Tooth Slice/Scaffold Model of Dental Pulp Tissue Engineering

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ABSTRACT

Multipotency is a defining characteristic of post-natal stem cells. The human dental pulp contains a small subpopulation of stem cells that exhibit multipotency, as demonstrated by their ability to differentiate into odontoblasts, neural cells, and vascular endothelial cells. These discoveries highlight the fundamental role of stem cells in the biology of the dental pulp and suggest that these cells are uniquely suited for dental pulp tissue-engineering purposes. The availability of experimental approaches specifically designed for studies of the differentiation potential of dental pulp stem cells has played an important role in these discoveries. The objective of this review is to describe the development and characterization of the Tooth Slice/Scaffold Model of Dental Pulp Tissue Engineering. In addition, we discuss the multipotency of dental pulp stem cells, focusing on the differentiation of these cells into functional odontoblasts and into vascular endothelial cells.

Advances in tissue engineering and stem cell biology have led to new strategies for the regeneration of damaged or lost tissues in the oral cavity. In recent years, the field of dental tissue regeneration has emerged as an exciting new area of research. However, few pre-clinical experimental models are available for mechanistic and translational studies involving the regeneration of the human dental pulp. Here, we will describe the development and characterization of the Tooth Slice/Scaffold Model of Dental Pulp Tissue Engineering and discuss recent findings in the areas of differentiation of stem cells into odontoblastic and endothelial cells, some of which were generated with the use of this model.

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DENTAL PULP TISSUE REGENERATION

Conventional root canal treatment is the therapy of choice for most necrotic permanent teeth. While the outcome of conventional endodontic treatment is usually excellent in mature teeth, the same is not true in immature permanent teeth with incomplete root formation. Such teeth pose technical challenges to conventional endodontic therapy (*e.g.*, difficult apical seal) and have poor long-term outcomes (Cvek, 1992). A frequent cause of failure is cervical root fracture, which is more frequent in endodontically treated immature teeth than in teeth with complete root formation (Cvek, 1992). A likely reason for these fractures is the fact that lateral dentin deposition along the developing root is halted at the time of pulp necrosis, which results in teeth with thin and fragile lateral walls. It has been proposed that the engineering of a new dental pulp can potentially result in the completion of lateral and vertical root formation in necrotic immature teeth (Nör, 2006), which could have a positive impact on their long-term outcome.

In general, there are two approaches for dental pulp tissue engineering: one in which host cells from the apical region are induced to migrate toward the interior of the root canal and differentiate into a vascularized pulp tissue (Trope, 2010); and the other based on the transplantation of stem cells into the root canal and the differentiation of such cells into a new dental pulp (Cordeiro *et al*., 2008; Huang *et al*., 2010). In both cases, stem cells appear to play a major role in the regeneration of the pulp tissue.

Dental pulp tissue engineering without stem cells would require the transplantation of multiple cell types (*i.e.,* odontoblasts, endothelial cells, neural cells, fibroblasts) in a spatially ordered manner (*e.g.*, odontoblasts in the periphery). Such an approach is technically challenging due to inherent complexities of culturing and transplanting multiple cell types. In contrast, stem cells can potentially constitute a singular cellular source for the engineering of a dental pulp.

It has been shown that dental pulp and apical stem cells exhibit multipotency and are therefore capable of undergoing differentiation into key cellular types of the dental pulp tissue,

Key Words

Endodontics, stem cells, odontoblasts, endothelial cells, angiogenesis, regenerative medicine.

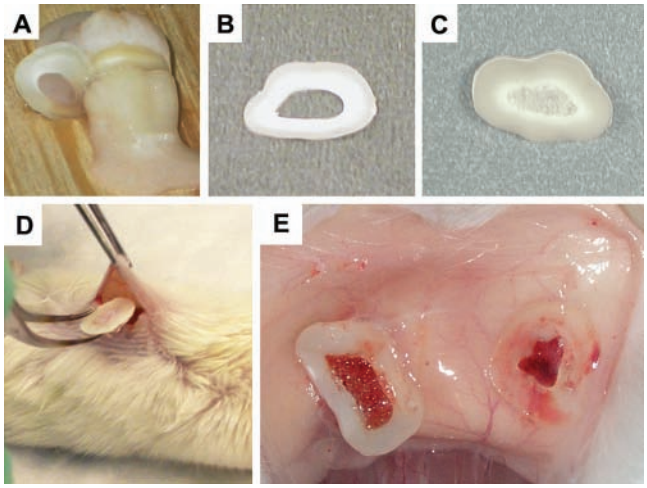

Figure 1. Tooth Slice/Scaffold Model of Dental Pulp Tissue Engineering. **(A)** A 1-mm-thick tooth slice is cut from the cervical region of a non-carious human third molar. **(B)** Tooth slice showing an empty pulp cavity after removal of the dental pulp. **(C)** A highly porous PLLA scaffold is cast within the pulp chamber. **(D)** Implantation of a tooth slice/scaffold seeded with dental pulp stem cells in the subcutaneous space of the dorsum of an immunodeficient mouse. **(E)** Bilateral tooth slice/scaffolds at the termination of the experimental period (*i.e.*, 3 wks after transplantation), showing a highly vascularized tissue in the pulp chamber.

