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REVIEW

Dental mesenchymal stem cells

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ABSTRACT

Mammalian teeth harbour mesenchymal stem cells (MSCs), which contribute to tooth growth and repair. These dental MSCs possess many *in vitro* features of bone marrow-derived MSCs, including clonogenicity, expression of certain markers, and following stimulation, differentiation into cells that have the characteristics of osteoblasts, chondrocytes and adipocytes. Teeth and their support tissues provide not only an easily accessible source of MSCs but also a tractable model system to study their function and properties *in vivo*. In addition, the accessibility of teeth together with their clinical relevance provides a valuable opportunity to test stem cell-based treatments for dental disorders. This Review outlines some recent discoveries in dental MSC function and behaviour and discusses how these and other advances are paving the way for the development of new biologically based dental therapies.

KEY WORDS: Dental, Incisor, MSCs, Pulp, Teeth

Introduction

Teeth are composed of two layers of mineral – an outer layer of enamel and an inner layer of dentine that encloses the soft inner fibroblast pulp tissue (Fig. 1). Multiple different stem cell populations have been described in teeth and their supporting structures, many of which share *in vitro* properties with bone marrow-derived mesenchymal stem cells (MSCs; see Box 1). For this reason, these stem cell populations are collectively referred to as dental MSCs, although not all dental MSCs are equal in terms of their phenotypic and functional properties (reviewed in Volponi and Sharpe, 2013).

Dental MSCs play an important role in tooth homeostasis and repair. In the dental pulp, these cells remain active throughout life and generate odontoblasts, which function to repair damaged dentine. In addition, dental MSCs located in the periodontal ligament also play a role in repair, and may also be involved in homeostatic turnover of this tissue. But not all tooth tissue can be repaired or replaced; the cells that form enamel, the epithelial-derived ameloblasts, are lost when teeth erupt and thus damage to enamel cannot be naturally repaired. Current clinical treatments to repair tooth damage involve the use of inorganic materials and clinical regeneration of periodontal ligament tissue is currently very difficult. An understanding of the properties of the different resident dental MSC populations can thus inform the development of novel, biologically based dental therapies.

Our understanding of dental MSCs – what characterizes them and how they might be used in the clinic – has grown in recent years, as several discoveries have shed light on various aspects of dental MSC function and behaviour. This Review brings together these advances and provides an overview of some of the key findings in identification and heterogeneity of dental MSCs. The role of dental MSCs in endogenous tooth repair is also discussed, as well as how these cells can be used to treat several different dental disorders: from restoration of tooth pulp to mineral formation. Finally, some future directions regarding dental MSC research and its application in the clinic provides an interesting perspective on how to move this field forward in order to realise the potential that these cells hold for regenerative medicine.

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Dental MSCs: what are they and where do they come from?

Most of what is known, and indeed the 'dogma' regarding adult MSCs has come from the bone marrow. In many respects the tooth pulp can be considered to be similar to bone marrow. Both are highly vascularized, innervated 'soft' tissues that are surrounded by mineral. In both bone marrow and dental pulp, the MSCs are capable of differentiating into cells that generate the mineral. In bone marrow, this function is performed by the osteoblasts, whereas in teeth it is performed by the odontoblasts, which are derived from the dental pulp stem cells (DPSCs). Importantly, however, DPSCs have more restricted differentiation than bone marrow cells *in vivo*. Dental pulp can therefore provide a simple model system to study mesenchymal stem cells that is easily accessible and has a defined structure.

The dental MSCs that are the topic of this review are neural crest-derived (ecto)mesenchymal cells that are located in the pulp of deciduous and permanent adult teeth and the periodontal ligament (Gronthos et al., 2000, 2002; Miura et al., 2003; Seo et al., 2004; Balic et al., 2010; Koyama et al., 2009; Waddington et al., 2009; Wang et al., 2012). Each dental MSC population is named according to its tissue of origin, e.g. stem cells from human exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSCs) and so on (Fig. 2). In mice, the relative contributions of mesenchymal stem cells to growth and/or repair can be distinguished by studying incisors, which grow continuously throughout life (Fig. 3A) and by studying molars, which do not grow at all in adult mice.

Identifying dental MSCs

When tooth pulp is removed and cultured, a population of cells can be rapidly established that shows all the characteristics attributed to MSCs such as clonogenicity, expression of defining markers such as CD90 (Thy1 – Mouse Genome Informatics), CD73 (Nt5e), CD105 (Eng) markers and multi-lineage differentiation following appropriate stimulation (Gronthos et al., 2000, 2002; Miura et al., 2003). Labelling of pericytes using *Ng2-Cre* (*Ng2* is also known as *Cspg4*) and reporter lines shows that cells with all the characteristics of odontoblasts are derived from pericytes following experimental damage to the pulp (Feng et al., 2011). Thus, with the limited genetic lineage tracing that has been carried out to date, it appears that DPSCs can be derived from pericytes. However, pericytes are

