

## Dental pulp and dentin tissue engineering and regeneration: advancement and challenge

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### 1. ABSTRACT

Hard tissue is difficult to repair especially dental structures. Tooth enamel is incapable of self-repairing whereas dentin and cementum can regenerate with limited capacity. Enamel and dentin are commonly under the attack by caries. Extensive forms of caries destroy enamel and dentin and can lead to dental pulp infection. Entire pulp amputation followed by the pulp space disinfection and filled with an artificial rubber-like material is employed to treat the infection -- commonly known as root canal or endodontic therapy. Regeneration of dentin relies on having vital pulps; however, regeneration of pulp tissue has been difficult as the tissue is encased in dentin without collateral blood supply except from the root apical end. With the advent of modern tissue engineering concept and the discovery of dental stem cells, regeneration of pulp and dentin has been tested. This article will review the recent endeavor on pulp and dentin tissue engineering and regeneration. The prospective outcome of the current advancement and challenge in this line of research will be discussed.

### 2. INTRODUCTION

Tooth enamel is incapable of self-repairing whereas dentin and cementum have limited capacity to regenerate. Tooth is the only organ that penetrates from the host internal tissue through the “oral integumentary layer” into the external environment – the oral cavity, which is a completely wet environment. Although the mucosal system is heavily protected by a strong immune defense mechanism, tooth is vulnerable to microbial invasion. When invasion occurs and manifests as a dental caries, the caries may become a gateway into the host internal tissues. Root canal infection develops if the caries is extensive and the infection often spreads into the jawbone. Before any microbial invasion is taken place, the root canal is occupied by dental pulp tissue whose main function is to produce dentin and maintain the biological and physiological vitality of the dentin. Additionally, it possesses highly responsive sensory nervous system that generates unbearable pain when tooth is inflicted by mechanical trauma, chemical irritation, or microbial invasion. Due to the small volume of the pulp tissue encased in dentin with

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blood supply, only from one opening end at the root apex, when pulp tissue is infected, it is difficult for the immune system to eradicate the infection without collateral blood supply.

Tooth is built by cells originated from both ectoderm and mesoderm. Enamel is made by ameloblasts derived from ectoderm and these cells are no longer present after the hard tissue formation is complete. Dentin is produced by odontoblasts derived from ectomesenchyme and these cells continue to exist in the pulp throughout life span. Recent advances in tissue engineering have drawn scientists to test the possibility of regeneration a whole tooth or part of the tooth structure (1-4). Tooth is a complex organ consisting of highly organized structures with various but specific shapes. It develops within the jawbone and after the eruption its root(s) are firmly anchored in the alveolar bone proper. Between the tooth and bone, there is a thin layer of membrane termed periodontal ligament (PDL), which plays a pivotal role in providing multiple functions to support the tooth including serving as a shock absorber or a cushion to take up forces transmitted via the tooth. Losing the PDL will lead to ankylosis and the root will be replaced by bone (5). Whole tooth engineering and regeneration is difficult and the technology is still at its very infancy (6-10).

When tooth is damaged but still repairable, regeneration of parts of the tooth structure can prevent or delay the loss of the whole tooth. Partially removing the infected pulp, a procedure termed partial pulpectomy, has been proven ineffective that infection may still be left behind. Therefore, this approach has not been a popular clinical practice. When pulp is diagnosed with irreversible pulpitis, i.e., no treatment can reverse the situation, regardless of the amount of remaining normal pulp tissue, the entire pulp is amputated by pulpectomy. The pulp space is then disinfected and replaced with a rubber-like material - gutta percha. This treatment protocol commonly known as root canal or endodontic therapy has been a common clinical practice for decades.

Regeneration of dentin relies on having vital pulps; however, regeneration of pulp tissue has been difficult as the tissue is encased in dentin without collateral blood supply except from the root apical end. Attempts to regenerate pulp tissue have been a long quest. With the advent of modern tissue engineering concept and the discovery of dental stem cells, regeneration of pulp and dentin has been tested. Moony and Rutherford [6-8] led the first team that initiated the testing of pulp tissue engineering (11-13). This endeavor halted due to the lack of isolation and characterization of pulp stem cells that potentially may differentiate into odontoblasts. Regenerated pulp tissue should be functionally competent, e.g., capable of forming dentin to repair lost structure. Reports have shown that isolated pulp cells can be induced to differentiate into odontoblast-like cells and generate dentin-like mineral structure *in vitro* (14-15). The *in vivo* evidence of pulp cells capable of generating dentin was demonstrated by Gronthos *et al.* (16) that pieces of human pulp/dentin complex can be formed ectopically in

