Dental Pulp Stem Cells, Niches, and Notch Signaling in Tooth Injury

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ABSTRACT

Stem cells guarantee tissue repair and regeneration throughout life. The decision between cell self-renewal and differentiation is influenced by a specialized microenvironment called the 'stem cell niche'. In the tooth, stem cell niches are formed at specific anatomic locations of the dental pulp. The microenvironment of these niches regulates how dental pulp stem cell populations participate in tissue maintenance, repair, and regeneration. Signaling molecules such as Notch proteins are important regulators of stem cell function, with various capacities to induce proliferation or differentiation. Dental injuries often lead to odontoblast apoptosis, which triggers activation of dental pulp stem cells followed by their proliferation, migration, and differentiation into odontoblast-like cells, which elaborate a reparative dentin. Better knowledge of the regulation of dental pulp stem cells within their niches in pathological conditions will aid in the development of novel treatments for dental tissue repair and regeneration.

Dental pathologies (*e.g.*, caries, genetic anomalies) and injuries are common problems. For esthetic, psychological, and medical reasons, tooth repair is an important social issue. Dental treatment requires considerable resources, and thus is very expensive for any health care system. Novel techniques have been used for the improvement of materials that can replace missing or damaged dental tissues, but their success is limited and depends on the integration and quality of the material used. The rapid progress made in stem cell, materials, and molecular biology sciences in the past 10 years has allowed scientists to imagine alternative and innovative strategies for tooth repair. Stem cells offer an amazing potential for tooth homeostasis, repair, and regeneration. Manipulating dental stem cells *in situ* and expanding them *ex vivo* by using specific signaling molecules is an exciting outcome. Nevertheless, stem-cell-based tooth repair is not devoid of challenges that need to be solved prior to any clinical application. For example, it is crucial to identify the different types of dental stem/progenitor cells and their niches in teeth in order to understand the mechanisms that support stem cell survival. This knowledge will guarantee the success of stem-cell-based therapeutic approaches in dentistry.

TOOTH DEVELOPMENT

The aim of regenerative dentistry is to re-create *in vitro* and *ex vivo* the processes of embryonic tooth development. This is a complex process in which, through a series of epithelial-mesenchymal interactions, cells of the oral epithelium and cranial neural-crest-derived mesenchymal cells (CNCCs) give rise to the various mineralized structures of the tooth. Epithelial cells give rise to the enamel-forming ameloblasts, while CNCCs form the dental follicle, dental pulp, and odontoblasts that are responsible for dentin matrix synthesis (Bluteau *et al*., 2008; Mitsiadis and Graf, 2009). For a better understanding of dental pathology and regeneration, it is necessary to conceive of teeth as part of an entity of tissues forming the craniofacial complex. Neural crest cells derived from the hindbrain and posterior part of the midbrain give rise to the majority of the mesenchymal tissue of the brachial arches (Mitsiadis and Graf, 2009). CNCCs destined to populate the first brachial arch, where the teeth are developing, migrate essentially from rhombomeres 1 and 2. These cells are embryonically distinct from other neural crest cell populations that are under control of the Hox genes. CNCCs are clonogenic cells, since they are able to differentiate into various cell types, such as odontoblasts, cementoblasts/cementocytes, osteoblasts, chondroblasts/chondrocytes, neurons, melanocytes, and muscles. One of the challenges in regenerative dentistry is to identify and characterize adult mesenchymal stem cells (or progenitor cells) that have the same properties and can successfully replace the clonogenic CNCCs.

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Key Words

stem cells, dental pulp, niches, tooth injury, regeneration, repair.

DENTAL STEM CELLS

Stem cells are able to continuously self-replicate (*i.e.*, produce daughter cells having the same characteristics as themselves), generate daughter cells with different and more restricted properties, and re-populate a host *in vivo* (Smith, 2006). Adult stem cells are undifferentiated cells found among differentiated cells in a tissue or organ that can renew itself. However, these stem cells have a limited replicative capacity. Dental stem cells are considered as a new source of adult stem cells that could be used for regenerative medicine. Dental stem cells could be removed from an individual's primary or permanent teeth, expanded, and put back into the same individual when repair becomes necessary. This autologous transplantation would remove the need for immunosuppression.

