CHAPTER EIGHT

Stem Cells in Tooth Development, Growth, Repair, and Regeneration

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Abstract

Human teeth contain stem cells in all their mesenchymal-derived tissues, which include the pulp, periodontal ligament, and developing roots, in addition to the support tissues such as the alveolar bone. The precise roles of these cells remain poorly understood and most likely involve tissue repair mechanisms but their relative ease of harvesting makes teeth a valuable potential source of mesenchymal stem cells (MSCs) for therapeutic use. These dental MSC populations all appear to have the same developmental origins, being derived from cranial neural crest cells, a population of embryonic stem cells with multipotential properties. In rodents, the incisor teeth grow continuously throughout life, a feature that requires populations of continuously active mesenchymal and epithelial stem cells. The discrete locations of these stem cells in the incisor have rendered them amenable for study and much is being learnt about the general properties of these stem cells for the incisor as a model system. The incisor MSCs appear to be a heterogeneous population consisting of cells from different neural crest-derived tissues.

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The epithelial stem cells can be traced directly back in development to a $Sox10^+$ population present at the time of tooth initiation. In this review, we describe the basic biology of dental stem cells, their functions, and potential clinical uses.

1. STEM CELLS IN TOOTH DEVELOPMENT

Mammalian dentition involves a complex sequence of reciprocal epithelial–mesenchymal interactions that are typically divided into initiation, morphogenesis, and differentiation phases. The mouse has a simple dentition with an incisor at the front and three molars at the back of the mouth in each quadrant. Humans have extra tooth types, such as canines and premolars, yet these teeth develop in a similar pattern as in the mouse. The main difference between humans and mice is that humans have one set of replacement teeth whereas mice only have a single set of teeth.

1.1 Developmental Origins of Dental Stem Cells

Two principal sources of cells are required for tooth development: the cranial neural crest (CNC)-derived mesenchyme and the oral ectoderm- or pharyngeal ectoderm-derived epithelium. The neural crest-derived mesenchymal cells give rise to most of the soft and hard tissues in and around the tooth, including dental pulp, dentin, alveolar bone, and periodontal ligament, as well as the facial and jaw skeletons (Bronner-Fraser, 1993; Chai et al., 2000; Lumsden, 1988). The epithelium mainly produces the enamel-secreting ameloblasts and their supporting cells (Chai et al., 2000; Rothova, Thompson, Lickert, & Tucker, 2012). Patterning of the mammalian dentition involves a three-dimensional process. The proximally expressed Fgf8 and distally expressed Bmp4 in the dental epithelium are critical for specifying the initiation sites of tooth formation as well as for the CNC cell-derived dental mesenchyme to become an incisor or molar tooth (Neubuser, Peters, Balling, & Martin, 1997; Tucker, Matthews, & Sharpe, 1998). FGF8 restricts Barx1, Dlx1, and Dlx2 expression, and BMP4 restricts Msx1 and Msx2 expression to the proximal and distal region of the ectomesenchymal domain, respectively (Thesleff & Sharpe, 1997; Tucker et al., 1998; Vainio, Karavanova, Jowett, & Thesleff, 1993).

Tooth development in the mouse begins around embryonic day (E) 10, when the first sets of signaling molecules involved in this process, such as Shh, Fgf8, and Bmp4, are expressed at the sites of tooth formation. The oral epithelium subsequently thickens into a placode, and the thickening grows

as the epithelium starts to invaginate into the underlying mesenchyme. The ability to induce tooth formation resides in the epithelium at this placode stage (Lumsden, 1988; Mina & Kollar, 1987). The mesenchyme responds by condensing around the invaginating epithelium to form the dental papilla and leads to the formation of a tooth bud around E13. The epithelium and the mesenchyme continue to proliferate to form a cap around E14. At this stage, the odontogenic potential switches from the epithelium to the mesenchyme (Kollar & Baird, 1969). A signaling centre called the primary enamel knot forms in the dental epithelium during the transition from bud to cap stage and regulates the future crown shape. During the late cap stage, significant differences between the developing incisor and molar arise. In the molar, the primary enamel knots are subsequently replaced by secondary enamel knots, which determine the position of epithelial folds that correspond to the tooth cusp formation (Jernvall & Thesleff, 2000), whereas the developing incisor begins to turn to grow parallel to the jaw axis. During the bell stage beginning at E16, the final tooth crown shape becomes apparent, and tooth-specific cell types, such as enamel-producing ameloblasts and dentin-producing odontoblasts, begin to differentiate. The differentiation of these cells is regulated, as in previous stages, by interactions between the epithelium and mesenchyme.

Ameloblasts are generated from epithelial cells residing in the inner enamel epithelium (IEE), and these cells secrete enamel matrix that later mineralizes. Signals such as FGFs and BMP2 from the IEE induce the formation of odontoblasts in the dental mesenchyme (Begue-Kirn et al., 1994; Thesleff, Keranen, & Jernvall, 2001). Odontoblasts differentiate from the outermost layer of the dental papilla and secrete dentin matrix. Signals arising from the odontoblasts are able to trigger the terminal differentiation of ameloblasts in the epithelium. It has been shown that the presence of functional odontoblasts or predentin matrix is required for ameloblast differentiation (Zeichner-David et al., 1995).

1.2 Neural Crest-Derived Stem Cells During Tooth Development

Dental mesenchymal cells are derived from two different origins: the CNC and the non-CNC mesoderm. Neural crest is a transitory population of cells, emerging from the lateral ridges of the neural plate during early stages of embryogenesis and subsequently differentiating into neurons, glia, melanocytes, and mesenchymal cells. During craniofacial development, neural crest cells migrate ventrally and proliferate to form the first branchial arch. These

CNC cells can differentiate into odontoblasts, cementoblasts, fibroblasts, osteoblasts, and chondroblasts (Bronner-Fraser, 1993; Chai et al., 2000; Lumsden, 1988).