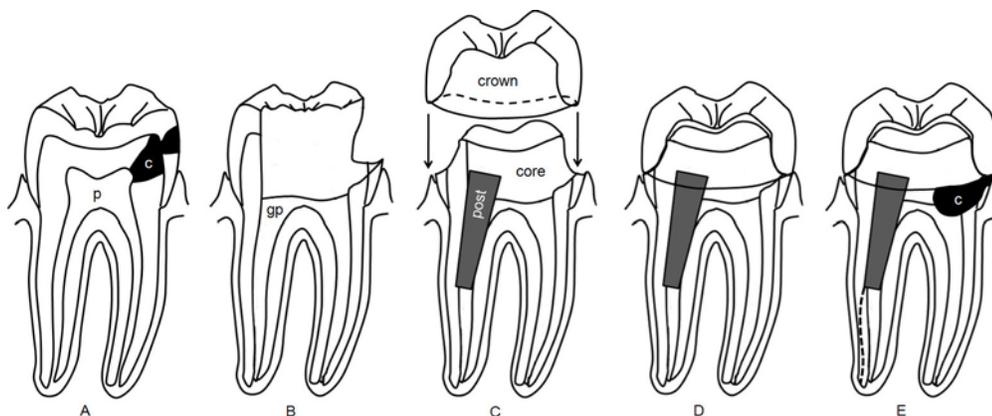
immunocompromised mice (16). This discovery has promoted the investigation on the stem cell-based regenerating pulp/dentin for clinical applications.

## 3. THE DIFFICULTY OF ENGINEERING AND REGENERATION OF A WHOLE TOOTH

Losing teeth leads to multiple consequences, although it is not life threatening. In an advanced society, lacking quality of life such as caused by missing teeth is an undesirable situation. Artificial dentures, especially the removable type, are a primitive approach trying to solve the missing teeth problem. Currently, the advancement of dental implants has provided a great service to those with missing teeth. However, similar to any artificial prosthesis, dental implants can never replace the functions and physiological normalcy of natural teeth (17). The fundamental pitfall is the lack of a natural structural relationship with the alveolar bone, i.e., absence of PDL. In fact, it requires a direct integration with bone onto its surface, which is prerequisite for success, an unnatural relation with bone as compared to a natural tooth. Furthermore, the unnatural contours and its structural interaction with the supporting structures create other problems, such as severe food impaction and peri-implantitis (18).

The alternative is tooth regeneration. As mentioned, the complexity of the tooth in terms of its structure and anatomical location makes it difficult to engineer and regenerate. Tooth is in fact a continuous structure of the jaw alveolar bone during and after its formation. This continuity is evidenced by two pieces of tissues associated with the tooth: pulp and PDL. Extracting the tooth out of the socket severs the continuity, causing a permanent damage to the pulp as well as various levels of damage to the PDL. The outcome of PDL healing depends on how quickly the tooth is replanted back to the socket. If replanting the tooth immediately back to the socket after extraction, the PDL is likely to recover. Any loss of viability of PDL due to drying or other damage, ankylosis will occur after replantation and the root will eventually be replaced by bone (replacement resorption). The blood and nerve supply of the pulp tissue is completely severed after extraction. If the tooth is matured with a small canal foramen at the apex, replantation is not likely to restore the vascularity or innervations of the pulp, therefore, the pulp will undergo necrosis. If the tooth is immature with a wide opening apex, there will have various extents of revascularization and reinnervation. However, the long-term recovery of the pulp tissue is usually poor and resulting in calcification. Because of these reasons, transplantation of a tooth from another site to replace a missing tooth cannot lead to a favorable outcome. These characteristics further indicate the inseparable structural nature between teeth and their supporting tissues.

Therefore, to engineer and regenerate a completely functional tooth with normal anatomic relationship to the alveolar bone, it needs to start from the very beginning of the tooth development – tooth germ or bud stage. Using isolated tooth bud cells and engineering



**Figure 1.** Tooth structure loss resulting from caries, root canal treatment and restoration. (A) Tooth is decayed (c) causing irreversible pulpitis that requires root canal therapy. p, pulp. (B) Tooth decay is completely removed and the root canal space enlarged (cleaned and shaped) and filled with gutta percha (gp). (C) A post is placed in a canal and core material placed to fill the coronal space. The natural crown is then prepared/cut into a specific configuration and an artificial crown is manufactured for insertion. (D) The artificial crown is cemented onto the tooth to restore its morphology and function. (E) If without rigorous care, tooth decay can recur. Tooth may also undergo fracture (dashed line) due to loss of structure that weakens its capacity to withstand mechanical stress. In this condition, the tooth is not salvageable and must be extracted.