such as odontoblasts, vascular endothelial cells, and neural cells (Gronthos *et al*., 2000; Miura *et al*., 2003; Sonoyama *et al*., 2008; Nakashima *et al*., 2009; Sakai *et al*., 2010). The exploitation of the multipotency of stem cells facilitates the pursuit of effective strategies for dental pulp tissue engineering. However, one of the critical issues that remain to be resolved is that the engineering of a complex and functional dental pulp requires a tightly controlled (in time and space) process of differentiation of stem cells into specific progenies. Studies designed to understand the regulation of dental pulp stem cell differentiation require an approach that allows for well-controlled mechanistic *in vitro* studies in combination with *in vivo* validation studies, ideally in the same experimental model. Furthermore, when one considers the significant differences observed in the way that stem cells from different species respond to stimuli, it is likely that studies with human cells would constitute the ideal approach for mechanistic experiments that are more relevant to human therapies. The tooth slice/scaffold model presented here constitutes our attempt to develop an experimental model that is suited for mechanistic and translational studies that are focused on the use of stem cells for dental pulp tissue engineering and for the understanding of dental pulp biology.

TOOTH SLICE/SCAFFOLD MODEL OF DENTAL PULP TISSUE ENGINEERING

The Tooth Slice/Scaffold Model of Dental Pulp Tissue Engineering (Cordeiro *et al*., 2008) was conceptually inspired by the Tooth Slice Organ Culture Model (Sloan *et al*., 1998) and the SCID (Severe Combined Immunodeficient) Mouse Model of Human Angiogenesis (Nör *et al*., 2001). In short, the Tooth Slice/Scaffold Model of Dental Pulp Tissue Engineering allows for the generation of a dental-pulp-like tissue *via* the transplantation of human dental pulp stem cells seeded in a biodegradable scaffold cast within the pulp chamber of a human tooth slice.

The development of this model began by the adaptation of the Tooth Slice Organ Culture Model (Sloan *et al*., 1998) for the evaluation of angiogenesis-based therapeutic strategies for revascularization of severed human dental pulps (Gonçalves *et al*., 2007; Mullane *et al*., 2008). We observed that it was possible to maintain the vitality of human tooth slices in the subcutaneous space of immunodeficient mice for several days (Gonçalves *et al*., 2007). We also observed that the application of vascular endothelial growth factor (VEGF) prior to transplantation of the tooth slices into the mouse enhanced the density of the dental pulp microvessel (Mullane *et al*., 2008). Notably, dental pulp blood vessels within the tooth slices appeared to contain blood cells, suggesting that the human vessels anastomized with the mouse vasculature. Analysis of these collective data demonstrated that it was possible to maintain human dental pulps in SCID mice and suggested that this approach could be useful for investigations of the biology dental stem cells and for studies on pulp tissue engineering.

For tooth slice/scaffolds, we prepare 1-mm-thick human tooth slices (cross-sections) from the cervical region of recently extracted non-carious human third molars (Fig. 1A). This tends to be the largest area of the pulp chamber, reaching volumes of 29-43 mm^3 in 1-mm-thick slices (Mullane *et al*., 2008). After the pulp tissue is carefully removed with forceps, without touching the predentinal walls (Fig. 1B), the pulp cavity is filled with a porogen (*e.g.*, sodium chloride with particles ranging from 250-450 μm in diameter) and with poly-L-lactic acid (PLLA) dissolved with chloroform, as described previously (Nör *et al*., 2001; Cordeiro *et al*., 2008). After 24 hrs, the salt is leached out with distilled water, and a highly porous biodegradable scaffold is observed in the pulp chamber. The goal here is that the PLLA scaffold occupies the entire space of the pulp cavity to ensure proximity between transplanted stem cells and the predentin (Fig. 1C). The tooth slice/scaffolds are disinfected and hydrophilized with sequential incubations in decreasing concentrations of ethanol (100-70%), then washed with sterile PBS overnight, at 4°C.