Development (2016) 143, 2273-2280 doi:10.1242/dev.134189

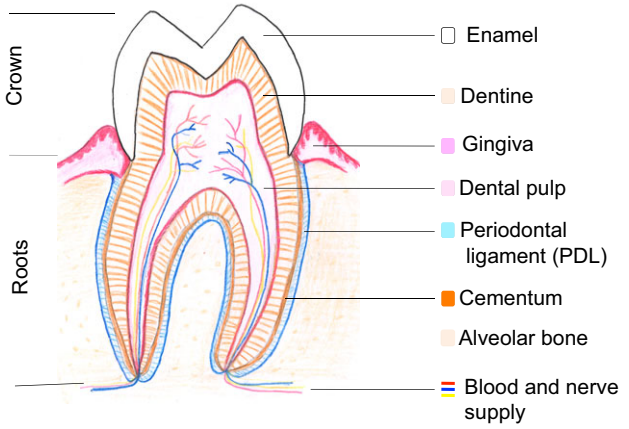

Fig. 1. Diagram of a section through an adult human molar tooth. Enamel and dentine encase the dental pulp, which is mainly fibroblastic and contains the blood and nerve supply for the tooth. MSCs can be derived from within dental pulp, as well as from the periodontal ligament and from gingival tissues.

not likely to be the only source of DPSCs: studies using the mouse incisor tooth as a model have revealed additional cell types that may function as dental MSCs. Rodent incisors undergo a process of occlusion and abrasion that occurs between the upper and lower teeth during feeding. In order to compensate for the loss of tissue from the tips during abrasion, the teeth need to be able to grow continuously, otherwise they would not occlude. Continuous growth is achieved by populations of stem cells located at the most proximal end of the incisor (often referred to as the cervical end) that continuously supply new cells for differentiation to replace those lost. For the mesenchymal cells, the odontoblasts and the pulp cells, a specific population of MSCs can be clearly identified, located between the lingual and labial aspects of the epithelial cervical loop, a structure that also houses the epithelial stem cells required for the generation of the epithelial cells of the incisor (Kuang-Hsien et al., 2014) (Fig. 3B).

Although the correlation between slow-cycling (nucleoside label-retaining) cells and stem cells remains controversial in some tissues, in the mouse incisor mesenchyme there is an exact and absolute correlation. Nucleoside labelling followed by long chase periods of up to 4 months identified a small population of slow-cycling mesenchymal cells between the epithelial cervical loops (Fig. 3B) (Zhao et al., 2014). Nucleoside labelling followed by a very short chase (24-48 h) identified a population of rapidly cycling cells distal to the slow-cycling cells that represent transit-amplifying cells or progenitors. In a separate study, Thy1 was shown to be expressed in the slow-cycling pulp mesenchymal cells of the mouse incisor (Kaukua et al., 2014). Thy1 (CD90) is a cell surface marker of all MSCs in culture but its expression is more widespread *in vivo* and in addition many mesenchymal stem cell populations do not express it (Lin et al., 2013). However, genetic lineage tracing using *Thy1-Cre* showed that the Thy1^{+} slow-cycling cells contributed to odontoblasts and pulp cells throughout the life of the incisor. This established that Thy1-expressing slow-cycling cells are dental MSCs responsible for incisor growth. Importantly, however, Thy1^{+} MSCs only contribute to a proportion of odontoblasts and pulp cells – around 10-20% – which correlates with the proportion of slow-cycling cells that express Thy1.

Following the identification of a population of melanocytes derived from neuronal glia, the possible contribution of glia to mesenchymal stem cells in the incisor was investigated (Adameyko et al., 2009; Kaukua et al., 2014). Using two different glial *ERT2-Cre* drivers (*Plp1* and *Sox10*), around 50% of pulp cells and odontoblasts were shown to be glia derived in the incisor. The Plp1/Sox10^{+} glia were located within the population of slow-cycling cells and although it is possible that the 50% contribution may be a result of variable tamoxifen Cre efficiency, it seems more likely that another non-glial population – possible pericytes – contributes the other 50%. Indeed, small numbers of pericyte-derived odontoblasts in mouse incisor growth have previously been identified (Feng et al., 2011). The use of a Confetti reporter line has enabled individual stem cell contributions to odontoblast and pulp cell differentiation to be quantified, revealing that the proximity of a stem cell (or more correctly, its fast-cycling progeny) to the periphery of the incisor influences differentiation (Kaukua et al., 2014). Thus cells closer to the periphery and the odontoblast layer give rise to more odontoblasts than pulp cells whereas those near the centre of the incisor and further away from the odontoblast layer form more pulp cells than odontoblasts (Fig. 3C).