technologies, tooth regeneration has been tested ectopically and orthotopically. In animal study models, cells isolated from tooth buds can be seeded onto scaffolds and form ectopic teeth *in vivo* (6, 19-21). Nakao *et al* reported engineering of teeth ectopically followed by transplantation into an orthotopic site in the mouse jaw (21). Tooth regeneration at orthotopic sites using larger animals such as dogs and swine has been tested (22-23). The study in dog failed to demonstrate root formation (22), while the swine model was able to show root formation with a 33.3% success rate (23). To ensure that the regenerated tooth is integrated with bone, an approach using hybrid tooth-bone constructs to engineer and regenerated tooth was developed and tested but much improvement is needed before a clinical application can be foreseen (7, 24). To date, the largest animal model used for tooth regeneration is minipig. The whole tooth may be regenerated in minipig jaws and reaches eruption (unpublished data presented by Dr. T.-F. Kuo, Taipei, Taiwan, 2009). However, the direction of the eruption is uncontrollable, i.e., the regenerated tooth may become impacted. The most difficult part of tooth regeneration lies in the lack of the source of autologous tooth bud cells. Unless the tooth buds (3<sup>rd</sup> molars) are removed and stored beforehand, no autologous tooth bud cells can be available when tooth regeneration is needed.

#### 4. PULP/DENTIN REGENERATION TO PREVENT TOOTH LOSS

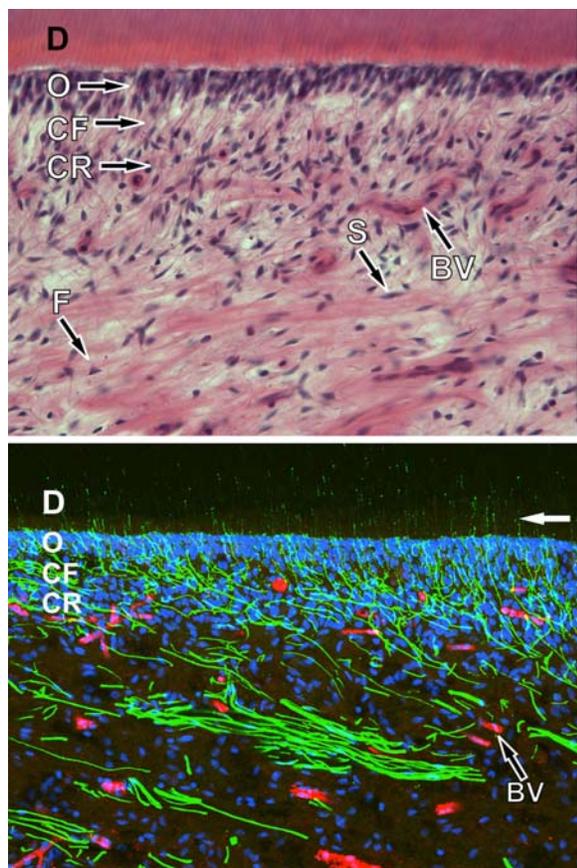
The potential of pulp tissue to self-regenerate lost dentin is well known. When pulp tissue is exposed due to the loss of the overlying dentin, direct pulp capping therapy can allow the pulp to form new dentin, which is termed dentin bridge. Using various cement-based materials for pulp capping such as calcium hydroxide and mineral trioxide aggregate (MTA) has been well documented and studied (25-26). When the tooth is further damaged, self-

regeneration of dentin becomes difficult, as it needs a healthy pulp, which may be compromised by the disease.

To remove the infection, first the entire pulp tissue has to be removed (pulpectomy). Subsequently, in order to thoroughly disinfect and fill the canal with gutta percha, the root canal space must be enlarged significantly causing further tooth structure loss (Figure 1). Although studies have shown that the success rate of endodontic treatment is relatively high (78%-98%) (27-30), other ramifications are normally not taken into the consideration in measuring the success. For example, i) endodontic procedures are technically sensitive that mishaps occur, including blockage of the root canal space, breakage of instruments in the canals, perforations, etc. (31-36); ii) after endodontic treatment of the immature tooth, no additional dentin tissue can be gained leaving behind a weak tooth susceptible to fracture from traumatic injuries (37-41); iii) significant amount of tooth structure is lost from endodontic treatment and more so from the subsequent restorative procedures (Figure 1) (42-44); and iv) pulpless teeth have no sensation to irritations, rendering caries progression unnoticed by patients. Long-term studies have shown that tooth loss is higher for endodontically treated teeth than non-treated due to secondary caries and complex restoration associated problems (45-48).

