induction conditions in culture, SCAP cells begin to downregulate their expression of CD24 while gaining expression of alkaline phosphatase, suggesting that SCAP represent a population of early progenitors that have at least some advantages for use in tissue regeneration. After transplantation into an immunocompromised mouse, SCAP cells could form functional dentinogenic cells capable of secreting dentin and expressing dentin sialoprotein (DSP), and when co-transplanted with PDLSC cells, they formed dentin and periodontal ligament respectively [34]. In addition to their dentinogenic potential, SCAP also exhibit adipogenic and neurogenic differentiation capabilities when treated with respective stimuli [35].

When compared with DPSC cells under the same culture conditions, SCAP cells express many different genes, a significantly higher rate of bromodeoxyuridine (BrdU) uptake, an elevated tissue regeneration capacity, an improved migration capacity in scratch assays and a higher telomerase activity, indicating that they form a distinct population of dental stem cells [34]. In addition, SCAP cells are a source of odontoblasts that produce primary dentin for the root [35] while DPSC are a source of odontoblasts that produce reparative dentin [11].

Other populations subsequently isolated from the root apical papilla cultured with porous hydroxyapatite (HA) scaffolds and transplanted into immunocompromised rats for 12 weeks exhibited the same characteristics, forming bone and dentin-like structures in the pores of the HA [36]. Further studies have shown that cells from the root apical papilla of third molars express the neural crest genes sex

determining region of the Y chromosome-related homeobox 9 (Sox9), Snail1, Snail2, Twist1, muscle specific homeobox homolog 2 (Msx2), and distal-less homeobox 6 (Dlx6). The cells differentiated into neurogenic, chondrogenic and osteogenic lineages and were shown to produce bone matrix in athymic mice. They were positive for CD49d, CD56 (neural cell adhesion molecule (NCAM)), and platelet-derived growth factor receptor alpha (PDGFR alpha) but were negative for the pericyte marker STRO-1 [37] which is a marker commonly found in other dental stem cell populations.

The root apical papilla is a tissue present only during root development but it is accessible in dental clinical practice especially from extracted wisdom teeth. The wisdom teeth develop later in life compared with the other human teeth, making it possible to access a developing tissue similar to those in embryonic development during adult life. In addition, an adult usually has four of these teeth and each one of their roots will present one apical papilla and therefore, it may be possible to bank these high-quality dental stem cells for future autologous use.

3.4 Stem cells from dental follicle

Dental follicle is the fibrous tissue that surrounds the developing tooth germ, and it has been implicated to contain progenitors for cementoblasts, periodontal ligament cells and osteoblasts. Dental follicle cells will give rise to the periodontal ligament by differentiation of the periodontal ligament fibroblasts. These fibroblasts secrete collagen, which interacts with fibers on the surfaces of adjacent bone and cementum [38].

When dissociated cells from six-month-old porcine third molars tooth buds were seeded onto biodegradable polymers they formed a tooth structure composed of dentin, odontoblasts, pulp tissue, root epithelial cells (Hertwig's epithelial root sheath (HERS)), cementoblasts and an enamel organ containing enamel [39]. The process of cell reorganisation seems to parallel that of natural tooth development with evident epithelial-mesenchymal interactions. A similar result using the same approach showed the formation of enamel-covered dentin and cementum-covered dentin [40].

Dental follicle cells isolated from cattle can form cementoblasts when transplanted into immunocompromised mice, indicating that cementoblast progenitors are present in this tissue [41,42]. Progenitor cells isolated from the dental follicle of human third molars have been shown to be positive for Notch-1 and Nestin. When compared with cells from bone marrow, periodontal ligament and osteoblasts, cells from dental follicle demonstrate higher levels of IGF-2. After induction they were capable of forming compact calcified nodules or appeared as plain membrane structures of different dimensions and after transplantation in immunocompromised mice, they differentially express osteocalcin (OCN) and bone sialoprotein (BSP) without any sign of cementum or bone formation [43].

The presence of heterogeneous cell populations in the dental follicle has been shown by different clones under the same culture conditions having different activities of alkaline

phosphatase and different potentials for differentiation [44]. In a clone of porcine dental follicle cells, after transplantation experiments, differences in the expression of BSP and periostin were found under the influence of collagen 1, which facilitated the mineralization process [45]. An understanding of the mechanisms that controls the pathways involved in dental follicle cell differentiation, which, in turn, will result in a mineralised or a fibroblastic cell type (bone or periodontal ligament), imply that in the future, the procurement of periodontal ligament cells will not necessarily require an extracted tooth.