Sonic hedgehog (Shh) signaling plays a role in numerous developmental and stem cell processes and in the incisor it is evident that Gli1-expressing mesenchymal cells colocalize with slow-cycling cells (Zhao et al., 2014). Moreover, lineage tracing of Gli1-expressing cells with *Gli1*^{ERT2Cre} shows that close to 100% of odontoblasts and pulp cells are derived from these cells during growth (Zhao et al., 2014). Gli1 expressing cells are located in neural-vascular bundles at the proximal end of the incisor, supporting the origins of the stem cells as glial and also possibly pericytes. The essential role of innervation either as a source of stem cells or Shh signals is supported by denervation studies that confirm that nerves are required for incisor growth (Zhao et al., 2014). Thus the long-established requirement of nerves to maintain tissue 'vitality' may well include the provision of stem cells.

Box 1. Mesenchymal stem cells – what's in a name?

The term mesenchymal stem cell, which is used to describe cells isolated from adult stromal tissues that have certain stem cell properties *in vitro*, has become established in the stem cell literature (Caplan, 1991). However, the extent to which this generic term should be used, if at all, has been questioned. The term skeletal stem cells has recently been adopted to describe cells in the bone marrow that were previously called bone marrow mesenchymal stem cells or mesenchymal stromal cells (Bianco and Robey, 2015). However these skeletal stem cells are capable of differentiating into non-skeletal cells such as adipocytes, both *in vitro* and *in vivo* (Bianco et al., 1988). Further confusion and controversy that permeates the field is that the definition of mesenchymal stem cells is based solely on their *in vitro* characteristics. A consequence of this is that all mesenchymal stem cells are now considered to be the same, regardless of their tissue of origin, to the extent that mesenchymal stem cells from widely different tissues are often considered equivalent in clinical applications. A defining feature of these cells is their generic multipotent differentiation *in vitro* into osteoblasts, chondrocytes and adipocytes; however, recent *in vivo* research clearly shows that mesenchymal stem cells have different origins, properties and functions in different tissues that are not well reflected *in vitro*. Thus, the crude directed differentiation of mesenchymal stem cells *in vitro* misses key subtleties that exist *in vivo*. It is only recently that genetic lineage tracing has been used to identify mesenchymal stem cells and their properties *in vivo*. These approaches are now the 'gold standard' for identifying stem cells and this needs to be recognized over their *in vitro* properties. *In vitro* definitions are still important for standardising cell characteristics, particularly in relation to possible therapeutic uses, but such definitions are not appropriate for cells *in vivo*.

Development (2016) 143, 2273-2280 doi:10.1242/dev.134189

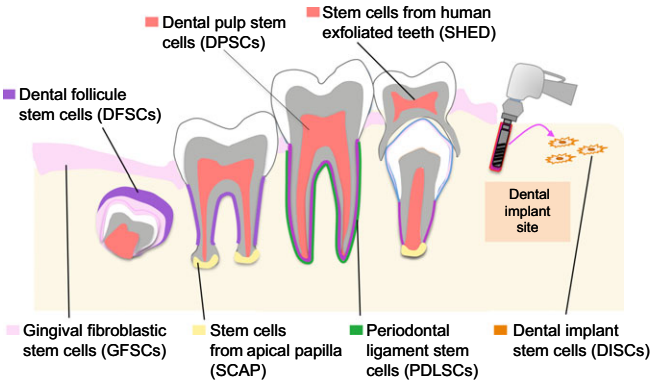

Fig. 2. The dental and associated tissues from which different populations of dental MSCs can be isolated. Different subpopulations of dental MSCs can be categorized according to their tissue of origin. From the tooth proper, dental pulp stem cells (DPSCs) can be derived from the inner tooth pulp of adult molars (red), from the pulp of deciduous exfoliated teeth (red) and from the apical papilla (yellow). From the supporting tissues, dental MSCs can be derived from the dental follicle (shown in purple), the periodontal ligament (green), the gingiva (pink) and also from tissue taken during dental implants (orange; see Box 2).

Heterogeneity of dental MSC populations

An obvious question is why do there appear to be multiple populations of dental MSCs (at least, cells that express different genes) to carry out a simple bipotential differentiation into odontoblasts and pulp cells. In fact, it could be argued that differentiation is unipotent because the transit-amplifying cells are all fibroblastic pulp cells and thus differentiation of these cells only involves formation of odontoblasts. A potential dual origin of neural glia and pericytes makes some sense in that both these mesenchymal cell types share the common features of being omnipresent in most tissues and being located on the outside of elongated structures. Thus, the dual origin may merely be a consequence of both cell types being present and capable of forming MSCs. The existence of the Thy1-expressing subpopulation of MSCs is more difficult to explain, but may be an indication of some hitherto unknown odontoblast heterogeneity or specialization. Interestingly, bone marrow MSCs – that is, skeletal stem cells (see Box 1) – are also surprisingly heterogeneous. A recent study using a series of independent lineage-tracing experiments on skeletal stem cells revealed a similar linear hierarchy of cell populations to that known for haematopoiesis (Chan et al., 2015). In the study, a single skeletal stem cell population was shown to give rise to at least eight different downstream cell populations identified by specific combinations of cell surface protein expression (Chan et al., 2015). The skeletal stem cells form two populations of more lineage-restricted progenitor cell populations that are all capable of differentiating into bone, cartilage and stroma. The end point of this hierarchy is the formation of several populations that have restricted differentiation into bone, cartilage or stromal cells.