Therefore, if lost pulp and dentin can be regenerated via tissue engineering process as part of endodontic therapy, decayed tooth is likely to defer the fate of extraction. Depending on the clinical situations, two types of pulp regeneration can be considered: i) Partial pulp regeneration: It has been observed that pulpal infection and inflammation is compartmentalized until the entire pulp tissue undergoes necrosis (49-50). Before the complete pulp necrosis, the remaining pulp tissue may be recoverable after disinfection and help regenerate the lost portion. To enhance the regeneration, engineered pulp tissues may be

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**Figure 2.** Micrographs of human dental pulp. (Top) Light micrograph of the pulpodentin complex from the pulp horn region of a decalcified human tooth following staining with hematoxylin and eosin. The pulpodentin complex consists of a highly differentiated tissue with a consistent morphological pattern that includes dentin and dentinal tubules (*D*), the odontoblast layer (*O*), the cell free zone (zone of Weil; *CF*), and the cell rich zone (*CR*). Other components of the pulp include Schwann cells (*S*) that enwrap nerve fibers, blood vessels (*BV*), and fibroblasts (*F*). (Bottom) Confocal micrograph of the pulpodentin complex from the pulp horn region of the same sample as seen above shows immunofluorescence for N52 (green; identifies nerve fibers) and von Willebrand factor (red; identifies endothelial cells associated with blood vessels), whereas cellular nuclei are stained with ToPro-3 (blue). The nerves fibers form an extensive plexus just below the odontoblasts. Some nerve fibers pass through the odontoblastic layer, where they enter and continue within dentinal tubules for about 100 microns (white arrow) before terminating. Both images courtesy of Dr. Michael Henry, University of Texas Health Science Center at San Antonio).

inserted into the pulp space to facilitate the entire recovery of pulp tissue and the generation of new dentin. ii) *De novo* synthesis of pulp: When the entire pulp tissue is lost, *de novo* synthesis of pulp must take place in order to regenerate the tissue. The volume of the mature pulp tissue is very small (~10-100  $\mu$ l), therefore, regeneration of pulp should be relatively simpler than that for larger organs or

tissues. However, it has been considered a difficult task to engineer and regenerate the entire pulp and its product, dentin, due to the following situations: 1) unique anatomical location of the pulp tissue - encased within dentin having mainly one apical foramen to allow angiogenesis for the engineered tissue; 2) unique microstructure of pulp tissue, i.e., different types of cells (e.g., odontoblasts) in different layers or zones and complex innervation (Figure 2); and 3) specific location of dentin located only peripherally of the pulp tissue and the highly organized dentin structure with well-aligned dentinal tubules (Figure 2).

## 5. DISCIPLINE OF TISSUE ENGINEERING FOR PULP/DENTIN REGENERATION

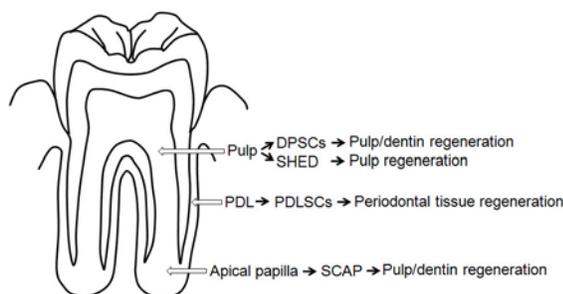
Regeneration of any tissue back to its original condition has been a long quest in any medical disciplines. To enhance the regeneration, blood clot has been used as a rich source of growth factors to help tissue repair. Creating hemorrhage to fill into the surgical site has been a routine practice for certain conditions in surgery. In endodontics, this idea was tested by Ostby in the 1960s (51) and in the 1970s by another group (52). They observed limited tissue regeneration in the canal space, and it was not pulp tissue.

The discipline “tissue engineering” emerged in the late 1980s and one of the key components of this modern concept is the utilization of synthetic biocompatible/biodegradable polymers configured as scaffolds seeded with *ex vivo* expanded viable cells (53). The scaffold provides a three-dimensional environment for cells to attach and grow, therefore mimicking the *in vivo* condition. Additionally, these synthetic matrices can be fabricated such that it may form any desired shape and carry needed growth factors to guide the process of cell differentiation and tissue formation (54-55). Generally, tissue-engineering technology involves generating tissue or organ constructs *in vitro* for subsequent implantation. The biodegradable material can be synthetic polymers, e.g., poly(D, L-lactide-co-glycolide) (PLG) or processed biological products, e.g., collagen matrix or gel (54-56).

Using this modern tissue engineering concepts, research groups led by Mooney and Rutherford tested the pulp regeneration by growing pulp cells onto a synthetic polymer scaffold of polyglycolic acid (PGA) and performed *in vitro* and *in vivo* analyses (11-13). Their approaches are a proof-of-principle to test whether cultured pulp cells can grow well and produce matrix on PGA, and whether the engineered pulp can be vascularized using *in vivo* study models. However, due to the lack of the technology to isolate stem cells in pulp tissues that can give rise to odontoblasts and make dentin *in vivo*, this line of research was discontinued until pulp stem cells were isolated and characterized.

A type of mesenchymal stem cell-like cells residing in human dental pulp was reported in a series of papers by Shi and colleagues (2000-2003). These cells were named postnatal dental pulp stem cells (DPSCs) and exhibited the ability to form pieces of ectopic human pulp

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**Figure 3.** Source of dental stem cells and use for dental tissue regeneration. Note DPSCs are from pulp of permanent teeth, SHED from exfoliated primary teeth.