Stem cells from exfoliated deciduous teeth (SHED; Miura *et al*., 2003) or dental pulp stem cells from permanent teeth (DPSC; Gronthos *et al*., 2000) are re-suspended in a 1:1 mixture of Matrigel (BD Biosciences, Bedford, MA, USA) and cell culture medium. Tooth slice/scaffolds containing stem cells are incubated for 30 min at 37°C to allow for the setting of the Matrigel. Of note, Matrigel serves as an immediate source of growth factors and nutrients for the transplanted cells and to retain the cells in the scaffolds during the transplantation process, as demonstrated previously (Nör *et al*., 2001). Negative controls are typically tooth/slice scaffolds containing a 1:1 Matrigel/culture medium mixture devoid of cells. Tooth slices in which the pulp tissue was not removed are frequently used as positive controls.

For study of the fate of the dental pulp stem cells after transplantation, cells can be stably transduced with LacZ with a

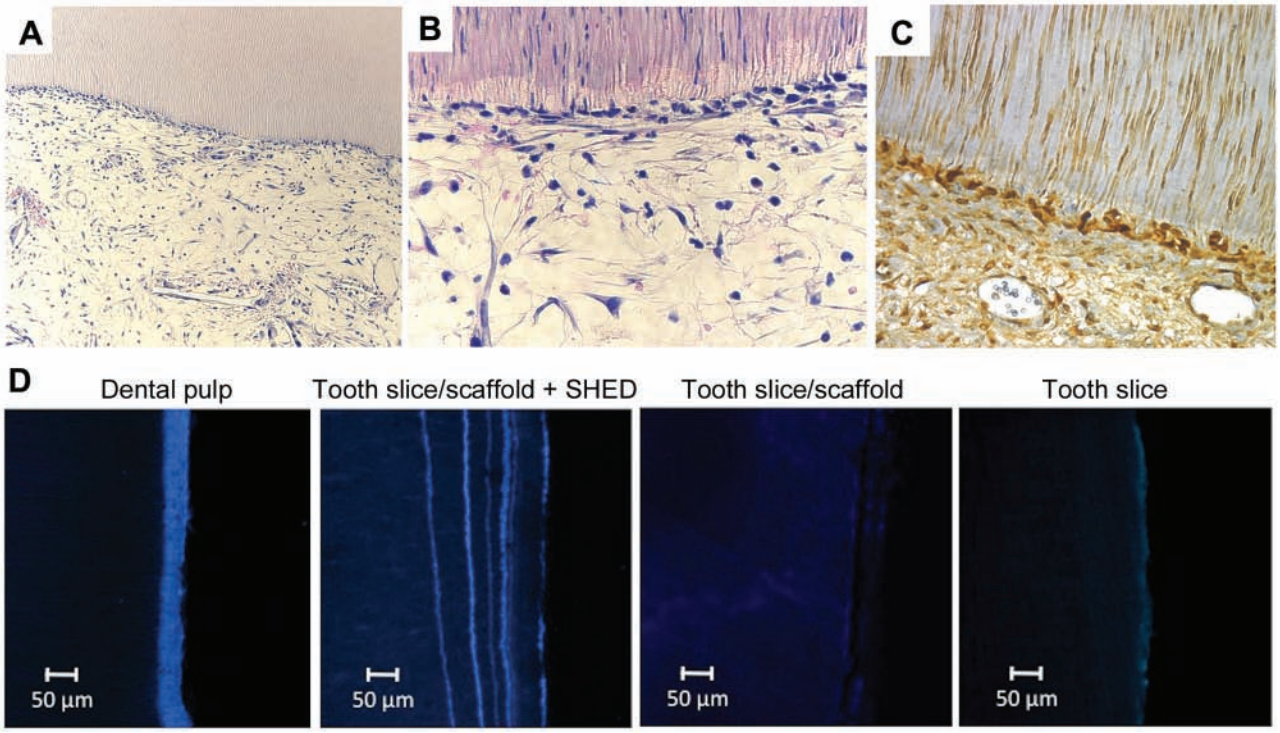

Figure 2. Dental pulp stem cells differentiate into dentinogenic cells. **(A,B)** Representative photomicrographs of dental pulps engineered with stem cells from human exfoliated primary teeth (SHED) seeded in a tooth slice/scaffold and transplanted into an immunodeficient mouse. Hematoxylin and eosin staining at 200x (A) and 400x (B). **(C)** Cells aligned close to the pre-dentin showed strong staining for human DSP (brown) at 400x. **(D)** Photomicrographs of confocal microscopy performed in tooth slices retrieved from immunodeficient mice 32 days after transplantation. The experimental conditions were as follows: dental pulp (tooth slice of a freshly extracted non-carious human molar from which the pulp was not removed); tooth slice/scaffold + SHED (tooth slice/scaffold seeded with SHED); tooth slice/scaffold (tooth slice/scaffold without SHED); and tooth slice (tooth slice without scaffold and without SHED). All mice received 4 to 5 intraperitoneal injections of 41.6 nmol/g of body weight of tetracycline hydrochloride.