4. Dental stem cells for whole body tissue engineering

Many of the same genes are concurrently involved in the development of both the facial skeleton and teeth. Selective change of the dentition may well be genetically correlated with other changes in the face and skull [46] and therefore, research into tooth development provides an important tool to address general questions of orofacial hard tissue development and organogenesis.

Although the initiation and early morphogenesis of teeth occurs before bone formation starts in the jaws, the development of the teeth and the surrounding bone is later tightly co-ordinated. Postnatally, the teeth have a central role in regulating the extent and direction of the growth of the alveolar processes [8].

As previously described, the dental pulp contains cells analogous to the bone as they express osteogenic markers and respond to same growth factors for differentiation into bone [47]. Therefore, they are one of the most promising sources of autologous MSCs to be used in craniomaxillofacial surgery. In fact, many studies are focusing on this capacity of dental stem cells.

A population selected from human third molars provided evidence that dental pulp is extremely rich in stem cells, that are *c-kit*⁺/*CD34*⁺/*STRO-1*⁺/*CD45*⁻ and capable of differentiation toward several stromal-derived differentiated cells and mainly osteoblasts [48]. Following the temporal expression pattern of specific markers when dental pulp cells are differentiating into osteoblasts, it is been shown that they express osteocalcin and fetal liver kinase 1 (*flk-1*), vascular endothelial growth factor receptor 2 (*VEGF-R2*) and 30% of them express specific antigens for endothelial cells (*CD54*, *von-Willebrand 1* and *2*, *CD31* and *angiotensin-converting enzyme*). These stem cell populations synergically differentiated into osteoblasts and endotheliocytes, with *flk-1* exerting a pivotal role in coupling osteoblast and endotheliocyte differentiation, suggesting that osteogenesis and angiogenesis mediated by human dental pulp stem cells are regulated by distinct mechanisms [49]. This mechanism is probably specific to dental stem cells as the osteogenesis mediated by the dental cells cannot recruit hematopoietic marrow elements seen in the osteogenesis generated by bone marrow mesenchymal cells. But a substantial repair resulted from transplantation

of SHED cells with hydroxyapatite/tricalcium phosphate into large calvarial defects in mice indicating that they are a suitable resource for orofacial bone regeneration [50].

Human dental pulp stem cells have also been used as a source of muscle cells after injections in dogs presenting muscular dystrophy. Analyses of cell migration engraftment, myogenic potential and expression of human dystrophin have shown that chimeric muscle fibers containing human cells were formed. No signs of immune rejection were reported [51].

The immunosuppressive function of dental pulp stem cells was tested by co-culturing phytohemagglutinin (PHA)-stimulated allogeneic T cells with or without mesenchymal stem cells from tooth and bone marrow. The addition of DPSCs resulted in a higher inhibition of T cell response assessed by a 3H-thymidine assay. This particular immunoregulatory characteristic may prompt future applications in the treatment or prevention of T-cell alloreactivity in hematopoietic or solid organ allogeneic transplantation. Under appropriate conditions, DPSCs can abolish T-cell alloreactivity more effectively than BMMSCs [52].

The cardiac muscle shares a few similarities with skeletal muscles, with both differing significantly from smooth muscle cells. It is a finely specialised muscle that has a large number of mitochondria and myoglobulins and an extensive blood supply provided by the coronary arteries. DPSC may provide a novel alternative cell population for cardiac repair, since when injected intramurally in infarcted nude rats, the animals showed an improvement in cardiac function, with a reduction in infarct size, possibly by increasing angiogenesis in the lesion site. Co-culture of DPSCs with neonatal rat cardiomyocytes lead to expression of connexin 43 and the cardiac-specific markers troponin I, atrial natriuretic peptide and sarcomeric actinin. Expression of these differentiation markers increased over time and were accompanied by translocation of the transcription factors neurokinin 2 transcription factor related, locus 5 (*NKX2.5*) and GATA binding protein 4 (*GATA4*) to the cell nuclei which resulted in an increase in myofibril organization and cardiomyocyte-like cells [53,54].