A number of signaling molecules and homeobox gene targets are involved in regulating the specification of CNC cells. FGF8 signaling from the isthmic organizer controls branchial arch patterning; transforming growth factor-β (TGF-β) regulates the differentiation of CNC cells to glial cells; and TGF-β, BMP, and Wnt together control chondrocyte differentiation (Chai, Ito, Han, 2003; Trainor, Ariza-McNaughton, & Krumlauf, 2002; Tucker, Grigoriou, Pachnis, & Sharpe, 1999).

Clonal culture experiments and *in vivo* lineage tracing have demonstrated that neural crest cells are capable of self-renewal and display unique characteristics of stem cells (Bronner, 2015; Stemple & Anderson, 1992). Neural crest cells are developmentally plastic, and thus their progenitors must be instructed by signals from other tissues to determine their fate after reaching their final destination. Baggiolini et al. traced single neural crest cells and demonstrated that the vast majority of the premigratory and migratory neural crest cells are multipotent using the Wnt1-CreER^{T} and Sox10-CreER^{T2} R26R-Confetti mouse models. Multipotent neural crest cells have also been identified in sites where postmigratory neural crest cells reside (Baggiolini et al., 2015; Lo & Anderson, 1995). Postmigratory CNC cells have been identified as MSCs. BMP and TGF-β signaling control the differentiation and fate of postmigratory CNC cells, and these cells support ectopic tooth germ survival and development via BMP signaling. Moreover, transplanted CNC cells can mediate the development of alveolar bone and teeth as a functional unit (Chung et al., 2009).

The contribution of the CNC cells to dental mesenchyme has been demonstrated by DI labeling, as well as transgenic approaches marking the neural crest cells with a LacZ reporter gene (Chai et al., 2000; Imai, Osumi-Yamashita, Ninomiya, & Eto, 1996; Serbedzija, Bronner-Fraser, & Fraser, 1992). Tissue recombination studies, in which CNC cells as well as CNC-derived ectomesenchyme but not mesoderm-derived mesenchymal tissue supported tooth formation when recombined with dental epithelium, provided additional evidence for the contribution of the CNC cells to mammalian tooth development (Lumsden, 1988). The CNC cells that migrate into the first arch between the 10- and 12-somite stage are odontogenic, either possessing instructive odontogenic potential to initiate tooth development or being able to respond to odontogenic signals such as FGF8 to support tooth formation (Zhang et al., 2003).

The shift in odontogenic potential coincides with a shift in BMP signaling from the epithelium to the mesenchyme. When Smad4 is conditionally inactivated in the neural crest cells, dental epithelium thickenings appear to be normal; however, tooth development arrests at the dental lamina stage, suggesting that Smad4-mediated BMP signaling in the CNC-derived ectomesenchyme is required for tooth development to advance into the bud stage (Ko et al., 2007).

1.3 Sox2+ Epithelial Stem Cells

Sox2 is a transcription factor expressed in the embryonic and adult stem cells and plays an important role in the maintenance of stem cell in various organs. *Sox2* is expressed in the primary dental lamina, where dental epithelial stem cells (DESCs) reside, and gives rise to all dental epithelial tissues (Juuri, Isaksson, Jussila, Heikinheimo, & Thesleff, 2013; Juuri et al., 2012; Smith, Fraser, & Mitsiadis, 2009). In the developing molar, Sox2-positive cells are detected by *in situ* hybridization from E11 in the lower jaw, and by E12, the expression is strongest along the lingual side of the dental epithelium. At E14–E16, *Sox2* expression is found in the oral epithelium of the lingual side of the tooth germ, including the dental cord and the enamel organ (Juuri, Isaksson, et al., 2013).

In the developing incisor, the labial cervical loop (CL) starts to show distinct *Sox2* expression from E14.5, and the expression in the lingual CL decreases. From E16 to E18, *Sox2* becomes increasingly restricted to the tip of the labial CL. The location of the most intense *Sox2* expression corresponds to that of the putative epithelial stem cell population, suggesting an early function for Sox2 in the specification of stem cells in the epithelium (Harada, et al., 1999; Juuri et al., 2012). Sox2 is subsequently expressed in the successional dental lamina, which is an epithelial sheet that connects the deciduous tooth germs and is embedded on their lingual side, where the replacement teeth form in all animals (Jarvinen, Tummers, & Thesleff, 2009; Smith et al., 2009). SOX2 expression has been detected in the lingual side of the dental lamina of a deciduous premolar of a 13-week human fetus (Juuri, Jussila, et al., 2013). In addition, dynamic Sox2 expression is found when mouse posterior molars develop in succession, indicating that Sox2 expression is associated with the posterior extension of dental epithelium where new teeth form. Using genetic fate mapping experiments, Sox2-positive cells of the mouse M1 were found to give rise to all epithelial cell lineages of the successionally developing M2 and to the bud of M3 (Juuri,

Jussila, et al., 2013). Furthermore, the conditional inactivation of Sox2 in the epithelium results in aberrant formation of the dental epithelium in the developing M2 and M3 of the E17.5 mouse mutant, suggesting that Sox may regulate the maintenance of the dental epithelial progenitors (Juuri, Jussila, et al., 2013).

1.3.1 Stem Cells in Tooth Pulp

Teeth have been called a "treasure chest" of MSCs since all tooth and support tissues appear to contain cells with MSC-like properties when cultured *in vitro* (Fig. 1; Balic, Aguila, Caimano, Francone, & Mina, 2010; Gronthos et al., 2002; Gronthos, Mankani, Brahim, Robey, & Shi, 2000; Huang, Gronthos, & Shi, 2009; Jo et al., 2007; Koyama, Okubo, Nakao, & Bessho, 2009; Volponi & Sharpe, 2013; Waddington, Youde, Lee, & Sloan, 2009). As with all *in vitro* studies of MSC-like cells, the differentiation potential attributed to these cells should be viewed with caution since most often formation of mineralized matrix is used to describe cells as more or less osteogenic. However, in the absence of detailed analysis of the mineral formed or *in vivo* studies, such conclusions mean very little.