Other genetic lineage-tracing experiments have identified populations of leptin receptor (LepR)- and gremlin 1-positive cells that give rise to a range of skeletal cell types *in vivo* (Worthley et al., 2015; Zhou et al., 2014). The full extent to which these populations are related to the eight subpopulations described by Chan et al. (2015) is not completely clear, but the fact that $LepR^+$ cells form bone but not cartilage *in vivo* and gremlin 1^+ cells form bone and stroma suggests that gremlin 1^+ cells lie higher in the hierarchy than $LepR^+$ cells. The extent to which any of these populations are associated with the vasculature (pericytes) is also unclear. Certainly $LepR^+$ cells are distinct from $Ng2^+$ perivascular cells, but Ng2 is not expressed on all pericytes. Earlier observations identified a population of $nestin^+$ cells in the bone marrow that appeared to represent a stem cell population but these are distinct from gremlin 1^+ and $LepR^+$ cells (Méndez-Ferrer et al., 2010). Whilst at present this paints a rather confusing picture, it probably reflects the existence of intermediate cell populations and, as more surface markers are studied, this confusion will be resolved and a clear hierarchy established. The extent to which DPSC biology overlaps with that of skeletal stem cells remains to be established, but it seems unlikely that the cell interactions in the tooth pulp niche will be as complex, given the more restricted differentiation and the additional role of skeletal stem cells in supporting haematopoiesis.

Incisor transit-amplifying cells

Although the majority of emphasis has been placed on understanding the biology of DPSCs, less attention has been paid to the transit-amplifying progenitor cells that are their progeny. This rapidly dividing population in the incisor represents the cells that differentiate, and thus the transition from a slow-cycling stem cell to a rapidly cycling progenitor is a critically important yet poorly understood process. The transit-amplifying cells are the most rapidly cycling cells in the incisor pulp and they are located immediately distal to the slow-cycling stem cells (Lapthanasupkul et al., 2012). These cells express specific genes, most notably those of the polycomb repressive complex 1 (*Prc1*), that are generally not expressed in the surrounding cells and which are important for cell proliferation (Lapthanasupkul et al., 2012). Targeted deletion of genes integral to Prc1, such as *Ring1a* (*Ring1*) and *Ring1b* (*Rnf2*), results in the arrest of incisor growth and loss of transit-amplifying cells, suggesting that a functional Prc1 complex is essential to maintain proliferation in these cells.

Cell signalling-mediated control of incisor MSC and transit-amplifying cell behaviour remains poorly understood. Whereas it is established that signals emanating from the mesenchymal cells such as FGFs and TGF-β are essential for the function of the epithelial stem cells, targeted mutations in cell signalling genes in the epithelial stem cell niche appear to have little, if any, impact on MSC behaviour (Klein et al., 2008; Yang et al., 2014) (Fig. 3B). Wnt/β-catenin signalling is exclusively restricted to mesenchymal cells; thus, expression of axin 2, which is a downstream target of Wnt/β-catenin signalling, is not detected in epithelial cells, but it can be seen in mesenchymal transit-amplifying cells (Lohi et al., 2010). The generic role played by Wnt/β-catenin signaling in stem cell dynamics across multiple tissue types indicates that this pathway may also play a role in incisor mesenchymal transit-amplifying cell

Box 2. Oro-dental tissues: an MSC treasure chest

In addition to tooth pulp and periodontal ligament, mesenchymal stem cells can be isolated following culture of developing tooth roots and soft tissues of the mouth such as the oral mucosa gingiva (Stephens and Genever, 2007; Zhang et al., 2012). Surgical tooth extraction usually includes a small amount of attached gingival tissue and thus freshly extracted teeth provide an easily accessible source of different mesenchymal stem cell populations. Another, as yet fully explored source of dental and bone mesenchymal stem cells is the material that is discarded during dental implant surgery. Dental implants are metal prostheses that are inserted into holes drilled into the jaw bone at the sites of lost teeth. This drilling removes bone and soft tissue that is collected via suction onto sterile filters and normally discarded. This material is easily cultured and can provide an excellent source of MSCs (Volponi et al., 2015).