Dental stem cells may also have other possible uses in neural repair and regeneration, although it is not clear the extent to which dental cells can form neural tissues. Populations of dental stem cells express several neural markers such as β III tubulin, glutamic acid decarboxylase, neuronal nuclear antigen (*NeuN*), nestin, neurofilament M and neuron-specific enolase [20,34,55,56]. Glial cell line-derived neurotrophic factor (*GDNF*) mRNA is highly expressed prior to the initiation of dental pulp innervation [56] and dental pulp cells can interact with trigeminal neurons and produce several others neurotrophic factors. When co-cultured with trigeminal neurons, they promote survival and a specific and elaborate neurite outgrowth pattern from trigeminal neurons. Moreover, dental pulp tissue becomes innervated when transplanted ectopically into the anterior chamber of the eye in rats, and upregulates the catecholaminergic nerve fiber density of the irises. Grafting the dental pulp tissue into hemisectioned spinal cord increases

the number of surviving motoneurons, indicating a functional bioactivity of the dental pulp-derived neurotrophic factors *in vivo* [55]. Dental pulp cells from both rats and humans produce *in vitro* nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and GDNF mRNAs, promote the survival and phenotypic characteristics of embryonic dopaminergic (DA) neurons and protect DA neurons against the neurotoxin 6-hydroxy-dopamine (6-OHDA) *in vitro* [56]. These characteristics make the dental pulp cells a potential source of autologous cell transplantation therapies with potential benefits to improve neuronal function in both the central and peripheral nervous system.

5. Tooth tissue regeneration – cells replacing artificial dental fillings

A tooth is a complex organ composed basically of enamel, dentin/pulp complex and periodontal ligament attached to the alveolar bone. Each one of these tissues has several cell types participating in its formation and/or maintenance making tooth regeneration an intricate and multifaceted process.

Dental decay is the most common tooth pathology and is a consequence of acidic destruction of the mineral component of the teeth, both enamel and dentin. The lesions may eventually lead to pulp inflammation and/or tooth loss, and treatment for these pathologies currently involves the substitution of the natural/physiological dental tissue by an artificial material.

Regeneration is a result of tissue reconstitution not only based on cell aggregation, but also based on proliferation and differentiation of precursors. Dentin is the major component of the tooth and is completely surrounded by enamel in the crown and by cementum in the root. It is a mineralized tissue, formed of a network of collagen type I fibrils and carbonated apatite crystals organized in a tubular pattern [8]. Odontoblasts continue to deposit dentin throughout life. The primary dentin forms until the completion of root development, secondary dentin forms at the roof and floor of the pulp chamber after root formation and, reactionary dentin (tertiary dentin) is formed by the original odontoblasts in response to some stimuli such as dental decay or restorative treatment. When external factors irreversibly affect the odontoblast layer in a given region, new odontoblast-like cells arise to form another subtype of tertiary dentin called reparative dentin which is morphologically different from the reactionary dentin showing an 'osteodentin' appearance [57,58].

The concept of dental tissue regeneration is not new and the capacity of the human dental pulp to react after injury to form tertiary dentin has been recognized previously, but, the mechanism underlying this process is still not well understood [10]. Calcium hydroxide has been used as a major pulp-capping agent to induce dentin formation for decades. It is highly alkaline and consequently leads to a local inflammatory process that promotes pulp cell recruitment [59]. The use of a mouse

genetic approach provided evidence that the dental pulp contains progenitors responsible for the differentiation into odontoblast-like cells which express dentin sialophosphoprotein (DSPP) and secrete reparative dentin [60].

As described above, many different dental stem cell populations are capable of forming dentin-pulp like complex both *in vitro* and *in vivo*. This complex can be more or less mature and more or less organized depending on the population of dental stem cells used. When DPSC and SHED primary cultures are analyzed for the presence of DSPP [61], no expression is found [11,13,15], however, when xenogeneic transplants are analyzed, DSPP is found in early stages of mineralization when DPSCs and SHEDs are used, but not when bone marrow stem cells are transplanted. This suggests that adult dental pulp stem cells populations represent an undifferentiated pre-odontogenic phenotype under non-inductive conditions [13,62]. SCAP populations behave differently in many aspects, assessed by histological, immunohistochemical, cellular and molecular criteria, from DPSC and SHED and evidence is accumulating to support the hypothesis that SCAP cells are the source of primary odontoblasts that are responsible for the formation of root dentin, whereas DPSCs are probably the source of replacement odontoblasts [33]. In clinical practical terms, it seems that the most powerful capacity of dental stem cells with no chemical induction is the formation of dentin; however, whilst DPSCs and SHEDs form reparative/tertiary dentin, SCAPs form primary dentin.