Deciduous teeth are a "perfect" source of stem cells since they are plentiful and are naturally exfoliated. Culture of deciduous tooth pulp tissue results in rapid expansion of cells that have the *in vitro* characteristics of MSCs, expressing appropriate markers and being capable of multilineage

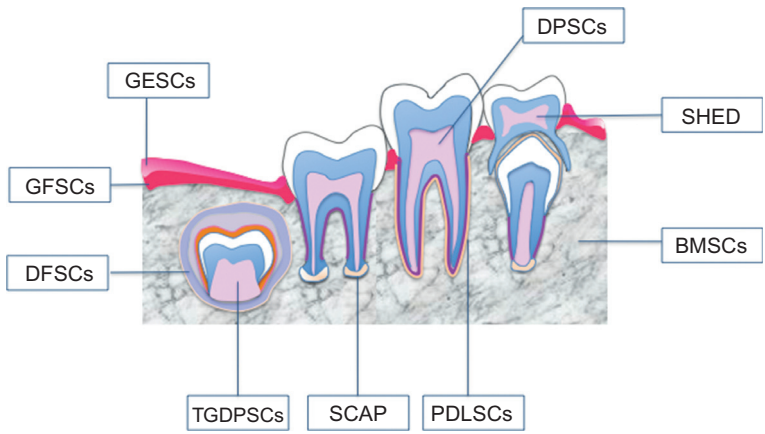

Figure 1 Teeth and supporting tissues are source for different stem cell populations. DPSCs, dental pulp stem Cells; SHED, stem cells from human exfoliated deciduous teeth; BMSCs, bone marrow stem cells; PDLSCs, periodontal ligament stem cells; SCAP, stem cells from apical papilla; TGDPSCs, tooth germ dental pulp stem cells; DFSCs, dental follicle stem cells; GFSCs, gingival fibroblast stem cells; GESCs, gingival epithelial stem cells.

mesenchymal cell differentiation into osteoblasts, chondrocytes, and adipocytes upon appropriate stimulation (Miura et al., 2003; Wang et al., 2012). These cells, called SHED (stem cells from human exfoliated deciduous teeth), can also be stimulated to form cells with properties that resemble odontoblasts and produce a dentin-like mineral (Miura et al., 2003).

Cells with virtually identical properties and of the same embryonic origin (CNC) can be isolated from the pulp of adult teeth and are generally referred to as Dental Pulp Stem cells (DPSCs). The role of these cells is to provide a source of cells to differentiate into odontoblast-like cells following dentin damage to produce reparative dentin (Feng, Mantesso, De Bari, Nishiyama, & Sharpe, 2011). It is plausible that SHED and DPSCs are the same cells. Although numerous studies have attempted to identify the origin of pulp stem cells, only one has used the accepted "gold standard" of genetic lineage tracing and shown that pericytes are a source of stem cells that can differentiate into odontoblast-like cells following dentin damage (Feng et al., 2011).

The relative ease with which pulp cells can be expanded *in vitro* suggests an obvious application in endodontics. *In vitro* experiments using tooth slice cultures suggest that pulp-like tissue can easily be generated from cultured pulp cells (Smith et al., 1995; Smith & Lesot, 2001), and *in vivo* studies similarly suggest restoration of pulp tissue and root dentin by DPSCs is possible (Huang et al., 2010; Murakami et al., 2013).

1.3.2 Periodontal Ligament Stem Cells

MSC-like cells can be isolated from periodontal ligament tissue and expanded in culture to generate populations with the properties expected of MSCs. *In vivo* studies suggest that these cells can be stimulated to differentiate into cells that are able to form cementum/PDL-like (Peiodontal Ligament) structures (Seo et al., 2004). PDL stem cells seeded as cell sheets can form PDL-like structures *in vivo* (Hasegawa, Yamato, Kikuchi, Okano, & Ishikawa, 2005), and the addition of PDL stem cells to dental implants to form an artificial PDL has been explored (Lin et al., 2011). Paradoxically, however, any PDL-like tissue around a metal implant is more likely to severely weaken rather than improve implant function. This highlights a major problem with cell-based PDL restoration: the PDL is a highly complex tissue comprising different cell types and intricate connections between the tooth and bone that are hard to imagine being fully reconstructed with cells alone. To this end, combinations of PDL stem cells and scaffolds are being tested (Morsczeck et al., 2005).

1.3.3 Root Apical Papilla and Dental Follicle Stem Cells

Not surprisingly, developing tissues such as third molar roots are an excellent source of highly proliferative MSC-like cells (often called SCAP cells) (Huang et al., 2009). These cells probably act as a source of root odontoblasts as the root grows. Cells isolated from the follicular sac, a loose connective tissue surrounding the enamel organ, of human third molars have MSC-like properties (Handa, Saito, Tsunoda, et al., 2002; Handa, Saito, Yamauchi, et al., 2002; Morsczeck et al., 2005; Park et al., 2010). These cells can differentiate into cementoblasts *in vivo* and can form cementum and PDL-like structures when cocultured with Hertwig's epithelial root sheath cells *in vitro* (Bai et al., 2011).

1.3.4 Gingival Stem Cells

The gingival tissue is composed of both epithelial and mesenchymal cells, and MSC-like cells have been cultured from gingival tissue (Fournier et al., 2010; Hsu, Huang, & Feng, 2012; Mitrano et al., 2010; Widera et al., 2009). Gingival wound repair shows similarities to scarless fetal wound repair (Schor et al., 1996; Sempowski, Borrello, Blieden, Barth, & Phipps, 1995), and the heterogeneous cell population of gingival fibroblasts plays a crucial part in the process. Gingival fibroblasts are also a convenient source of cells for iPS cell generation (Wada et al., 2011) and gingival epithelial cells are capable of forming teeth when combined with embryonic tooth mesenchymal cells (Angelova Volponi, Kawasaki, & Sharpe, 2013). Stem cells isolated from the oral epithelium have been successfully used to treat severe ocular surface disorders, and a treatment known as cultivated oral mucosal epithelial transplantation (COMET) has been introduced (Nakamura et al., 2003, 2004; Nakamura, Takeda, Inatomi, Sotozono, & Kinoshita, 2011).