Historically, dental stem cells were first isolated by Gronthos and co-workers from the dental pulp (DPSCs) (Gronthos *et al*., 2000) and exfoliated deciduous teeth (SHED) (Shi and Gronthos, 2003). Dental stem cells can also be extracted from the apical papilla of shed primary teeth (SCAP) (Bluteau *et al*., 2008). DPSCs exhibit a multipotent character since they are capable of differentiating into various cell types, such as chondrocytes (Waddington *et al*., 2009), adipocytes (Waddington *et al*., 2009), osteoblasts (de Mendonca Costa *et al*., 2008), myocytes (Kerkis *et al*., 2008), neuronal cells (Nosrat *et al*., 2001), and cardiomyocytes (Gandia *et al*., 2008). Adult DPSCs can divide only a finite number of times (according to the age of the individual) (Mitsiadis *et al*., unpublished observations), and they may accumulate genetic changes over time (Feki *et al*., unpublished observations; C. De Bari, personal communication; Gronthos *et al*., 2002). Dental pulp cells can be reprogrammed into induced-pluripotent stem cells (iPS) at a higher rate compared with other cell types of human origin tried so far (Yan *et al*., 2010).

Animal studies have shown the great potential of DPSCs for repair and regeneration of various tissues, such as bone (Graziano *et al*., 2008a,b), heart (Gandia *et al*., 2008), muscles (Kerkis *et al*., 2008), and teeth (Onyekwelu *et al*., 2007; Cordeiro *et al*., 2008; Nedel *et al*., 2009). However, DPSCs need to be further investigated for their clinical usefulness. For example, the composition of the culture medium in which DPSCs are grown can dictate their differentiation into either osteoblasts/osteocytes or chondroblasts/chondrocytes. This property has made DPSCs an attractive choice for bone and cartilage tissue engineering, especially when they can be used as autologous transplants. Recently, the first clinical trial of DPSC application in patients for bone reconstruction was successfully carried out by Papaccio and co-workers (d'Aquino *et al*., 2009).

Adult stem cells present at a low frequency (*e.g.*, roughly one stem cell *per* 100,000 bone marrow cells), indicating that isolation of real DPSCs could be problematic. It is of prime importance to show that dental pulp-derived cells are indeed stem cells. The precise identity of DPSCs remains a challenge because of the lack of a single specific stem cell marker. Standard assays to identify DPSCs rely on their morphology, selective adherence to a solid surface, proliferative potential, capacity to differentiate, and ability to repair tissues. The most common and efficient method of cell purification to enrich the population of a specific cell type is by labeling cell lineages with fluorescent antibodies and then purifying them by FACS. This requires the identification of specific cell-surface markers for a particular cell type. DPSCs are characterized by their negative expression of hematopoietic antigens (*e.g.*, CD45, CD34, CD14), and positive expression of stroma-associated markers (*e.g.*, CD29, CD73, CD105, CD44) and extracellular matrix proteins such as collagen, vimentin, laminin, and fibronectin. Some dental pulp cell types do not exhibit any specific cell-surface marker, and their purification is not yet possible. Evidence provided by different studies agrees that dental pulp cells have a mesenchymal character based on their ability to differentiate into many cell types (Karaoz *et al*., 2009). However, it remains a challenge to produce homogenous DPSCs types as required.

STEM CELL NICHES

Stem cells are distributed around the body in various niches. These niches consist of specific anatomic locations housing stem cells and enabling them to self-renew. The particular microenvironment of the niche regulates how stem cell populations participate in tissue maintenance, repair, and regeneration. Specific signals derived from precise areas of the niche permit stem cells to stay alive, and to change their number and fate (Scadden, 2006; Djouad *et al*., 2009). Soluble molecules such as the Wnt, Notch, fibroblast growth factor (FGF), and Hedgehog proteins are important paracrine regulators of stem cell function, with various capacities to induce proliferation or differentiation (Mitsiadis *et al*., 2007). The function of adult stem cells is often limited outside the niche.

It is widely admitted that heterologous cell types comprise the niches, but analysis of recent data suggests that some stem cell populations might have a niche composed exclusively of extracellular matrix and other non-cellular components (Scadden, 2006; Hynes, 2009). Therefore, the basement membrane might also participate in the specialized microenvironment and could influence the function of a stem cell pool. In addition, non-protein components of the local microenvironment, such as inorganic ions and metabolic products, might affect stem cell function. For example, high ionic calcium concentrations might influence stem cell behavior in the niche that will allow cells to respond to the changeable conditions of a given tissue. Similarly, oxidative stress affects stem cell function and is therefore critical in modifying stem cell fate (Uccelli *et al*., 2008).