retroviral vector and selected with G418 sulfate, as described previously (Cordeiro *et al*., 2008; Sakai *et al*., 2010). As empty retroviral vector controls, dental pulp stem cells can be transduced with pLXSN and selected (Nör *et al*., 1999). This constitutes a powerful approach for studies on the differentiation potential of the stem cells transplanted into the tooth slices, and has already led to the unexpected observation that dental pulp stem cells can differentiate into functional human blood vessels that anastomize with the mouse vasculature (as discussed below). The tooth slice/scaffolds containing stem cells and their controls are typically implanted bilaterally in the subcutaneous tissue of the dorsum of 5- to 7-week-old SCID mice (Figs. 1D, 1E). Tooth slice/scaffolds can be retrieved 14-32 days after transplantation and processed for histology, immunohistochemistry, and confocal and/or electron microscopy. The entire pulp tissue, or selected cell populations, can be microdissected by Laser Capture Microscopy (LCM), and analyzed for gene expression by PCR methods (Kaneko *et al*., in press). Alternatively, tooth slice/scaffolds seeded with cells transduced with LacZ can be processed for β-galactosidase staining for evaluation of the fate of transplanted stem cells.

We believe that the Tooth Slice/Scaffold Model provides a valuable approach to study the biology of dental pulp stem cells, as well as to examine mechanisms underlying dentin and pulp regeneration. It may also allow for the study of molecular and cellular events involved in the decisions of dental pulp stem cell fate. Notably, the same experimental platform (*i.e.,* the tooth slice/scaffold) has been successfully used for parallel mechanistic *in vitro* and translational *in vivo* studies (Casagrande *et al*., 2010; DeMarco *et al*., 2010; Sakai *et al*., 2010). This constitutes a unique approach for the validation of bench results in preclinical trials performed in an animal model with human cells and human tooth structures.

ODONTOBLASTIC DIFFERENTIATION OF DENTAL PULP STEM CELLS

We consistently observe that the tissue generated in the pulp chambers of tooth slice/scaffolds seeded with dental pulp stem cells is well organized (Figs. 2A, 2B), showing architectural and histological characteristics that closely resemble those of human dental pulps (Cordeiro *et al*., 2008). Ultrastructural analysis showed that cells adjacent to the predentin of tooth slice/scaffolds seeded with SHED present eccentric polarized nuclei positioned at the basal part of the cell body, well-developed Golgi's complex, and rough endoplasmic reticulum with multiple cytoplasmic vesicles, suggesting that these cells are actively engaged in secretory activity (Cordeiro *et al*., 2008).

Cell differentiation involves the progressive specialization of cell morphology and function that ultimately results in specialized cells. This process is mediated by exquisitely regulated differential expression of specific genes in a time- and space-specific manner. Odontoblasts secrete type I collagen and other non-collagenous proteins, such as osteonectin, osteopontin, osteocalcin, bone sialoprotein, dentin matrix proteins (DMP)-1, -2, and -3, dentin sialoprotein (DSP), and dentin phosphoprotein (DPP) (Butler and Ritchie, 1995; Papagerakis *et al*., 2002; Arana-Chavez and Massa, 2004; Priam *et al*., 2005; Narayanan *et al*., 2006). These matrix proteins have been extensively used as differentiation markers for odontoblastic cells (Gronthos *et al*., 2000; Miura *et al*., 2003; Alliot-Licht *et al*., 2005; Liu *et al*., 2005). We have observed a certain concentration of DSP staining in the cells that are close to the predentin in dental pulps engineered with SHED (Fig. 2C). However, it is becoming increasingly evident that no single marker can be used individually as an unequivocal demonstration of odontoblastic differentiation, since most of these proteins are also expressed by other cell types (*e.g*., osteoblasts).

It is well known that dental pulp cells are capable of differentiating into mineralizable nodule-forming cells (Tsukamoto *et al*., 1992; About *et al*., 2000). Alkaline phosphatase is believed to play an important role in the initiation of connective tissue mineralization by allowing for local enrichment of inorganic phosphate, which leads to hydroxyapatite nucleation. Using an alkaline phosphatase assay, we evaluated the mineralization potential of SHED when cultured in inductive medium containing dexamethasone, ascorbic acid, and β-glycerophosphate (Sakai *et al*., 2010). The first signs of mineralization were detected in the second week of culture, and gradually increased up to 35 days. No sign of mineralization was observed in the cells cultured with regular cell culture medium at any time-point. This study confirmed previous observations that dental pulp stem cells can be induced to differentiate into cells that secrete mineralizable matrices. However, this analysis does not constitute definitive proof of odontoblastic differentiation, since other cell types can certainly have high mineralization potential in response to appropriate stimuli.