2. STEM CELLS IN DENTIN REPAIR: TERTIARY DENTINOGENESIS

The dentin–pulp complex has the ability to naturally repair itself by re-initiating dentinogenesis to protect against external injuries and insults, such as caries and mechanical trauma (Smith et al., 1995). The dentin formed in response to pathological conditions is called tertiary dentin and is deposited by two distinct mechanisms, namely, reactionary and reparative dentinogenesis, depending on the severity of the injury (Fig. 2). In response to mild damage, postmitotic primary odontoblasts located at the periphery of the pulp respond by upregulating dentin secretion (reactionary

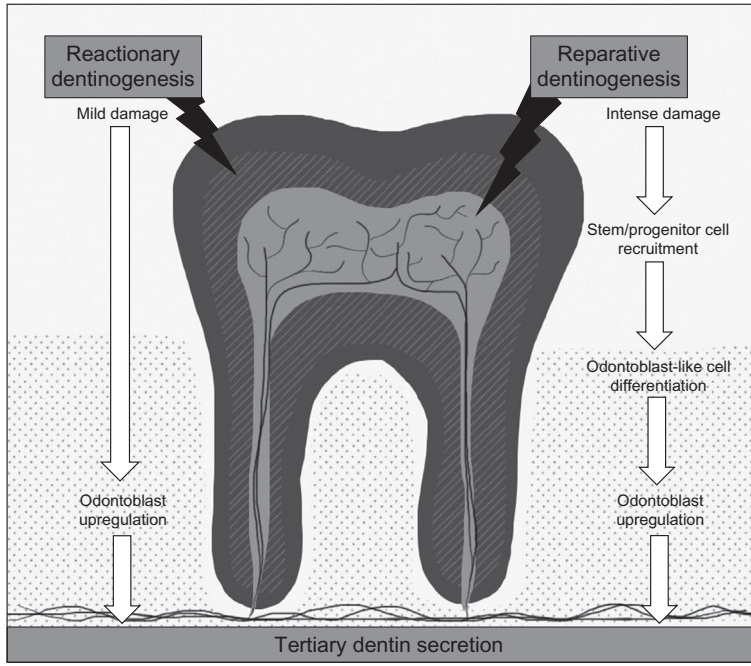

Figure 2 Diagram of a tooth showing possible sources of dental pulp stem cells in reparative dentinogenesis.

dentinogenesis). If the damage is extensive and the pulp is exposed, resulting in the death of local resident odontoblasts, stem/progenitor cells are recruited, differentiate into new odontoblast-like cells and subsequently secrete dentin.

The ontogeny, anatomical localization, and precise identity of odontogenic stem cells remain largely unknown. The cell-rich layer of Höhl, adjacent to the primary odontoblasts, contains undifferentiated MSCs that may be one stem/progenitor source of odontoblast-like cells. However, these cells are unlikely to provide a source of new odontoblasts, as their survival is closely linked to the survival of primary odontoblasts (Goldberg & Smith, 2004). Two cell niches within the dental pulp, the perivascular and neural stem cell niches, have been proposed to provide a source of stem/progenitor cells capable of generating reparative odontoblasts. Genetic-based linage tracing of pericytes using NG2, a proteoglycan that is commonly used as a marker for pericytes, revealed that differentiated odontoblasts originate from perivascular cells during incisor repair following damage (Feng et al., 2011). These $NG2^{+}$ pericyte-derived odontoblasts

produce reparative dentin in response to damage. It has further been shown that $NG2^+$ pericytes represent a dental stem cell subpopulation derived from $Gli1^+$ cells, which are located on arteries and are mobilized in response to incisor damage (Zhao et al., 2014). However, not all differentiated odontoblasts are pericyte derived, indicating that the dental pulp may also contain MSC-like cells of a nonpericyte origin. Some dental stem cells have been shown to differentiate from peripheral nerve-associated glial cells (Kaukua et al., 2014). Linage tracing of Schwann cells, the principal glial cells, demonstrated that reparative odontoblasts originate from neural crest-derived Schwann cells and Schwann cell precursors. It is important to note that Schwann cells do not express pericyte markers, signifying that pericytes and Schwann cells represent two distinct dental stem cell populations.

2.1 Signaling Events Mediating Reparative Dentinogenesis

The underlying molecular and cellular events that control the recruitment, proliferation, and differentiation of dental stem/progenitor cells into reparative odontoblasts at the site of damage are poorly understood. However, from what is known, it appears that the regeneration process does not exactly recapitulate what occurs during embryogenesis to reconstitute the original tissue architecture and biological function. During embryogenesis, sequential and reciprocal interactions between the stomadial epithelial and CNC-derived mesenchyme regulate tooth morphogenesis and differentiation. The odontogenic potential that directs the differentiation of odontoblasts resides in the dental epithelium. During tooth eruption, the enamel epithelium is lost, so an alternative signaling source is required to stimulate the formation of new odontoblasts during reparative dentinogenesis.