Combination of cultured cells and growth factors may be a functional method to improve dentin regeneration. Growth/differentiation factor 11 (GDF11; BMP11) is a member of the BMP/TGF β family expressed by odontoblasts [63]. Recombinant BMP11 can induce DSPP in organ cultures of mouse dental papilla and when transfected into the dental papilla mesenchymal cells stimulated the reparative dentin formation during pulpal wound healing by increasing the differentiation of pulp stem cells into odontoblasts [64]. It had been shown previously that other members of the BMP/TGF β family (BMP-2 and BMP-4) induce differentiation of adult pulp cells into odontoblasts [65].

This differentiation can be induced *in vitro* with the use of conditioned medium. By culturing rat's dental pulp mesenchymal cells using a medium conditioned by developing tooth germ cells, an odontogenic microenvironment was created and the dental pulp cells start to present many characteristics of odontoblasts such as synthesis of dentin sialoprotein and expression of dentin matrix protein 1 and DSPP genes [66]. Additional studies have shown that the induced dentin can further induce cementum and PDL formation. When PDL cells were seeded on the bioengineered dentin and transplanted into immunocompromised mice, cementogenesis and condensed PDL arranged perpendicularly to the dentin could both be seen [67].

Dentin and pulp cells form a complex and for its regeneration both cell types must be formed. Recent literature has shown that

a negative response to pulpal testing with the presence of periradicular pathoses of endodontic origin is not always indicative of pulpal necrosis as previously believed. Moreover, sporadic case reports in the literature show the potential of root maturation even in the presence of endodontic lesions [68-72]. The SCAP population may explain this clinical phenomenon as due to its apical location, this tissue may benefit from collateral circulation, which enables it to survive even during the process of pulp necrosis [33], providing a new paradigm to the treatment of young teeth.

A complete rescue of the dental pulp is the main goal and current literature suggests that it is a real possibility not only with the use of the stem cell populations mentioned above (DPSC, SHED and SCAP), but also using biomaterials where cells can be cultured until they form a high-density tissue similar to the native pulp [73] that can be further driven to the right position in the root chamber. Recently, a side population of dental pulp cells from canines has been shown to repopulate amputated pulp tissue in only 14 days, showing angiogenesis and vasculogenesis with the formation of capillaries and neural cells [74].

Enamel is the hardest tissue in the human body and differs from dentin and bone, as it is mainly composed of carbonated hydroxyapatite and contains little or no matrix proteins. Amelogenesis is the result of highly orchestrated processes involving matrix molecules, proteases, and mineral ion fluxes that collectively regulate nucleation, growth and organization of forming mineral crystals [75].

After crown formation, the only remaining epithelial-derived cells are the epithelial cell rests of Malassez (ERM) that remain quiescent with no other function in life; therefore, no new ameloblasts can be generated, making this tissue a real challenge in terms of regeneration that requires identification of alternative sources for these cells. ERM are remnants of HERS that are involved in the formation of tooth roots [8].

Promising new attempts demonstrate in pigs, that the ERM could generate ameloblast-like cells and enamel-like structures. ERM cultured with dental pulp cells showed expression of the enamel marker amelogenin. When this combination was seeded onto scaffolds, an enamel-like tissue with well-developed ameloblasts was formed [76]. Moreover, a population of c-Kit⁺ enriched bone-marrow derived cells mixed with embryonic dental epithelial cells and cultured in re-association with dental mesenchyme gave rise to polarized and secretory ameloblast-like cells. Some of the bone-marrow cells also form odontoblast-like cells [77].

The periodontium is an unusually complex tissue comprised of two hard (cementum and bone) and two soft (gingiva and periodontal ligament) tissues. The periodontal ligament, which is a highly fibrous and vascular tissue, has one of the highest turnover rates in the body [78]. Periodontal disease leads to destruction of the connective tissues responsible for restraining teeth within the jaw. To date, various conventional therapies for periodontal regeneration have shown limited and variable clinical outcomes.

PDLSC and SCAP cells were used in co-transplantation experiments in order to form a functional bio-root in a minipig model. A HA/TCP block was assembled to have a root shape of an incisor. PDLSCs were seeded outside and SCAP cells were loaded inside the carrier which was subsequently transplanted into an empty space of an extracted root. After 3 months, a pre-fabricated porcelain crown resembling a minipig incisor was installed. CT and histological analysis confirmed that the root/periodontal structure had regenerated after 4 weeks. This newly formed bio-root demonstrated a significantly improved compressive strength compared with that of original HA/TCP carriers after 6 months. Although it showed a lower compressive strength than that of a natural swine root dentin, it seemed capable of supporting the porcelain crown and resulted in normal functions [34].