In vivo, it has been shown that dental pulp stem cells mixed with hydroxyapatite/tricalcium phosphate and transplanted into immunodeficient mice generate tooth-like structures within 6 wks (Gronthos *et al*., 2000). When DPSC were seeded onto human dentin surface and implanted into immunodeficient mice, scattered reparative dentin-like tissues were deposited onto the dentin surface (Batouli *et al*., 2003). Previous work from our laboratory provided initial evidence that SHED can differentiate into odontoblast-like cells *in vivo* (Cordeiro *et al*., 2008). However, we believe that the definitive proof that a stem cell has differentiated into an odontoblast is the demonstration that this cell is capable of generating tubular dentin.

The ability of SHED to differentiate into odontoblasts was evaluated through the combined use of confocal microscopy and light microscopy in tooth slices implanted into mice that were injected with tetracycline (Sakai *et al*., 2010). It is well known that tetracycline stains structures that are actively undergoing mineralization (Iinuma *et al*., 2002). It incorporates itself into mineralizing tissues (Sánchez *et al*., 2004) and generates auto-fluorescence that can be detected by confocal microscopy. Confocal microscopy revealed well-defined lines in the tooth slice/scaffolds seeded with SHED (Fig. 2D), with the number of fluorescent lines coinciding with the number of injections of tetracycline. In control tooth slices containing human dental pulps, barely distinguishable successive labeled lines of tetracycline were observed (Fig. 2D). Analysis of these data indicates that SHED differentiated into cells that secrete a mineralizable matrix at a rapid pace in the engineered dental pulp (Sakai *et al*., 2010). In contrast, control tooth slices containing human dental pulps showed a slower pace of dentin formation. We hypothesize that the variation observed in the dentin deposition rate between the engineered pulp and the normal pulp (control) might be related to the fact that the odontoblasts from these two tissues are in distinct phases of their life cycle (Sloan and Smith, 2007; Larmas, 2008). In other words, while the odontoblasts differentiated from SHED would be "younger" cells with high secretory activity, the odontoblasts present in the control dental pulps would be "older", less active cells (Sakai *et al*., 2010). Light microscopic analysis of the area of dentin within the tetracycline lines showed unequivocally that the dental pulp tissue engineered with SHED generated tubular (not osteoid) dentin (Sakai *et al*., 2010).

Collectively, these studies revealed that dental pulps engineered with SHED within tooth slices generate tubular dentin *via* a process that appears to mimic primary or secondary dentinogenesis, in which the dentin is deposited layer by layer, following a centripetal growth pattern. We believe that these studies demonstrated that SHED indeed differentiate into fully functional odontoblasts capable of recapitulating the process of dentinogenesis observed during tooth development.

Notably, the localization of the stem cells within the dental pulp plays an important role in the process that results in odontoblastic differentiation. It has been shown that pulp cells seeded onto dentin surfaces established an odontoblast-like morphology with cytoplasmic processes extending into dentinal tubules (Huang *et al*., 2006). It is also known that precursor cells align perpendicularly in the periphery of the pulp chamber, and the daughter cells adjacent to the basement membrane undergo terminal odontoblastic differentiation (Goldberg and Smith, 2004). Analysis of such data suggests that dentin-derived signaling events are critical for the terminal differentiation of stem cells into odontoblasts. Indeed, it has become increasingly evident that a milieu of growth factors and bioactive molecules sequestered within the dentin matrix provides critical signals for the induction of odontoblast differentiation and dentinogenesis (Smith *et al*., 1990; Tziafas *et al*., 2000; Goldberg and Smith, 2004; Graham *et al*., 2006). We have recently shown, with the tooth slice/scaffold model, that dentin-derived morphogenic signals are necessary and sufficient to induce the differentiation of SHED into odontoblasts (Casagrande *et al*., 2010). Notably, bone morphogenetic protein (BMP)-2 (but not BMP-7) inhibition prevented the dentin-induced differentiation of SHED into odontoblasts, suggesting a critical role for dentin-derived BMP-2 in this process (Casagrande *et al*., 2010).

The understanding of mechanisms underlying the processes that result in odontoblastic differentiation of stem cells is critical for the field of dental tissue engineering. Such knowledge is

certainly a prerequisite for the translation of stem-cell-based tooth regeneration to the clinic. We believe that *in vitro* experiments using the tooth slice/scaffold model, combined with the use of the same experimental template in *in vivo* studies, constitute an important addition to the experimental armamentarium for research on mechanisms underlying the odontoblastic differentiation of stem cells, and should accelerate the process of translation of this knowledge from the "bench to chairside".