An assortment of growth factors is fossilized within the dentin matrix during tooth development (Smith et al., 2012). These bioactive molecules include members of the TGF-β superfamily, insulin-like growth factor-1 and 2 (IGF-1 and -2), fibroblast growth factor-2 (FGF2), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF). The release of these molecules into the pulp environment in response to tissue injury triggers signaling events that act on stem/progenitor cells to stimulate their recruitment, proliferation, and differentiation into reactionary odontoblasts. One particular member of the TGF-β superfamily, TGFβ-1, is a potent stimulator of odontoblast differentiation and promotes tertiary dentinogenesis in response to damage *in vivo* (Arany et al., 2014). Recently, the Wnt/β-catenin signaling pathway has been implicated in reparative

dentinogenesis. Wnt signaling has been proposed to promote odontoblast survival, proliferation, and differentiation (Han et al., 2014; Hunter et al., 2015). The source and the specific Wnt ligand(s) that mediate tooth repair are currently unknown. The activation of the p38 MAP signaling pathway in primary odontoblasts is an important event that triggers the secretion of new dentin (Simon, Smith, Berdal, Lumley, & Cooper, 2010). The tissue microenvironment also plays a crucial role in determining whether reparative dentinogenesis can take place; in particular, the degree of inflammation and infection has a strong impact on the tissue environment. Inflammation is not only required to remove dead cells and debris from the site of damage, it also facilitates the repair process, although the precise nature of the interplay between repair and inflammation is unclear. If inflammation is chronic, tertiary dentinogenesis will not occur, and the tissue will not repair. Inflammatory cells recruited to an injury site release cytokines and growth factors, notably TNF-α and ROS, at low levels to promote reparative dentinogenesis (Cooper, Holder, & Smith, 2014; Cooper & Smith, 2013). MSCs have recently been shown to possess immunomodulatory properties, which they exert through direct cell–cell contact with immune cells and the secretion of soluble factors, including prostaglandin E2, indoleamine 2,3-dioxygenase, and TGF-β, to promote tissue regeneration. DPSCs have been demonstrated to elicit an anti-inflammatory effect on innate and adaptive immune cells, supporting tissue regeneration (Li et al., 2014).

3. THE CONTINUOUSLY GROWING INCISOR

3.1 A Model System for Stem Cell Biology: The Mouse Incisor

Rodent incisors, including those of mice, are capable of continuously growing throughout the lifetime of the animal. This is achieved by the stem cells resident at the proximal end of the incisors, which provide a source of cells for compensation of tissue loss and homeostasis. The existence of continuously growing rodent incisors was first documented by Fougeroux de Bondaroy (1768). In 1962, Hwang and colleagues identified histidine as being highly expressed in the enamel in growing mouse incisors using autoradiography (Hwang, Tonna, & Cronkite, 1962). A decade later, Smith identified the renewal of the cell population in rat incisors using ′H-thymidine radioautography (Smith, 1980; Smith & Warshawsky, 1975). Since the landmark article on stem cell concepts by Lajtha (1979) and

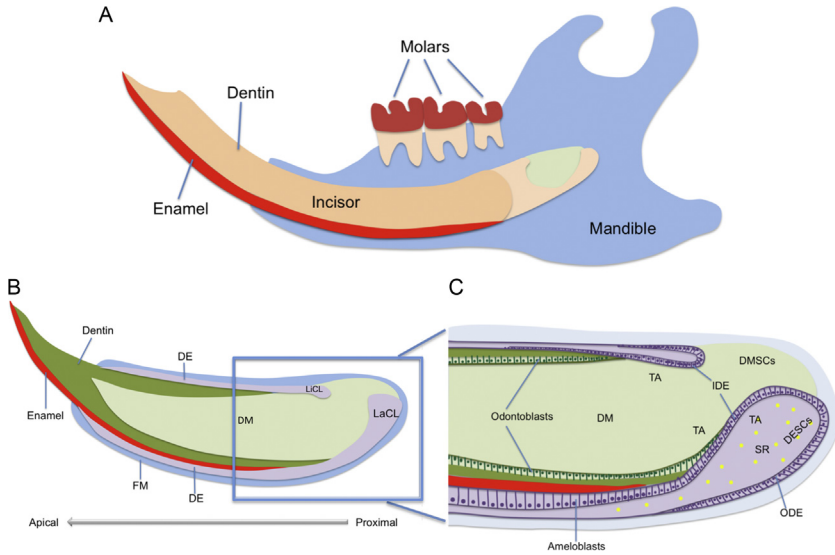

Figure 3 Schematic diagrams of the continuously growing mouse incisor. (A) Basic structure showing the mandible, incisor, and molars in adult mouse. (B) Illustrator of tissue distribution of mouse incisor on a sagittal section, and the enlarged region (C) at the proximal end of incisor showing the individual cells that constitute mouse incisor. (LaCL, labial cervical loop; LiCL, lingual cervical loop; DM, dental mesenchyme; DE, dental epithelium; FM, follicle mesenchyme; TA, transit-amplifying cells; IDE, inner dental epithelium; ODE, outer dental epithelium; SR, stellate reticulum; DESCs, dental epithelial stem cells; DMSCs, dental mesenchymal stem cells).

especially over the past 15 years, interest in rodent incisor stem cells has increased as an attractive model system for the study of adult stem cell biology (Kuang-Hsien Hu, Mushegyan, & Klein, 2014). Two basic types of stem cells are found in the mouse incisors: neural crest-derived ectomesenchymal and ectodermal epithelial (Koussoulakou, Margaritis, & Koussoulakos, 2009). Dental mesenchymal stem cells (DMSCs) give rise to pulp cells and odontoblasts which produce dentin, while DESCs only give rise to ameloblasts that produce enamel (Fig. 3).

3.2 Gene Expression in the Epithelial Stem Cell Niche of Mouse Incisors

Several signaling pathways including FGF, BMP, TGF-β, Notch, Shh, and Wnt, as well as adhesion molecules and microRNAs, have been identified as important for maintaining the continuous growth of mouse incisors (Fig. 4).