6. Whole tooth tissue engineering – The third dentition

Organogenesis involves positional information, morphogenesis and differentiation. All these processes must be synchronized to generate the organ with the right coordinates (position, size, shape and structure temporally and spatially regulated). Tissue engineering of teeth requires the coordinated formation of correctly shaped crowns, roots, periodontal ligaments and interaction with alveolar bones.

One mechanism for dental engineering is to replicate tooth development *in vitro*. The idea is to induce tooth formation in the laboratory to achieve the initial stimuli for dental development; and afterwards, this primary structure would be transplanted in the required position in the mouth.

To simulate tooth development *in vitro* is necessary to find epithelial and mesenchymal sources of tissues since dental development is based on epithelial/mesenchymal interactions. The oral epithelium from E9.0 – E11 mouse embryos can induce non-dental neural-crest-derived mesenchyme to form a tooth. Heterotypic recombinations of mandibular arch and second branchial arch tissues showed that early mandibular arch epithelium, before day 11 has odontogenic potential and can elicit the formation of a dental papilla in non-odontogenic, neural-crest-derived mesenchymal cells of the second arch. However, the mandibular mesenchyme must interact with mandibular epithelium in order to have the competence to induce teeth in non-odontogenic epithelium [79]. Further studies have shown that teeth can be formed in birds by recombining E9 – E11 days mandibular arch epithelium with nondental ectomesenchyme [80], implying that with the right odontogenic signal, a non odontogenic tissue can be used to form a tooth.

In 2004, the first report showing that adult mesenchymal stem cells from a non-dental source could be used in a dental tissue engineering context was published by Ohazama *et al.* [81]. Recombinations between a non-dental-cell-derived mesenchyme (adult bone-marrow-derived cells) and embryonic

oral epithelium stimulated an odontogenic response in the mesenchymal cells. Transfer of recombinations into adult renal capsules resulted in the development of tooth structures and associated bone. Moreover, transfer of embryonic tooth primordia into the adult jaw resulted in development of tooth structures, showing that an embryonic primordium can develop in its adult environment [81]. This study illustrated the potential of different approaches for tooth tissue engineering and offered considerable hope that biological teeth may eventually become a reality.

Different types of reassociations between epithelial and mesenchymal tissues and/or cells from mouse embryos have shown that, while in culture, reassociated tissues developed and resulted in tooth-like structures that exhibited normal epithelial histogenesis and allowed the functional differentiation of odontoblasts and ameloblasts. After transplantation, they formed roots and periodontal ligament, the latter connected to developing bone. Interestingly, the shape of the crown, initially suspected to depend on the integrity of the mesenchyme, could be modulated by adjusting the number of dissociated mesenchymal cells reassociated with the epithelial compartment [82].

Another mechanism for dental engineering was first described in 2002 and consists in combining dental cell populations with scaffolds made of biomaterials. Dissociated porcine third molar tooth buds were seeded into a tooth-shaped bio-degradable scaffold. The cells were able to re-organise themselves into small tooth germs that developed into recognizable tooth structures with dentin, odontoblasts/pulp chamber, cementoblasts, and a morphologically correct enamel organ containing fully formed enamel [39]. Experiments using dental epithelium dissociated into individual cells then reassociated with dental mesenchyme, have shown that despite a complete loss of positional information, cells rapidly underwent typical dental epithelial histogenesis [83]. Confirmation of these first studies was produced using a 3D organ-germ culture methods with dissociated third molar [84].

Each of these approaches has advanced this early research field and provided the framework for further investigations. However, at this point in time it is important not to lose sight of their limitations such as the very large numbers of cells required to form small tooth structures and inductive embryonic tissue still being required. In reality none of these or any other currently published methodologies is applicable as a clinical therapy. Harvesting cells from hundreds of embryos or third molars is clearly out of the question and even using adult bone marrow is not a particularly attractive option.

Finally, it is important to consider that many obstacles must be overcome to generate a bio-tooth. The very complex epithelial-mesenchymal interactions have to be fully recreated in the lab; the crown morphology and function is different depending on the tooth (incisors, canines, pre-molars and molars) and the organ formed must be functional with further development in the correct site after transplantation.

7. Expert opinion: dental stem cells – hope or hype?

Research in the field of dental stem cells is only commencing and a clinical application is merely a possibility at some point in the future. Some of the challenges that must be overcome will be presented and discussed in the following paragraphs.