ENDOTHELIAL DIFFERENTIATION OF DENTAL PULP STEM CELLS

The success of most tissue-engineering efforts relies on the establishment of an adequate blood supply to allow for the survival, proliferation, and differentiation of transplanted cells (Brey *et al*., 2005). Indeed, without the rapid establishment of an effective vascular network, most cells will not survive the early post-transplantation stage. Notably, an adequate local oxygen and nutrient supply is critical for sustaining the high metabolic activity of cells that are engaged in tissue regeneration (Iohara *et al*., 2008; Nakashima *et al*., 2009). Due to the anatomical characteristics of the root canal, the development of strategies that provide the rapid establishment of a functional vascular network is a major challenge in the field of dental pulp tissue engineering. Ideally, one would like to see the rapid differentiation of a subpopulation of stem cells into endothelial cells to establish a vascular network in the engineered pulp, and then the differentiation of stem cells into functional, dentin-making odontoblasts. The tooth slice/scaffold model proved to be a versatile experimental approach to evaluate mechanisms regulating the endothelial and the odontoblastic differentiation of dental pulp stem cells (Sakai *et al*., 2010).

The angiogenic potential of specific cell populations can be studied *in vitro* with capillary tube formation assays (Hirschi *et al*., 2008). Although the capillary sprouting assay in 3-D collagen matrices has been typically used for analysis of the angiogenic phenotypes of endothelial cells (Nör *et al*., 1999), this assay is also useful for evaluation of the endothelial differentiation potential of stem cells (Sakai *et al*., 2010). Vascular endothelial growth factor (VEGF) is the prototypic pro-angiogenic factor, and studies have shown that VEGF induces stem cell differentiation into endothelial cells (Oswald *et al*., 2004; Jazayeri *et al*., 2008). VEGF induced dental pulp stromal stem cells (DP-SC) to acquire endothelial-cell-like features when cultured in a 3-D fibrin mesh, displaying focal organization into capillary-like structures (Marchionni *et al*., 2009). We observed that SHED cells cultured in collagen matrices are capable of organizing themselves into capillary structures that resemble microvessels (Figs. 3A, 3B). VEGF significantly enhanced the numbers of capillary-like structures, as compared with controls (Sakai *et al*., 2010). Analysis of these data supported the notion that VEGF enhances the angiogenic potential of dental pulp stem cells.

However, more definitive evidence for the differentiation of dental pulp stem cells into endothelial cells was provided by the detection of endothelial-cell-specific markers and the formation of functional blood vessels *in vivo* in the tooth slice/scaffold method (Sakai *et al*., 2010). Endothelial differentiation is typically detected through the analysis of markers such as platelet endothelial cell adhesion molecule 1 (PECAM-1), vascular endothelial growth factor receptor-2 (VEGFR2), tyrosine kinase receptor Tie-2, vascular cell adhesion molecule-1 (VCAM1), intercellular adhesion molecule-1 (ICAM-1), and vascular endothelial cadherin (VE-cadherin) (Jazayeri *et al*., 2008; Marchionni *et al*., 2009; Sakai *et al*., 2010). We have recently analyzed the effect of VEGF on SHED cultured in tooth slice/scaffolds (Sakai *et al*., 2010). VEGFR2 expression was observed after the first day of $rhVEGF_{165}$ stimulation. After 28 days in culture, SHED cultured in tooth slice/scaffolds and exposed to $rhVEGF_{165}$ began to express PECAM-1 and VE-cadherin. Analysis of these data suggested that SHED progressively acquired an endothelial-like phenotype. Notably, VEGFR2 is considered a marker of early stages of differentiation, while PECAM-1 and VE-cadherin tend to be expressed in more mature endothelial cells (Kim *et al*., 2008).

In our experience, the expression of the 3 markers of endothelial differentiation (*i.e.,* VEGFR2, PECAM1, and VE-cadherin) occurred only when SHED were cultured in the tooth slice/scaffolds in the presence of medium supplemented with $rhVEGF_{165}$ (Sakai *et al*., 2010). Analysis of these data suggested that angiogenic factors derived from the intact dentin are not sufficient to induce the angiogenic differentiation of SHED, at least in this model. This appears to be in contrast to the process leading to the acquisition of odontoblastic phenotypes by SHED, in which dentin-derived signals were sufficient to induce differentiation (Casagrande *et al*., 2010). From a translational standpoint, these observations support the concept of local delivery of VEGF as a means to enhance the angiogenic potential of SHED used in dental pulp tissue engineering.