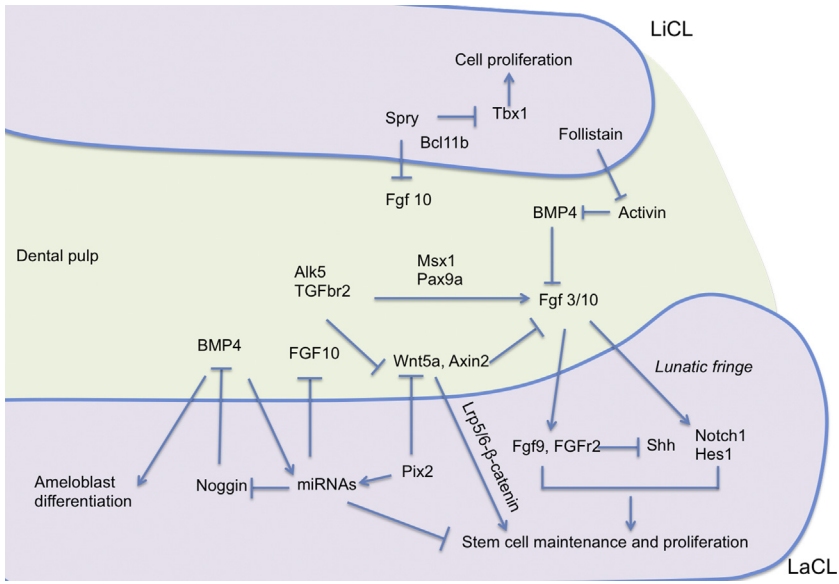

Figure 4 Gene regulatory network in a stem cell niche in the mouse incisors. Signaling molecules, transcription factors, and miRNAs expressed in the dental epithelium and mesenchyme regulate and maintain stem cells proliferation and differentiation.

Among these, FGF signaling is the most understood as crucial for epithelial stem cell proliferation and maintenance during tooth development and in adults (Kuang-Hsien Hu et al., 2014). *Fgf3* and *Fgf10* expression is restricted to the mesenchyme underlying the basal epithelial cells. FGF signaling regulates the Notch pathway in DESCs via stimulation of *lunatic fringe*, a secretory molecule that modulates Notch signaling expression (Harada, et al., 1999). Incisors in $Fgf3^{-/-}$ knockout mutants have a normal CL and $Fgf10^{-/-}$ mice die at birth, whereas $Fgf3^{-/-}$;$Fgf10^{-/-}$ compound mutants show a smaller labial CL, indicating that FGFs function cooperatively during tooth development and growth (Wang et al., 2007). *Fgf10* has been suggested to play a role in the regulation of incisor epithelial stem cells mainly through fibroblast growth factor receptor 2β (FGFR2β) (Klein et al., 2008). An inducible and reversible attenuation of FGFR2β signaling by rtTA transactivator/tetracycline promoter approaches identifies the role of FGFR2β signaling during development and adult mouse incisors. The results demonstrate that FGFR2β signaling regulates both the establishment of the incisor stem cell niche in the embryo and the regenerative capacity of incisors in the adult (Parsa et al., 2010). Other *Fgfs*, such as *Fgf9*, are

expressed in the incisor epithelium during development (Porntaveetus et al., 2011) but have not yet been studied in the adult incisors.

Members of the TGF-β family and their receptors play a role in the regulation of proliferation and maintenance of incisor epithelial stem cells. BMP4 is expressed in the dental mesenchyme and represses *Fgf3* (but not *Fgf10*) expression in the lingual mesenchyme. Activin, which is strongly expressed in the labial mesenchyme, inhibits the repressive effect of BMP4 and restricts *Fgf3* expression to the labial dental mesenchyme, resulting in increased stem cell proliferation (Wang et al., 2007). *Follistatin*, an extracellular antagonist of Activin and BMP, is expressed in the mesenchyme and at higher levels in adjacent dental epithelium. *Follistain* inhibits *Fgf3* expression and limits the number of lingual stem cells, further contributing to the characteristic asymmetry of mouse incisors (Wang et al., 2007). Both TGF-β type I and type II receptors play a role in the maintenance of incisor epithelial stem cells in the apical end of the CL. Loss of TGF-β type I receptor (*Alk5*) in the mesenchyme severely affects the proliferation of transit-amplifying (TA) cells in the dental epithelium, whereas loss of *Alk5* in the dental epithelium has no effect on the CL (Zhao, Li, Han, Kaartinen, & Chai, 2011), indicating the directionality of the signaling in these mesenchymal–epithelial cell interactions. TGF-β type II receptor (*Tgfbr2*) is expressed in the dental mesenchyme and its deletion results in upregulated expression of Wnt5a and downregulated expression of *Fgf3/10* in mesenchyme, both of which synergistically enhance *Lrp5/6*-β-catenin signaling in the CL epithelium, thus causing malformed postnatal incisors (Yang et al., 2014).

The Notch pathway regulates stem cells and progenitor maintenance and proliferation in several tissues including bone marrow, intestinal epithelium, and neuronal tissues (Breunig, Silbereis, Vaccarino, Sestan, & Rakic, 2007; Fan et al., 2006; Fre et al., 2005; Sakata-Yanagimoto et al., 2008). Studies on Notch signaling in mouse incisors suggest a role of Notch receptors, ligands, and modulators in the regulation of epithelial stem cells in the continuous regeneration of the mouse incisors. *Notch1, -2,* and *-3* as well as Notch target gene *Hes1* are expressed in the stellate reticulum (SR) cells in the incisor epithelium (Felszeghy, Suomalainen, & Thesleff, 2010; Harada, et al., 1999). In addition *Notch3* is found also expressed in dental mesenchyme (Harada, et al., 1999). *Notch1* expressing SR cells in the CL of the continuously growing mouse incisor are stem cells that have the capacity to self-renew and to generate progeny that differentiate into various cell types of epithelium, especially the enamel-producing ameloblasts (Harada, et al., 1999). Using

organ cultures, BrdU and *DiI* labeling experiments demonstrate that the Notch pathway in DESCs is regulated by FGF signaling in dental mesenchyme via stimulation of *lunatic fringe* expression (Harada, et al., 1999). The expression of Notch ligand, *Jagged 2* (*Jag2*), and Notch signaling modulator *lunatic fringe* is regulated by FGF and BMP signaling (Harada, et al., 1999; Mitsiadis, Graf, Luder, Gridley, & Bluteau, 2010). Notch pathway inhibitor, DAPT, inhibits Notch target gene *Hes1* expression in the CL thus causing a significant reduction in the size of the CL and massive apoptosis in the epithelial stem cell niche, suggesting Notch signaling is required for epithelial stem cell survival and enamel formation in the continuously growing mouse incisors (Felszeghy et al., 2010).