Development (2016) 143, 2273-2280 doi:10.1242/dev.134189

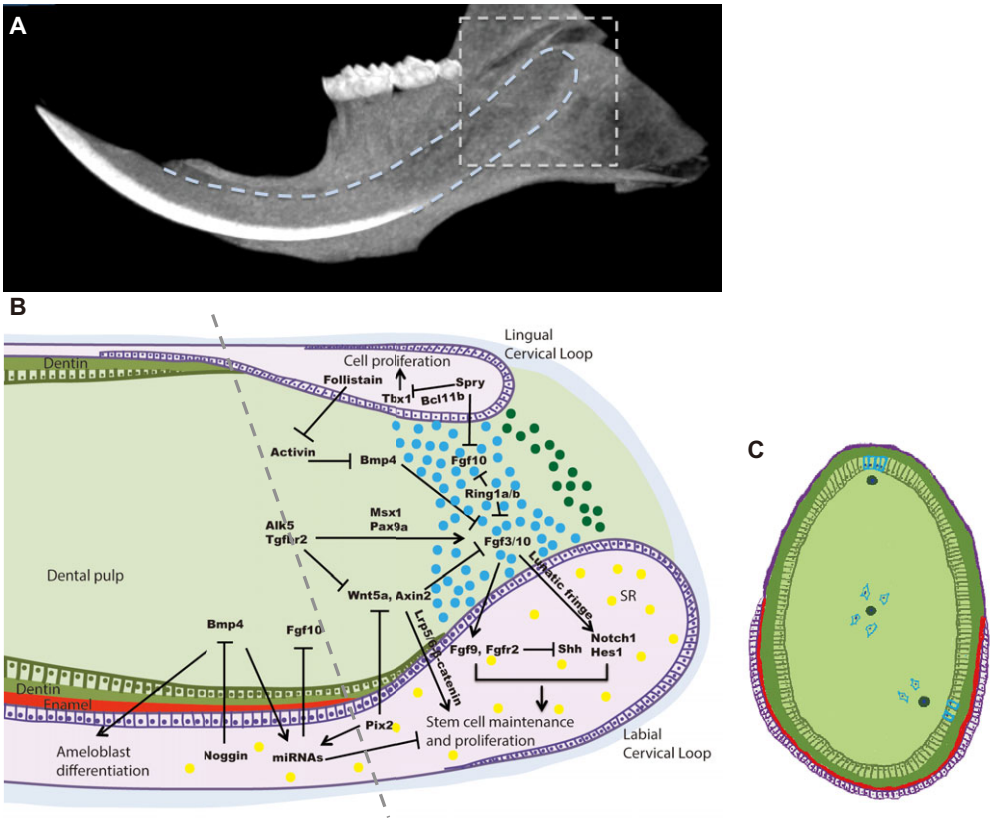

Fig. 3. Molecular interactions that regulate dental MSCs in the adult mouse incisor. (A) Micro CT scan of a continuously growing mouse incisor (extent is indicated by blue dashed line). Dashed grey box represents inset as shown in B. (B) Diagrammatic representation of the stem cell niches in the mouse incisor adapted from Yu et al. (2015) with permission from Elsevier. Slow-cycling MSCs (dark green dots) and fast-cycling transit amplifying cells (blue dots) are located between the lingual cervical loop and the labial cervical loop at the proximal end of the adult mouse incisor. Yellow dots indicate the epithelial stellate reticulum cells. The molecular network that controls the different dental MSC populations is complex, involving a range of different signalling, transcriptional and epigenetic pathways active in the pulp mesenchyme (light green) and epithelium (purple) that affect stem/progenitor cell maintenance, proliferation and differentiation (for detailed review, see Yu et al., 2015). Note that the enamel (shown in red) is generated by ameloblasts (purple cuboidal cells) and is present exclusively on the labial side, compared with dentine (dark green) which is made by odontoblasts (green cuboidal cells) and is present on both labial and lingual sides. (C) Schematic of a transverse section through the incisor as indicated by grey dashed line in B, representing the relative location and contribution of stem (progenitor) cells (green dots), which give rise to odontoblasts (columnar cells outlined in blue) and/or pulp cells (outlined in blue), depending on their location.

function. The extent to which cell signalling regulates maintenance of incisor MSC function is not understood beyond the role of Shh, as discussed above (Zhao et al., 2014).

MSCs in tooth repair

Adult teeth possess a natural repair process that acts to restore mineralized dentine following damage. Mild damage to dentine that does not penetrate into the underlying pulp stimulates odontoblasts in the immediate vicinity to generate new dentine, called reactionary dentine, in response to the damage. When lesions are more severe and penetrate the pulp, odontoblasts in the vicinity are destroyed and the tooth is at risk of infection. In this case, the resident MSCs are mobilized to differentiate into odontoblast-like cells that generate a form of dentine, called reparative dentine, that creates a bridge to protect the exposed pulp and repair the dentine (Smith et al., 1995; Sloan and Smith, 2007). Genetic lineage tracing in mice shows that reparative odontoblasts are formed from pericyte- and glia-derived MSCs in both molar and incisor damage repair (Feng et al., 2011; Kaukua et al., 2014). In both cases, the proximity of pericytes and glial cells to the damaged odontoblasts and pulp cells provides a local source of MSCs. Interestingly, in incisors, damage also stimulates the MSCs in the growth niche, which is at a distance from the site of damage, suggesting that all sources of these stem cells are able to receive and respond to signals released at the site of damage (Feng et al., 2011). Thus, it seems that damage provides a generic, indiscriminate stimulus to which all dental MSCs respond, regardless of origin, function or location. This presumably ensures that the damage is repaired quickly and suggests that the origin of the stem cell-derived odontoblasts that carry out the repair is irrelevant.