7.1 Finding the best culture conditions

Stem cells naturally locate in specialised niches in our body. The niche is a specific anatomic location that regulates how they participate in tissue generation, maintenance and repair. It must have anatomical and functional dimensions to enable cells to reproduce and self-renew allowing them to persist and to change in number and fate. It is the niche that provides modulation in stem-cell function needed under conditions of physiological challenge and therefore, the niche concept is particularly important and central to the realization of regenerative medicine [85].

It is clear that once removed from the niche, stem cells will react and some changes will occur. This is an important consideration for any cell in our body, but particularly important considering that the number of stem cells naturally occurring is small and therefore, it is necessary to expand these cells using *in vitro* techniques prior to clinical usage. *In vitro* culture is variable and unable to mimic the stem cell niche and, moreover, somatic cells with a mature phenotype *in vivo* are able to dedifferentiate [86] or differentiate in other cell types in culture [87].

With these concepts in mind, it is clear that the culture conditions can influence cell properties and basic information is still uncertain in many aspects. Is there a better culture medium and/or formulation? Should we purify the stem cells or is it better to work with a mixed cell population mimicking at least partially the niche function? Is there a better cell density? What is the proportion of cells necessary to allow/not allow correct differentiation?

From a clinical perspective, once cells are able to form the right tissue or react in the desired direction, such questions are not as relevant, but the lack of scientific knowledge may compromise their fully potential of usage.

Some dental stem cell populations have been isolated based on the expression of perivascular markers such as STRO-1, and mucin 18 (MUC-18) [15,26,34,88], while others have been isolated using different approaches [23,39]. Even using the same markers for isolation, the populations obtained behave differently depending on its primary location and function in the tissue. Moreover, different clones of the same population show diverse multilineage potential and cell kinetics. This instability obviously harms the reproducibility of some experiments.

7.2 Dental stem cells exist: True or false?

It is still unclear how the different dental stem cell populations behave and function and indeed the extent to which any of those can be considered to be mesenchymal stem

cells rather than progenitors remains controversial. However, the potential of these cells as being 'bankable' is unique since children lose up to 20 teeth naturally as the permanent dentition erupts, and most adults lose teeth at some point of life. In addition, the accessibility of the pulp chamber in an adult is simple and straightforward and does not result in morbidity to the patient. Even the access to the root apical papilla area is uncomplicated with the removal of the third molars. Therefore, teeth are undoubtedly a promising source of autologous stem cells for tissue engineering.

The majority of craniofacial cartilages and bones are formed by neural-crest-derived mesenchymal cells that after migration will further differentiate into neural, pigmented and smooth muscle cells, craniofacial cartilage and bone. In this context, a naturally exfoliated deciduous tooth or other dental tissues are similar in some ways to the umbilical cord, containing stem cells that may have potential to repair damaged structures in the craniofacial area or in other parts of the body.

Teeth are organs that present peculiar characteristics. Mainly, they are formed by the hardest tissue in the body and their soft tissue is completely surrounded by an external layer of hard tissue. Due to these characteristics, teeth have limited potential to react after an injury and eventually, an inflammatory process will end up in pulp tissue necrosis. In addition, it is known that the aging process of the pulp tissue results in the formation of pulp stones associated with intensive deposit of collagen fibres and with decrease in pulp cell

numbers. Therefore, in contrast to other parts of the human body known to have a constant repair rate such as hair or bone marrow, dental pulp has no constant repair ability and will react only after damage with a limited type of response.

Taking this into consideration, it not clear why cells related to the maintenance of the cell numbers and with a clear function related to injury response will be present in dental tissues, in particular in the pulp. Even the well known ability to form odontoblasts may be just a natural property of a pulp fibroblast that although it can differentiate, does not present other stem cell properties such as capacity for self-renewal. However, the peculiar characteristics of the dental stem cells show that the proliferation rates of multi-colony-derived DPSCs, SHED, and PDLSC are 30, 50 and 30% higher when compared with BMSSCs [11,15,20,26,89] and the dental cells also show better immunoregulatory action than bone marrow cells [52].

In conclusion, the understanding of dental stem cells and tooth repair is still elusive and further research, particularly using *in vivo* approaches as the gold standard are necessary to understand and better characterize the different populations of dental stem cells.

Declaration of interest

Research in the authors' laboratory is supported by the Medical Research Council (MRC).

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