The Sonic hedgehog (*Shh*) pathway also regulates incisor epithelial stem cells in adult mice. *Shh* reporter gene *Gli1* and Hedgehog receptor Patched Homolog 1 (*Ptch1*) are expressed in both the epithelial TA cells and the dental pulp cells in the developing and growing mouse incisors (Bitgood & McMahon, 1995; Seidel et al., 2010). To identify a role of *Shh* signaling in adult stem cells, $Gli1^{lacZ}$ and $Ptch1^{lacZ}$ reporter mice are used for genetic lineage tracing of Hh-responsive cells and the fate of their progenies following continuous growth of the mouse incisors. The results reveal that high levels of *lacZ* expression in the reporter lines are found in the labial and lingual CL epithelium and in the mesenchyme between the two aspects of the CL. Inhibition of *Shh* pathway demonstrates that Hh signaling is required for the stem cells to continuously generate ameloblasts in adults but not for stem cell survival (Seidel et al., 2010). Runt-related transcription factor (*Runx*) regulates epithelial stem cell continuous proliferation and differentiation in growing incisors by maintaining of *Fgf9* and *Shh* expression, and *Fgf9* acts as antagonist of *Shh* expression (Kurosaka et al., 2011).

The Wnt signaling pathway plays a critical role in the epithelial stem cell niche in many adult tissues such as the skin (Arwert, Hoste, & Watt, 2012), hair follicle (Fujiwara et al., 2011), and the intestine (Holik et al., 2014). BAT*gal*, TOP*gal*, and $Axin2^{lacZ/lacZ}$ reporter mice are used to gain insight into the role of Wnt signaling in the regulation of the continuously growing mouse incisors. Wnt/β-catenin activity is found mainly confined to mesenchyme but completely absent from the epithelial stem cell compartment (Suomalainen & Thesleff, 2010). *Lgr5,* a Wnt target gene, is a specific epithelial stem cell marker in the hair follicle (Fuchs, 2009; Jaks et al., 2008) and intestine (Barker et al., 2007) and has been found expressed in the region of putative epithelial stem cells niche in the labial CL of mouse incisors. However, Wnt activity is not found in the *Lgr5*-positive cells in the incisor stem

cell niche (Suomalainen & Thesleff, 2010). Wnt3a is expressed in the epithelial cells, and overexpression of Wnt3a inhibits amelobast formation in the postnatal mouse incisor (Millar et al., 2003). Constitutively expression of active β-catenin in the adult dental epithelium results in increased proliferation and oversized CL (Liu et al., 2010). Taken together, Wnt/β-catenin signaling may not directly regulate epithelial stem cell maintenance but might have an inhibitory role on stem cell proliferation and differentiation (Millar et al., 2003; Suomalainen & Thesleff, 2010). Recent studies suggest that mesenchymal *Tgfbr2* deletion activates dental epithelial *Lrp5*/6-β-catenin signaling by upregulation of Wnt5a and *Fgf3/10* (*Yang et al., 2014*), indicating a cross talk among TGF-β, Wnt, and *Fgf* signaling pathways in the epithelial CL.

Sprouty (*Spry*) genes are negative regulators of multiple receptor tyrosine kinases including receptors for FGF (Hacohen, Kramer, Sutherland, Hiromi, & Krasnow, 1998), EGF (Frank et al., 2009), and HGF (Lee et al., 2004). *Sprys* genes prevent the generation of lingual ameloblasts by inhibiting an FGF-mediated epithelial–mesenchymal signaling loop on the lingual side in adult mice (Boran et al., 2009; Klein et al., 2008). Transcription factors Tbx1 and *Bcl11b* have been found up- and downregulated, respectively, in $Spry2^{+/-};Spry4^{-/-}$ mutant incisors. Tbx1 maintains ameloblast progenitors in rodent incisors and loss of expression results in absence of enamel formation (Caton et al., 2009). $Bcl11b^{ko/ko}$ mice die at birth, but heterozygous $Bcl11b^{s82G/ko}$ mice suggest that attenuated *Bcl11b* activity impairs the maintenance of ameloblast progenitors in the mouse incisors (Katsuragi et al., 2013). The transcription factor Sox2 is an epithelial stem cells marker expressed in the labial aspect of the CL and contributes to ameloblast cell renewal regulated by *Fgf8* and fine tuned by specific miRNAs (Juuri et al., 2012).

Recent studies highlight the importance of adhesion molecules that are required for a given stem cell type to interact with their niche (Chen, Lewallen, & Xie, 2013). Deletion of integrin β3 resulted in a smaller CL and reduced proliferation in the TA region in adult mice (Yoshida et al., 2013). Conditional inactivation of E-cadherin causes decreased stem cell number and cell migration in the CL of the mouse incisors *via* FGF signaling regulation (Li et al., 2012). Gene microarrays identified the expression of miRNAs in the LaCL, LiCL, and ameloblasts in the adult incisors, suggesting a role of miRNAs in adult stem cell renew and differentiation during incisor growth (Jheon, Li, Wen, Michon, & Klein, 2011). Activated *miR200a-3p* is expressed in the preameloblasts and represses *Pitx2* and

β-catenin expression in the labial epithelial stem cell niche, whereas *Pitx2* reciprocally activates *miR200a-3p* expression to enhance epithelial cell differentiation (Sharp et al., 2014). *Pitx2* directly binds to miR-200c/141 upregulating miR-200c and miR-203 expression and inhibiting noggin and Bmper, two antagonists of BMP, thus activating BMP signaling and regulating epithelial stem cell differentiation in the mouse incisors (Cao et al., 2013).