/dentin-like complex as well as to form scattered dentin-like structures on existing human dentin surface in immunocompromised mice. (16, 57-58). Besides DPSCs, several other types of stem cells or progenitor cells from dental tissues have been isolated and characterized, these are stem cells from exfoliated deciduous teeth (SHED) (59), periodontal ligament stem cells (PDLSCs) (60), stem cells from apical papilla (SCAP) (10, 61) and dental follicle progenitor cells (DFPCs) (62). These different types of dental stem cells appear to be good cell sources for regenerating dental tissues (Figure 3). In the field of orofacial regeneration, these stem cells have been tested in animal models on their potential for clinical applications (3). Particularly, they promoted a new discipline in clinical endodontics, the term regenerative endodontics which focuses on the understanding of these stem cells and their use for the regeneration of endodontic tissues including pulp and dentin (17, 63-64).

### 5.1. Non-cell-based pulp/dentin tissue regeneration

For a sizable tissue defect, cell based therapy is inevitable for a complete regeneration of the lost tissue. Non cell-based approach is preferable if the tissue defect is not extensive because the utilization of cells, especially *ex vivo* expanded, as a therapeutic mode is much more complex. The source of cells is a major issue.

Application of recombinant growth factors to the injured site to enhance the regeneration of dentin has been investigated for repair of small amount of dentin lost (65). Utilizing growth factors to attract stem cells residing in the remaining pulp tissue to the defected site and to regenerate the lost part is considered a preferred approach. However, pulp tissue appears to lack the tendency to expand when there is space next to it. Our recent report showed that when pulp is partially damaged in an immature tooth with open apex, the remaining pulp tissue did not expand to the damaged site during healing process, instead, the site was filled in by periapical tissues, i.e., periodontal ligament and cementum (66) (Fig 4). This finding suggests that relying on attracting stem cells in the remaining healthy pulp tissue to the damaged site to regrow pulp using growth factors is unlikely to work. The damaged site was filled with blood clot, a rich source of growth factors, was not able to attract stem cells from the remaining pulp to the damaged site.

Evidence on more extensive regeneration using non-cell-based approach is lacking. As mentioned before, attempt to use blood clot to regenerate totally lost pulp tissue was not successful. A recent paper utilized a set of growth factors to regenerate pulp tissue without the inclusion of cells yielded formation of vascularized tissues in the root canal space (67). However, the nature of the regenerated tissue is questionable and the quality is far from being comparable to the regenerated pulp tissue using stem cell-based, described below.

### 5.2. Stem cell-based pulp/dentin tissue regeneration

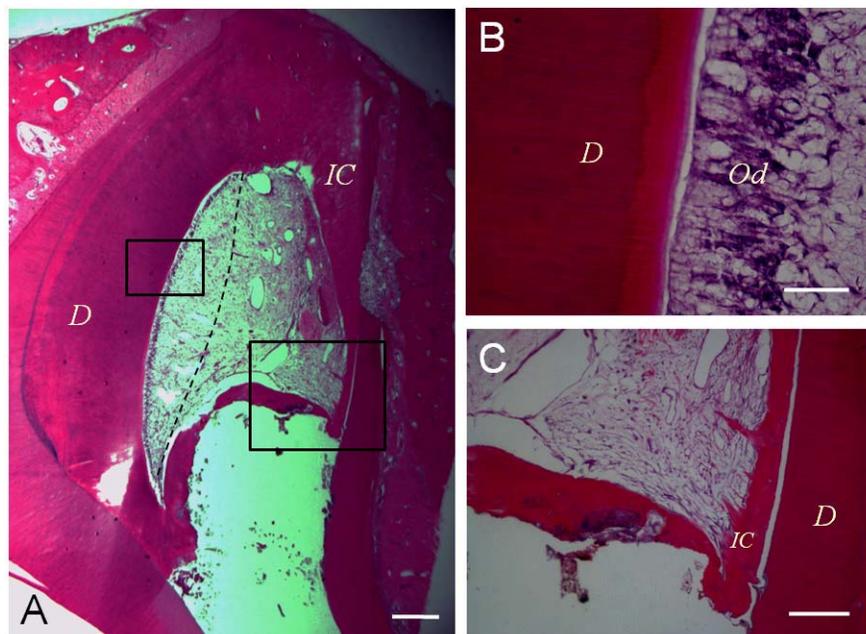
Cell-based therapy is effective for repairing defects of extensive size. Stem cell-based approach provides even more optimal results due to their potency in dividing and differentiating in response to microenvironmental cues. Because of this premise, stem cell biology has emerged as one of the fundamental underpinnings for regenerative medicine.