The tooth slice/scaffold model was used to test the potential of stem cells to differentiate into blood-vessel-forming endothelial cells *in vivo*. For this purpose, it was imperative that the transplanted cells be distinguishable from the host vascular cells. One such way is to transplant genetically tagged cells into the animal (*e.g*., GFP, LacZ). This strategy allows for the unequivocal determination of the presence of transplanted stem cells lining the walls of blood vessels (Hirschi *et al*., 2008; Sakai *et al*., 2010). It also allows for the evaluation of the functionality of the stem-cell-derived blood vessels, as determined by the presence (or not) of blood cells in the lumen. A functional engineered blood vessel requires that the stem cells align themselves as capillaries, and find a way to connect with the host vasculature through the process of anastomosis. We reported that human endothelial cells transplanted into mice generate functional human blood vessels that undergo a process of maturation, as determined by the gradual investment by host pericytes (Nör *et al*., 2001). Our group has also reported the presence of LacZ-positive cells (*i.e.,* transplanted SHED) lining the walls of capillaries in the pulp tissue engineered within tooth slice/scaffolds (Cordeiro *et al*., 2008; Sakai *et al*., 2010).

The fate of SHED seeded into tooth slice/scaffolds and transplanted into mice was evaluated through the use of cells stably transduced with LacZ (Figs. 3C-3E) (Cordeiro *et al*., 2008; Sakai *et al*., 2010). Beta-galactosidase-positive capillaries containing blood cells in the lumen were observed in the engineered dental