NOTCH SIGNALING AND STEM CELL NICHES

Notch receptors (Notch1, Notch2, Notch3, Notch4) are evolutionarily conserved transmembrane proteins that bind to Notch-specific ligands (Jagged1, Jagged2, Delta-like1, Delta-like3, Delta-like4). These complexes result in the activation of two sets of enzymatic activities. TACE/ADAM10 and γ-secretase presenilins cleave Notch proteins and release their intracellular domain (NICD) that contains nuclear localization signals (Radtke et al., 2005; Kopan and Ilagan, 2009; Artavanis-Tsakonas and Muskavitch, 2010). The NICD fragment forms a complex with the DNA-binding protein RBP-Jκ/CBF1 (Oswald *et al*., 2002). This complex activates the transcription of target

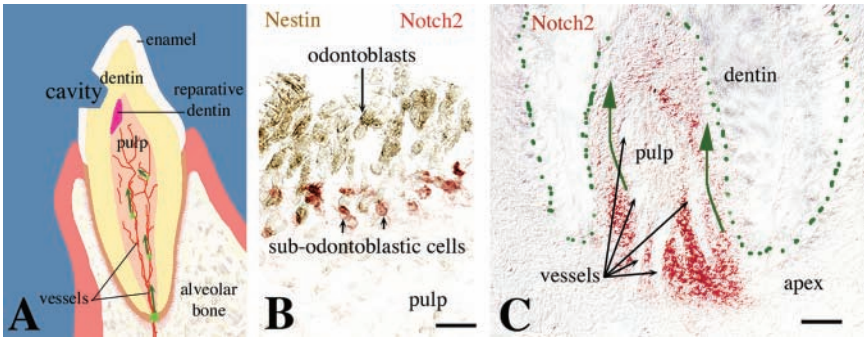

Figure. Cellular and molecular events following dental injury. **(A)** A hypothetical model summarizing the cellular events after dental injury. Cavity preparation activates stem cell niches located in different parts of the dental pulp (green asterisks). Stem cells/progenitor cells start to migrate toward the injury site and, once in place, differentiate into odontoblast-like cells and form a reparative dentin. **(B)** Cultured tooth slices. Mild injuries activate the expression of Nestin in odontoblasts and Notch2 in the layer of sub-odontoblastic cells. These Notch2-positive cells could replace apoptotic odontoblasts in severe injuries. **(C)** Dental injury at the crown level activates Notch2 expression in a subpopulation of undifferentiated cells (stem cells/progenitor cells) at the apex of the root. These cells may migrate to the injury site and contribute to the formation of a reparative dentin. Bars: (B) 20 μm; (C) 60 μm.

genes that maintain cells in a proliferative/undifferentiated state (Kageyama *et al*., 2007; Artavanis-Tsakonas and Muskavitch, 2010). Although Notch activation promotes stem cell survival (Androutsellis-Theotokis *et al*., 2006), Notch biological function depends on the developmental context, type, or state of the cell (Kopan and Ilagan, 2009; Artavanis-Tsakonas and Muskavitch, 2010).

Asymmetric stem cell division ensures stem cell renewal and a progeny of cells that will differentiate, thus ensuring repair and regeneration of tissues and organs. During asymmetric stem cell division, Notch signaling is inhibited in one of the two daughter cells while it is activated in the other, enabling two different cell types to be generated (Artavanis-Tsakonas and Muskavitch, 2010). Notch signaling plays a key role in cell fate determination and maintenance of stem cells, and its activation or inhibition can regulate the fate of stem cells. For example, the simultaneous loss of Notch1 and Notch2 leads to the disappearance of intestinal stem cells/progenitors and restricts their differentiation potential (Riccio *et al*., 2008). Similarly, Notch1 deletion in mice results in the differentiation of corneal cells into a skin-like epithelium that leads to blindness (Vauclair *et al*., 2007). Furthermore, Notch signaling controls the fate of pancreatic endocrine cells (Apelqvist *et al*., 1999), since mice deficient in either Dll1 or RBP-Jκ exhibit an accelerated differentiation of endocrine cells (Sommer *et al*., 1996; Jensen *et al*., 2000). Finally, deletion of RBP-Jκ in epidermis induces skin pigmentation defects, suggesting that Notch signaling determines the fate and survival of melanoblast/melanocyte stem cell populations (Moriyama *et al*., 2006). Indeed, melanocyte stem cells require Notch activation to remain in an undifferentiated state (Aubin-Houzelstein *et al*., 2008).

In bone marrow, the hematopoietic stem cells (HSCs) are located in two different niches (*i.e.*, endosteal and perivascular). Notch promotes HSCs self-renewal and inhibits cell differentiation, thus increasing the number of HSCs (Stier *et al*., 2002; Duncan *et al*., 2005). HSCs are regulated by signals that are derived from stromal fibroblasts and osteoblasts, which form the HSC niche and express Jagged1 (Mitsiadis *et al*., 2007).