#### 5.2.1. Suitable cell types for pulp/dentin engineering and regeneration

DPSCs, SHED and SCAP are potentially suitable cell sources for pulp/dentin regeneration because they are derived from pulp tissue (source of DPSCs and SHED), or the precursor of pulp (source of SCAP). DPSCs and SCAP can form pieces of pulp/dentin complex when transplanted into immunocompromised mice (10, 16, 57) whereas SHED form mineralized tissue without distinct pulp/dentin complex (59). Whether other types of stem cells, such as bone marrow (BM) MSCs, can differentiate into odontoblasts and make dentin is questionable. Hu *et al* showed that mouse crude bone marrow cells rarely give rise to dental cells and only c-kit<sup>+</sup>-enriched bone marrow cells can acquire the characteristics of odontoblasts. However, this phenomenon requires the interactions between oral epithelial cells and the enriched BM cells (68). Jin's research team further investigated this issue using a rat model in the following two studies. First, they compared the odontogenic capability between BM mesenchymal stromal cells (BMSSCs) and DPSCs by co-culturing these cells with apical bud cells (ABCs). They found that recombined DPSCs/ABCs formed typical tooth-shaped tissues with balanced amelogenesis and dentinogenesis, whereas BMSSCs/ABC recombinants developed into atypical dentin-pulp complexes without enamel formation (69). The team next observed that multipotent dermal cells incubated with conditioned medium of embryonic tooth germ cells can behave similarly to DPSCs by undergoing odontogenic differentiation (70). Therefore, other sources of MSCs may be a source of dentinogenic cells when guided by an appropriate environment. Nevertheless, these findings indicate that utilizing other cell types to regenerate pulp/dentin is a less straightforward approach than using DPSCs, SCAP and SHED.

#### 5.2.2. Stem cell source

The existing of stem/progenitor cells in pulp tissue that can give rise to newly differentiated pulp cells especially the highly specialized cells odontoblasts that produce dentin has been known (3). Dental stem cells are



**Figure 4.** Lost portion of pulp is replaced by periapical tissues. A dog tooth was accessed and the pulp tissue partially removed and infected. The root canal was then disinfected and the space filled with blood clot. (A) Histologic view of the healed pulp tissue 3 months after the disinfection. The pulp tissue on the left side healed and right side of the pulp tissue was lost and the space filled in by periodontal tissue including soft connective tissue and intracanal cementum (IC). Dashed line separates the healed pulp tissue (left) and the ingrown periapical connective tissue (right). (B) Higher magnification view of the odontoblast layer (od) from the left boxed region in (A). (C) Magnified view of the right boxed region in (A). Pulp space is filled with soft connective tissue. The hard tissue IC extends from the dentinal wall toward the opposite side of the canal forming a bridge. Scale bars: 500  $\mu\text{m}$  (A); 50  $\mu\text{m}$  (B); 200  $\mu\text{m}$  (C). Adapted with permission from (66).

considered a population of MSC-like cells, therefore, markers that have been used for identifying MSCs are also used for dental stem cells, such as the positive markers: STRO-1, CD13, CD44, CD24, CD29, CD73, CD90, CD105, CD106, CD146, Oct4, Nanog, beta2 integrin etc and the negative markers: CD14, CD34, CD45, HLA-DR, etc (71-77). Like all MSCs, dental stem cells are also heterogeneous and various markers listed above may be expressed by subpopulations of these stem cells (57). Subpopulations expressing  $c\text{-kit}^+/\text{CD}34^+/\text{STRO-1}^+$  DPSCs or SHED were reported to be multipotent stem cells, although  $c\text{-kit}$  and CD34 are known to be markers for hematopoietic lineages of cells (78-79). Side population cells exist in porcine dental pulp exhibiting stem cell properties with self-renewal and multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis (80). The human counterparts of these subpopulations of DPSCs have also been reported (82).

Using STRO-1, CD146 and pericyte associated antigen (3G5) as markers, the DPSC niche in human dental pulp was found to be localized in the perivascular and perineural sheath regions (77). Isolated  $\text{STRO-1}^+/\text{CD}146^+$  DPSCs form dentin-pulp-like complex *in vivo*, similar to the multiple colony-derived DPSCs. The STRO-1 positive region in the pulp of deciduous teeth is similar to that of permanent teeth, also in the perivascular regions. STRO-1 staining of apical papilla has shown that the positive stain is

located in the perivascular region as well as other regions scattered in the tissue (10).