Pericyte-derived odontoblast cells have also been shown to play a role in the continuous repair process that occurs in the adult mouse incisor (Pang et al., 2016). Continuous growth and wear are balanced in the mouse incisor to maintain the optimum tooth size. However an unfortunate consequence of the abrasion and sharpening of the tips is that the soft tissue pulp in the tooth core is exposed to the oral cavity and the tooth is thus prone to infection. To prevent this exposure, a process of continuous pulp mineralization occurs at the tip, which produces a layer of mineral covering the exposed pulp. The mineral is dentine-like in composition and has been termed restorative dentine (Pang et al., 2016). In common with dentine repair processes in non-growing teeth, restorative dentine is a rapidly generated mineral that is produced by pericyte-derived odontoblast-like cells. However, unlike other repair processes, the formation of restorative dentine does not appear to be specifically stimulated by tooth damage, but is

Development (2016) 143, 2273-2280 doi:10.1242/dev.134189

a continuous process that occurs even in the absence of damage (Pang et al., 2016).

The signals that mobilize the dental MSCs and stimulate their differentiation are poorly understood, but are known to involve the release of sequestered TGF-β following physical damage and Wnt signals that regulate pulp cell apoptosis, among other functions (Hunter et al., 2015). The presence of latent TGF-β proteins in dentine tubules and its activity in promoting reparative dentine formation has recently been exploited in a potential clinical device involving the delivery of low energy laser light to stimulate liberation of TGF-β (Arany et al., 2014). Canonical (β-catenin) Wnt signalling, which is induced immediately following tissue damage, is likely to play a major role in this mesenchymal stem cell mobilization (Whyte et al., 2012).

Clinical applications of dental stem cells

From the onset of their discovery in bone marrow, the potential of adult MSC populations that can readily differentiate into osteogenic and chondrogenic cells has attracted interest for their potential use in bone and cartilage repair. Similarly, dental MSCs and, more specifically, DPSCs have clinical potential for pulp restoration following endodontic treatment (otherwise known as root canals) and for biological enhancement of tooth repair via the formation of reparative dentine. The extent to which dental MSCs may be useful in the myriad other clinical contexts that have been suggested is debatable and beyond the scope of this Review. There are, however, several areas of research where dental stem cells show some promise.

Restoration of tooth pulp

The most obvious use of cultured DPSCs is to restore tooth pulp following root canal treatment. Infected root pulp needs to be physically removed and the current treatment involves the use of cements to refill the root canals following pulp removal and sterilization. Such treatments, although effective in combating infection, do not restore lost dental pulp tissue or tooth vitality. The use of DPSCs to regenerate healthy pulp tissue represents a simple and potentially very effective biological treatment. DPSCs can be easily expanded *in vitro* and have been shown to reconstitute a pulp-like tissue *ex vivo* and *in vivo* (Gronthos et al., 2002; Miura et al., 2003; Cordeiro et al., 2008; Huang et al., 2010). An ongoing small scale Phase I clinical trial is currently being conducted in Japan by Misako Nakashima to look at the feasibility of using dental pulp stem cells to replace infected pulp tissue. The trial involves the application of autologous 'mobilized' DPSCs to the teeth of patients with irreversible pulpitis, which is a condition characterized by prolonged sensitivity to certain stimuli. After 25 weeks, there were no adverse effects and the treated teeth show recovery of pulp (Nakashima and Iohara, 2014). Since current root canal treatments do not restore pulp vitality but rather replace tissue with an inert inorganic cement-like material pulp, restoration using autologous DPSCs is a genuine realistic alternative that may soon appear as a routine dental treatment in the near future.

Craniofacial skeletal repair

DPSCs provide a source of adult MSCs that may retain certain properties of the neural crest cells from which they originate. Other adult mesenchyme-derived stem cells retain a memory of their origin that may influence their differentiation, which is particularly relevant for applications related to bone tissue engineering (Leucht et al., 2008). A characteristic feature of cranial neural crest cells that form the face and jaw skeleton is that they do not express Hox genes, whereas neural crest cells that form the more caudal skeletal structures and mesodermal-derived skeletal cells do express Hox genes during development, suggesting that a memory of this expression is retained into adulthood (Creuzet et al., 2002; Wang et al., 2009). Cross-transplantation of osteoprogenitor cells from the mandible into injured tibia and from tibia into injured mandible revealed that Hox-positive tibia cells continued to express Hox genes in the mandible; however, they did not differentiate into osteoblasts, but rather formed a cartilaginous callus. Hox-negative mandible cells expressed Hox11 (Tlx1) in the Hox-positive environment of the tibia and formed bone with no evidence of cartilage formation (Leucht et al., 2008). The finding that Hox-positive cells continued to express Hox genes in the Hox-negative environment of the injured mandible may have implications for the use of bone-derived cells in craniofacial repair (Leucht et al., 2008).