3.3 Identification of a MSC Niche

The continuously growing mouse incisors need a constant supply of cells to compensate tissue loss from the abrading tips, and this process requires not only epithelium stem cells that form enamel-producing ameloblasts, but also mesenchyme stem cells that form dentin-producing odontoblasts and pulp cells (Fig. 5). BrdU/EdU (5-ethynyl-2′-deoxyruidine)-based label-retaining

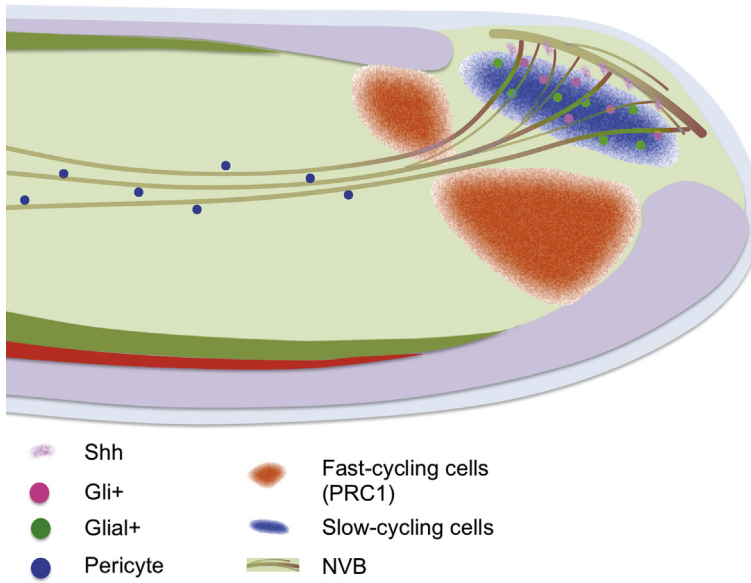

Figure 5 Identification of a mesenchymal stem cell niche in the mouse incisors. Slow-cycling stem cells locate in the mesenchyme between labial and lingual cervical loop region close to the NVB, represented by Gli+ and Glial+ expressing cells. PRC1 expressed in the mesenchyme closed to the labial and lingual cervical loop region, corresponding to the fast-cycling (TA) cells. Pericytes reside on the abluminal surface of endothelial cells in the vascularized connective tissues and contribute to MSC-derived mesenchymal cells responding to tissue damage (NVB, neurovascular bundle).

assays and H2B-GFP mice have been used to identify MSC populations in many tissues (Foudi et al., 2009; Kaukua et al., 2014; Tang et al., 2008; Tumbar et al., 2004; Zhao et al., 2014). The general concept is that stem cells divide infrequently to preserve their long-term proliferation potential and to prevent the errors occurrence during DNA replication (Fuchs, 2009), so such "slow cycling" can be identified by their ability to retain nucleosides. Progenitor cells in adult tissues divide fast and frequently, and those cells are called transit-amplifying (TA) cells or fast-cycling cells (Lapthanasupkul et al., 2012). Nucleoside labeling identifies a slow cycling cell population of mesenchymal cells that lies between the epithelial CL and a population of fast-cycling cells more distally (Kaukua et al., 2014; Lapthanasupkul et al., 2012; Zhao et al., 2014; Fig. 5).

Slow-cycling cells express Gli1 and lineage tracing using Gli1-Cre^{ERT2} mice shows that these cells gives rise to almost all odontoblasts and pulp cells during adult incisor growth. The Gli1 expressing cells are localized in a neurovascular bundle and respond to Shh ligand that appears to be neuronal derived (Zhao et al., 2014). Within this Gli1^{+} populations of slow-cycling MSCs there appears to be as yet unexplained heterogeneity. By using promoters that direct gene expression in peripheral glial cells (Sox10 and PLP), a population of slow-cycling cells that contributes to around 50% of odontoblast and pulp cell formation during incisor growth was identified (Kramann et al., 2015). Significantly, this study utilized confetti reporter mice to trace individual stem cell contributions and showed that all glial-derived MSCs are capable of forming both odontoblasts and pulp cells, but the relative proportions of each depend on the position of the stem cell in its niche. Thus, the mouse incisor MSC population consists of a glial-derived population and a nonglial-derived population both of which are Shh responsive in the neurovascular bundle. Surprisingly, an additional layer of heterogeneity also exists since a proportion of the Gli1+ cell and glial cells express Thy1 (CD90) and these cells contribute around 20% of the odontoblasts and pulp cells. The reasons for this unexpected complexity are not yet understood.

Polycomb Repressive Complex 1 (PRC1) proteins were first described as gene repressor for Hox genes in *Drosophila melanogaster* and have been identified critical roles in adult stem cells function (Luis, Morey, Di Croce, & Benitah, 2012; Morey, Aloia, Cozzuto, Benitah, & Di Croce, 2013; Morey et al., 2012). Ring1a/b are the core components of PRC1 expressed in the incisor apical mesenchyme closed to both labial and lingual CL, consistent with a location for TA (fast cycling) cells. Depletion of Ring1 in a Ring1a$^{-/-}$;Ring1b$^{cko/cko}$ incisors shows dramatically reduced

cell proliferation in the apical mesenchyme and CL epithelium, and a down-regulation of FGF singling as well as downstream targets (Lapthanasupkul et al., 2012). Interestingly, *Bmi*1, one of other component of PRC1, is expressed in the CL epithelial stem cells and also expressed in the BrdU-retaining mesenchyme, indicating that those cells are also stem cells (Biehs et al., 2013).

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