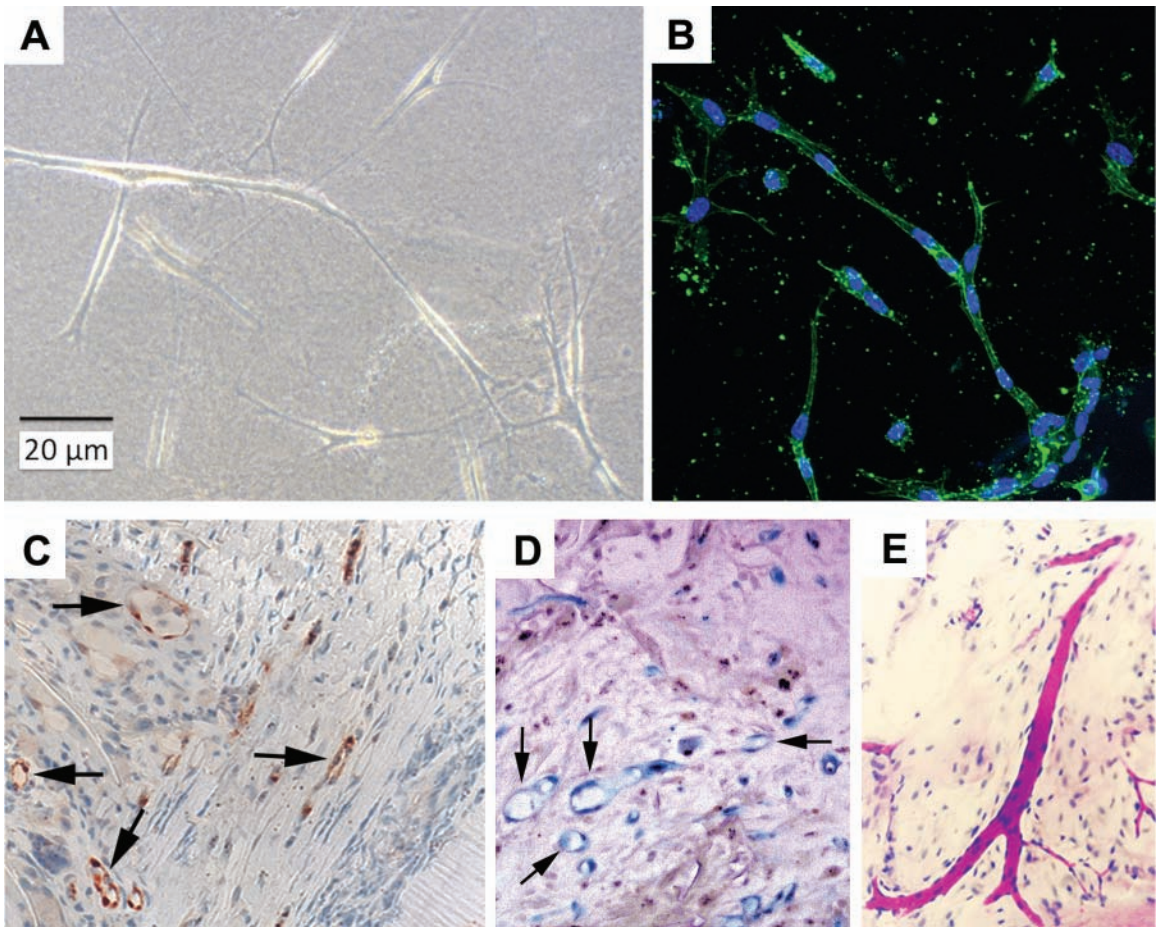

Figure 3. Dental pulp stem cells differentiate into vasculogenic cells. **(A)** Photomicrograph of SHED cells cultured in 3-D collagen type I matrices and stimulated with rhVEGF$_{165}$. **(B)** Confocal microscopy of SHED cells cultured in 3-D matrices and stimulated with rhVEGF$_{165}$. **(C)** Immunohistochemistry for human Factor VIII, a histological marker for identification of blood vessels at 200x. Arrows point to Factor VIII-positive (red) blood vessels. **(D)** SHED stably transduced with LacZ were seeded in tooth slice/scaffolds and implanted into immunodeficient mice. After 21 days, tooth slice/scaffolds were retrieved, demineralized, and stained with x-gal. Arrows point to β-galactosidase-positive blood vessels (blue) in the engineered tissue, confirming that the SHED cells differentiated into endothelial cells *in vivo*. Representative photomicrograph of hematoxylin-and-eosin-stained tissue section at 400x. **(E)** Blood vessel sectioned longitudinally within a dental pulp engineered within a tooth slice/scaffold seeded with SHED (200x).

pulps (Fig. 3D). We speculate that the sources of the angiogenic factors (*e.g.*, VEGF) required for the differentiation of SHED into endothelial cells are the surrounding dentin (Roberts-Clark and Smith, 2000) and host cells (Tran-Hung *et al*., 2006, 2008). Notably, areas within tissue-engineering constructs experience local hypoxia, which is a very well-described physiological condition in which angiogenesis is stimulated through the activation of transcriptional factors (*e.g.*, HIF-1 alpha) and induction of VEGF expression (Maxwell and Ratcliffe, 2002). Our laboratory is currently working on the optimization of strategies to allow for the use of the scaffold as a slow-release device for rhVEGF in attempt to accelerate the process of endothelial differentiation of SHED in engineered dental pulps.

CONCLUSIONS

The tooth slice/scaffold model was the methodological approach used to demonstrate that human dental pulp stem cells transplanted into immunodeficient mice differentiate into functional human odontoblasts capable of making tubular dentin, and functional vascular endothelial cells that organize themselves into blood vessels that anastomize with the host vasculature and carry blood (Cordeiro *et al*., 2008; Sakai *et al*., 2010). These results demonstrate that the tooth slice/scaffold model is uniquely suited for mechanistic and translational studies that aim at the understanding of the differentiation potential of dental pulp stem cells. Analysis of these data also suggests that dental pulp tissue engineering with stem cells may become an alternative strategy for the revitalization of necrotic immature permanent teeth, allowing for completion of vertical and lateral root formation.

We believe that the tooth slice/scaffold model constitutes a powerful approach for detailed studies of many aspects relevant to dental pulp tissue engineering, as follows:

(A) It is suitable for studies designed to understand mechanisms involved in dental pulp stem cell differentiation into odontoblasts and endothelial cells, as shown previously (Casagrande *et al*., 2010; Sakai *et al*., 2010).

(B) It allows for genetic manipulation of the stem cells *in vitro* prior to transplantation into the mice (Bento *et al*., unpublished observations). This constitutes a relatively easy approach for the evaluation of the biological consequences of these gene expression changes on the differentiation potential of stem cells, as compared with alternative methods such as the use of transgenic mice.

(C) It allows for the evaluation of cell fate by the transplantation of cells stably tagged with a marker, such as LacZ (Cordeiro *et al*., 2008; Sakai *et al*., 2010).

(D) The tooth slice/scaffold is suitable for the testing of the impact of scaffold design and composition on the differentiation of stem cells and generation of a dental pulp tissue, as shown (DeMarco *et al*., 2010).

In addition, the tooth slice/scaffold model has features that enhance the translational potential of the studies. It involves both human dentin and human pulp stem cells, and it utilizes the same conceptual framework for parallel *in vitro* and *in vivo* studies (*i.e.,* the tooth slice/scaffold).

In summary, the tooth slice/scaffold model constitutes a powerful and nimble approach for mechanistic studies designed to understand the processes of dental pulp stem cell differentiation. It allows for the evaluation of the impact of biological processes that regulate stem cell differentiation on the formation of tubular dentin and on the vascularization and innervation of engineered pulp tissues. We believe that the development and characterization of pre-clinical experimental models specifically designed for dental pulp tissue regeneration studies are required for the understanding of the biology of dental pulp stem cells and may ultimately define the long-term prospectus of the field of regenerative endodontics.

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