The majority of craniofacial bones are generated from neural crest-derived osteoblasts that have been suggested to have different characteristics from mesodermal-derived osteoblasts. Therefore, the use of skeletal stem cells, for example, in craniofacial bone tissue engineering may not be optimal to produce membrane bone. Similarly, the development of cell-based organ engineering systems to generate tooth primordia that can develop into teeth following transplantation may benefit from the use of mesenchymal cells of neural crest origin. The tooth pulp provides an easily accessible source of these cells. The extent to which tooth pulp-derived MSCs might be used to form skeletal tissue outside the craniofacial environment may depend on the extent to which they can be induced to adopt a non-neural crest-like state, either by manipulating their local microenvironment or by direct cell reprogramming. These and other proposed therapeutic applications of DPSCs have resulted in the commercial banking of these cells, either from naturally exfoliated teeth (children's deciduous teeth) or from extracted teeth. Pulp cells are cultured and stored for future use, providing a banked source of autologous cells obtained with minimal intervention or trauma.

Periodontal ligament

The periodontal ligament is a complex connective tissue that links tooth roots with alveolar bone in the jaws (see Fig. 1). Its role is to absorb the forces generated during mastication and it provides a seal around the teeth, protecting the roots. Periodontal disease is a major cause of tooth loss in the developed world and restoration of diseased or damaged periodontal tissue is difficult due to the complexity of the tissue. *In vitro* culture of cells derived from periodontal tissue can generate cells with mesenchymal stem cell-like properties – the PDLSCs – which are increasing being studied in relation to the treatment of periodontal disease (Seo et al., 2004; Lin et al., 2008; Park et al., 2010; Hasegawa et al., 2005). This cell population may include cells derived from the ligament connective tissue and also cementoblast precursors, the cells that form the mineralized cementum that links the periodontal ligament to the tooth. PDLSCs are the most likely candidates to give rise to the soft tissue cells of the ligament and possibly also to cementoblasts and osteoblasts during repair. They may also be involved in normal homeostatic tissue turnover, although this has yet to be demonstrated by lineage tracing. In general, *in vivo* characterization studies of PDLSCs are sparse and basic questions regarding their origin, location and heterogeneity as well as their *in vivo* differentiation potential remain unanswered. Cell proliferation rates in the periodontal ligament increase following injury and during orthodontic treatment, but resting rates in adults are low and decrease with age, suggesting that PDLSCs, similar to DPSCs, are mobilized by specific stimuli (Lim et al., 2014). The extent to which

Development (2016) 143, 2273-2280 doi:10.1242/dev.134189

exogenous addition of PDLSCs or their endogenous mobilization can be used to treat periodontitis requires a more detailed understanding of the *in vivo* characteristics of these cells. In pig models of periodontal lesions, surgical application of PDLSCs led to improved restoration of periodontal tissue (Liu et al., 2008) and such cellular therapies are therefore of significant interest for treatment of a disease that is the major cause of tooth loss (Bassir et al., 2015).

Immune 'modulation'

The ability of MSCs to affect T cell proliferation *in vitro* has long been established and has formed part of the basis of clinical therapies that utilize adult MSCs as an aid to modulate certain immune responses (Le Blanc et al., 2008). The *in vitro* effects of MSCs on T cell proliferation are most likely a reflection of their *in vivo* behaviour, where they may act to suppress inflammation during tissue repair. Although to date most clinical applications of this property have utilized bone marrow-derived cells, it is clear that DPSCs are equally potent *in vitro* (Yamaza et al., 2010; Wada et al., 2009). The extent to which any *in vivo* effects on immune processes involve the same mechanisms as those attributed to *in vitro* activity, such as tryptophan depletion by indoleamine 2,3-dioxygenase (IDO), for example, remain contentious. Although effects on T cell proliferation as observed in *in vitro* assays are consistent and substantial, the extent to which such inhibition of proliferation is sufficient to suppress all T cell function *in vivo* is unclear, especially as T cell numbers will increase at the sites of tissue damage via the circulation. Therefore, it is likely that additional interactions between MSCs and T cells as well as other immune cells occur *in vivo* (François et al., 2012; Noone et al., 2013; Reading et al., 2015). Of particular interest is the concept that MSCs are able to promote the conversion of pro-inflammatory M1 macrophages towards M2 anti-inflammatory macrophages and thus regulate inflammation (Németh et al., 2009; Kim and Hematti, 2009; Maggini et al., 2010; Zhang et al., 2010). In addition to these specific functions, MSCs secrete a large array of cytokines and chemokines, far beyond any conceived role in differentiation (Caplan and Correa, 2011). Immune regulation at the site of tissue damage is likely to play a significant role in repair and, for dental MSCs, during the repair of damaged dentine and soft tissues. The extent to which immune modulatory mechanisms identified *in vitro*, act *in vivo* clearly warrants detailed study, not only to inform the clinical use of MSCs, but also for implications in a wider context of immune cell biology.