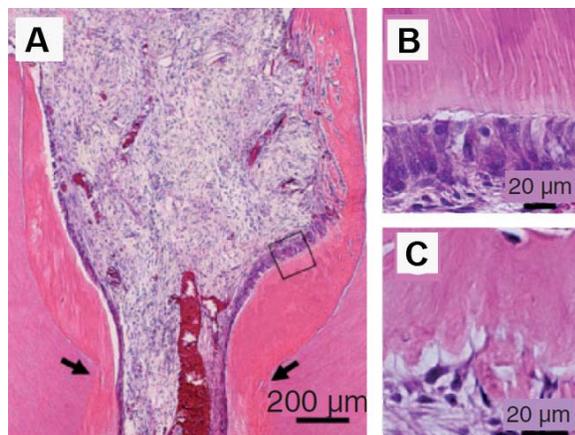
### 5.2.3. Partial regeneration of pulp

Nakashima & Akamine illustrated the possibility of generating a piece of pulp/dentin complex *in vitro* as a filling material (83). This approach appears difficult as engineering and generating a three-dimensional structure of pulp-dentin complex *in vitro* is very technically challenging. However, Nakashima's research team was able to show partial regeneration of pulp using a dog model by two approaches: i) Autologous DPSCs was grown as a three-dimensional pellet treated with the growth factor BMP-2 and implanted into the space of partially amputated pulp chamber. This approach was able to stimulate reparative dentin formation by the newly differentiated odontoblasts (84). ii) A subfraction of side population of pulp cells ( $\text{CD}31^+/\text{CD}146^-$ ) were mixed with a collagen scaffold and inserted into the pulp chamber space where tissues were removed by pulpotomy. Formation of regenerated pulp tissue with good vascularity and new dentin deposition was observed in the pulp chamber (81) (Figure 5).

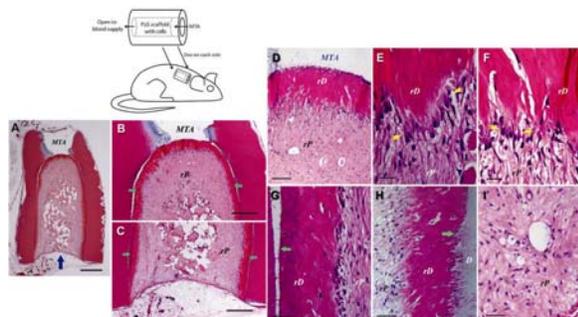
### 5.2.4. De novo regeneration of dental pulp and dentin

To regenerate pulp tissue from an empty root canal space, pulp stem cells seeded into a scaffold and inserted into the canal space appears to be a clinically practical approach. To simulate the clinical situation, a

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**Figure 5.** Partial pulp regeneration in dogs after autologous transplantation of CD31-/CD146- side population cells. (A) Regenerated pulp tissue in the cavity on the amputated sites (arrows). Note the tubular dentin and/or osteodentin only along the dentinal wall. (B) Tubular dentin formation along the dentinal wall in the cavity. Image originated from boxed area in (A). (C) Osteodentin formation at the top of the cavity under cement. Adapted with permission from (81).



**Figure 6.** *De novo* regeneration of human dental pulp/dentin. Illustration at upper left depicts SCID mouse subcutaneous study model for pulp/dentin regeneration. The canal space of human tooth root fragments (~6-7 mm long) was enlarged to ~2.5 mm in diameter. One end of the canal opening was sealed with MTA cement. (A-I) Histological analysis of *in vivo* pulp/dentin regeneration using SCAP. A root fragment was prepared and the canal space inserted with SCAP/PLG and transplanted into a SCID mouse for 3 months. The sample was harvested and processed for H&E staining. D, original dentin; rD, regenerated dentin-like tissue; rP, regenerated pulp-like tissue. Blue arrow in (A) indicates the blood supply entrance; green arrows in (B&C) indicate continuous layer of uniform thickness of rD; yellow arrows in (E&F) indicate the region of well-aligned odontoblast-like cells with polarized cell bodies; green arrows in (G&H) indicate junctions between D and rD. Scale bars: (A) 1 mm; (B&C) 500  $\mu$ m; (D) 100  $\mu$ m; (E & F) 20  $\mu$ m; (G-I) 50  $\mu$ m. Adapted with permission from (4).

tooth fragment model was employed by our team. A section of human tooth roots of ~ 6-8 mm in length was obtained.

The canal content was totally removed and the space enlarged to 1-3 mm in diameter with one end of the canal opening sealed with a cement – MTA (Figure 6, black and white illustration). PLG was utilized as a scaffold to carry heterogeneous population of SCAP or DPSCs, packed into the canal space and the constructs transplanted subcutaneously into SCID mice. Three-four months later, the emptied canal space was filled with a good quality of vascularized pulp-like tissue and more importantly, a uniform thickness of a newly generated dentin-like layer was deposited onto the canal dentin wall as well as onto the MTA cement (Figure 6) (4).