TOOTH INJURY, NOTCH SIGNALING, AND DENTAL STEM CELLS

Mechanisms that contribute to dental injury include induction of apoptosis, activation of immune responses, and alterations in dental tissue physiology (Tziafas *et al*., 2000; Mitsiadis and Rahiotis, 2004; Mitsiadis *et al*., 2008). Under most circumstances, clearance of apoptotic cells occurs with remarkable rapidity, without eliciting an inflammatory response (Geske and Gerschenson, 2001). A structural remodeling of the pulp chamber takes place during tooth repair with reparative dentin deposition. Apoptosis is significantly higher in the odontoblastic layer than in the rest of the pulp. It is possible that the elimination of odontoblasts by apoptosis may produce death signals, leading to the simultaneous elimination of the neighboring progenitor cells (*i.e.*, sub-odontoblastic cells).

Upon injury, stem cells are recruited from remote storage sites to areas of wound healing, where they are engrafted in high numbers (Fig. A). Elimination of odontoblasts and the neighboring odontoblast progenitors by apoptosis will elicit migration of an important number of DPSCs to the injury site, where they will differentiate into odontoblast-like cells, thus ensuring the regenerative capacity (plasticity) of the pulp. DPSCs are thought to reside in one or more specific niche(s), being activated and utilized in the repair mechanism against dental damage. Cells located in putative dental pulp perivascular niches exhibit mesenchymal stem cell properties (Gronthos *et al*., 2000; Shi and Gronthos, 2003). These cells are activated after injury, proliferate, and finally differentiate into odontoblast-like cells.

Numerous significant physiological changes (*i.e.*, levels of signaling molecules, arterial oxygen content) normally accompany regeneration of the dentin-pulp complex. Signaling molecules such as bone morphogenetic proteins (BMPs) are released from the dentin after injury and play a role in reparative dentin formation (Tziafas *et al*., 2000; Mitsiadis and Rahiotis, 2004). In addition, it has been shown that apoptosis is accompanied by up-regulation in the expression of transforming growth factor beta (TGFβ) (Kobayashi *et al*., 2000; Pollack and

Leeuwenburgh, 2001), which is also essential for reparative dentin formation (Tziafas *et al*., 2000; Mitsiadis and Rahiotis, 2004).

Given the importance of Notch signaling in regulating stem cell behavior and fate in many other tissues and organs, we hypothesized that this signaling pathway might also be important for tooth homeostasis and repair. Indeed, previous studies have shown that Notch receptors were absent in adult rat pulp tissue, but their expression was reactivated after dental injury (Mitsiadis *et al*., 1999; Lovschall *et al*., 2005; Zhang *et al*., 2008). Notch receptors have been detected in cells of the sub-odontoblastic layer (*i.e.*, Notch2), pulp fibroblasts in the vicinity of the injury (*i.e.*, Notch1), numerous pulp cells at the apex of the root (*i.e.*, Notch2), and cells correlated with blood vessels (*i.e.*, Notch1 and Notch3) (Mitsiadis *et al*., 1999) (Figs. B, C). Similar results have been obtained for the receptor Notch2 in injured human permanent teeth (Mitsiadis *et al*., 2003). More recent findings suggested that pericytes may represent an alternative source for DPSCs, since Notch3 protein has been detected in pericytes of injured rat molars (Lovschall *et al*., 2007). The ligand Delta-like1 has been found in odontoblasts of injured molars (Lovschall *et al*., 2005). The activation of Notch signaling by either Jagged1 or N1ICD inhibits odontoblast differentiation without affecting dental pulp cell proliferation (Zhang *et al*., 2008). In contrast, studies on human DPSCs have shown that activation of Notch by Delta-like1 stimulates both cell proliferation and differentiation (He *et al*., 2009). Together, these results suggest that Notch signaling may act as either a negative (through Jagged1 activation) or a positive (through Delta-like1 activation) regulator of odontoblast differentiation. They also reveal the context-dependent function of Notch signaling (*i.e.*, intact/physiological status *vs*. injury/pathological status, rodent *vs*. human tissues). However, it has not yet been proven that these Notch-positive pulp cell populations participate in the repair process and are capable of differentiating into odontoblast-like cells after injury.

CONCLUSIONS

Although the prospect of dentin-pulp engineering is very attractive, we are far from being able to perform routine clinical procedures. Despite the significant interest in this field, no clinical trials have been performed for dentin-pulp repair and regeneration. Cell and materials sciences have to define conditions for manufacturing consistent and reproducible products, which are quality-controlled for safety and efficacy. The use of expanded cell populations needs to take into account the possibility of genetic and epigenetic instability. The possibility of autologous cell transplantation and the use of cells naturally occurring in the injury site may minimize the risk of side-effects. In addition, a better understanding of the biology of dentin-pulp regeneration opens the exciting prospect of the development of cell-based approaches.

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