Mineral formation

The minimal criteria described for defining cells with MSCs characteristics *in vitro* has unfortunately led to the erroneous assumption that all cells with such properties are 'equal' in their ability to form osteoblasts and generate bone. The standard assays of mineral production are growth of cells in osteogenic-inductive medium followed by assay of osteogenic gene expression (for example, alkaline phosphatase and Runx2) and/or Alizarin Red staining for mineral deposition. *Ex vivo* approaches to identify mineral formation are rarely used, but when done, they clearly highlight major differences in mineral formation from MSCs of different origins (Gronthos et al., 2002). A recent comparison using Raman microspectroscopy of the mineral composition generated by MSCs isolated from oral tissues has shown major differences in both inorganic and organic composition (Fig. 4) (Volponi et al., 2015). Although such measurements from *in vitro* differentiation do not reflect exact differences in mineral composition *in vivo*, since cells transplanted from tissue to tissue may adopt a host-like environment, they do give an indication that cells isolated from different tissues are predisposed to differentiate into cells with different functional characteristics. Thus, for example, MSCs from the pulp of exfoliated teeth (SHED cells) produced a mineral of markedly different composition *in vitro* from MSCs isolated from adult tooth pulp (DPSCs) (Fig. 4). Such differences, even between closely related cell populations, are indicative of the importance of selection of appropriate origins of cells for clinical applications.

Conclusions

The historical reliance on the use of *in vitro* properties of MSCs to define their *in vivo* functions and potential therapeutic applications has inevitably led to misunderstanding of these remarkable cells. The concept that one MSC source will suit all applications is clearly misleading at best. These cells behave in similar ways *in vitro* because their differentiation is forcefully directed, whereas in their natural environment, their behaviour and differentiation is highly regulated and restricted. What is needed is a much better understanding of the *in vivo* regulatory mechanisms that restrict differentiation to specialized cell types, for example, odontoblasts from DPSCs and osteoblasts from skeletal stem cells. This information will not only guide future progress in the modulation of *in vivo* repair mechanisms but it will also aid the use of MSCs in therapeutic applications.

Dental MSCs have already entered clinical trials. The area in which they currently hold the most promise is in the restoration of tooth pulp, although additional fields of application are quickly emerging, depending on the specific type or origin of the dental MSC population. One area where the source of mesenchymal stem

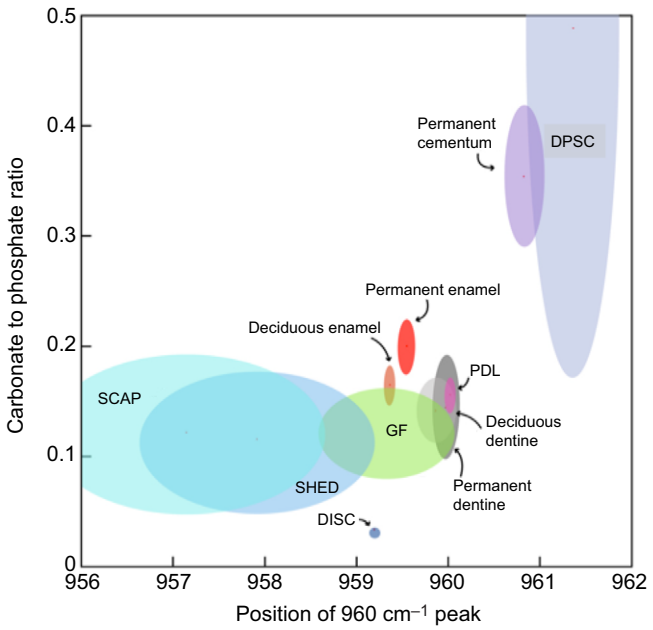

Fig. 4. Different dental MSC populations form mineral of varying composition. Analysis of the mineral produced from different dental MSC populations *in vitro* reveals that each population produces mineral with a slightly different carbonate to phosphate ratio. Data were obtained by Raman microspectroscopic analysis. Permanent dentine is represented by dark grey oval and deciduous dentine by pale grey oval. GF, gingival fibroblastic (stem cells); DISC, dental implant stem cells; DPSC, dental pulp stem cells; PDL, periodontal ligament cells (pink oval); SCAP, stem cells from the apical papilla; SHED, stem cells from human deciduous teeth. Image reproduced with permission from Volponi et al. (2015).

Development (2016) 143, 2273-2280 doi:10.1242/dev.134189

cells is unlikely to impact efficacy is their use for immune modulation. Mesenchymal stem cells from many different sources express multiple cytokines and it is these that most likely mediate their ability to modulate immune responses (Murphy et al., 2013). Regardless of the specific application, dental MSC-based therapies must still overcome significant hurdles to ensure quality control and efficacy in the clinic, such as the appropriate *in vivo* mobilization and selection of cell origin for *ex vivo* therapies. In that way, dental MSC-based therapies can be considered as still being in their infancy and much more research is required before they can fulfil the promise that they hold.

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Competing interests

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