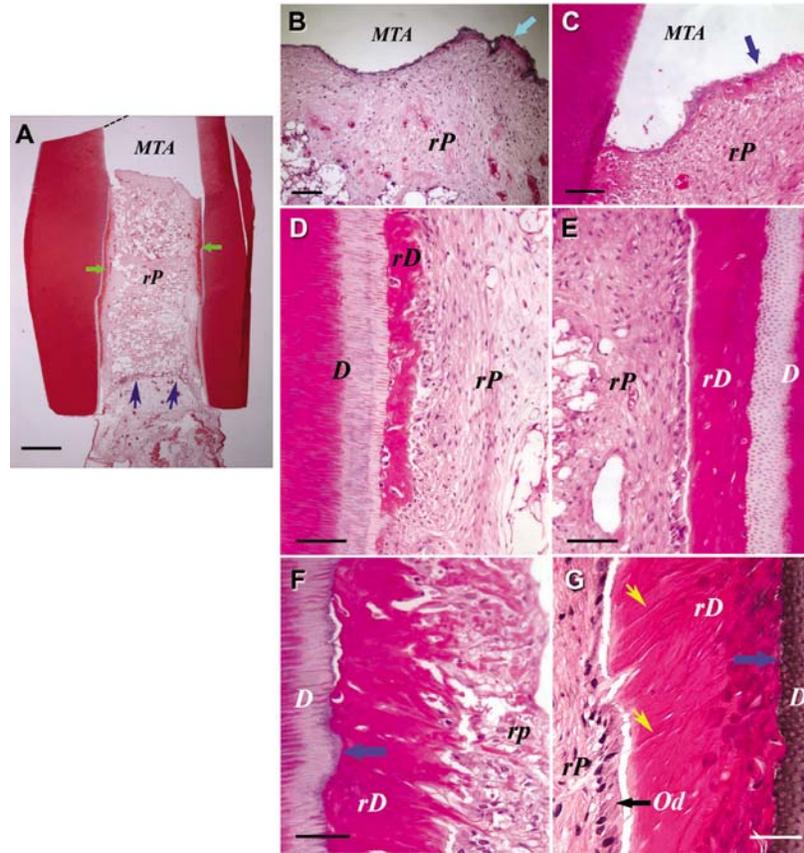
Higher magnification observations show that a layer of odontoblast-like cells is lined against the mineralized dentin-like tissue. There are also scattered cells embedded within the dentin-like structure. Unlike the natural dentin, the pulp side of the regenerated dentin-like tissue is not smooth; instead, there are projections of the structures into the pulp side. The odontoblast-like cells are not well organized, nor well aligned as the natural counterparts, and it is difficult to observe these typical characteristics that natural odontoblasts display (e.g., polarized cell bodies). Nonetheless, some regions of the odontoblast-like cells are of somewhat typical odontoblast characteristics. Groups of odontoblast-like cells are well aligned, such that each cell is spatially juxtaposed to adjacent cells with overt polarized morphology (Figure 6E&F and Figure 7G). The regenerated dentin-like tissue does not form observable well-organized dentinal tubules, except in a few regions where odontoblast-like cells were better aligned (Figure 7G). No different cell or structural zones like the natural pulp tissue are seen. Nonetheless, these well-organized cell and structural zones are normally seen in the pulp chamber of the natural teeth, less so or none in the radicular pulp. Therefore, the data presented in Figs 6 & 7 represent a real possibility that pulp and dentin can be *de novo* regenerated using a cell-based engineering approach (4).

## 6. CHALLENGE AND FUTURE PROSPECT ON REGENERATION OF FUNCTIONAL PULP/DENTIN

The most challenging part of tissue regeneration is perhaps the functional tissue engineering and regeneration. Although pulp tissue is very small, it is highly organized and complex. Regenerated pulp tissue in a tooth should be: i) vascularized, ii) containing similar cell density and architecture of the extracellular matrix to those of natural pulp, iii) capable of giving rise to new odontoblasts lining against the existing dentin surface and produce new dentin, and iv) innervated.

Vascularization may be difficult for teeth that the apical canal opening for blood vessel entrance is small (< 1mm). The size of apical opening would affect the ingrowths of blood vessels into the engineered pulp tissue. The larger the opening, the more likely the angiogenesis can occur. Therefore, immature teeth with open apices are the best candidates for pulp tissue regeneration. It was considered that the use of angiogenic inducing factors such

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**Figure 7.** Histological analysis of *in vivo* pulp/dentin regeneration using DPSCs. Samples were prepared using the same procedures as described in Figure 6, except that the sample was harvested from the SCID mouse 4 months post-implantation. D, original dentin; rD, regenerated dentin-like tissue; rP, regenerated pulp-like tissue. Green arrows in (A) indicate rD; blue arrows in (A) indicate the entrance of blood supply; blue arrow in (B&C) indicate the thin layer of rD under MTA cement; blue arrows in (F&G) indicate the junction of D and rD; black arrow in (G) indicates well-aligned odontoblast-like cells. Scale bars: (A) 1 mm; (B) 200  $\mu$ m; (C-E) 100  $\mu$ m; (F&G) 50  $\mu$ m. Adapted with permission from (4).

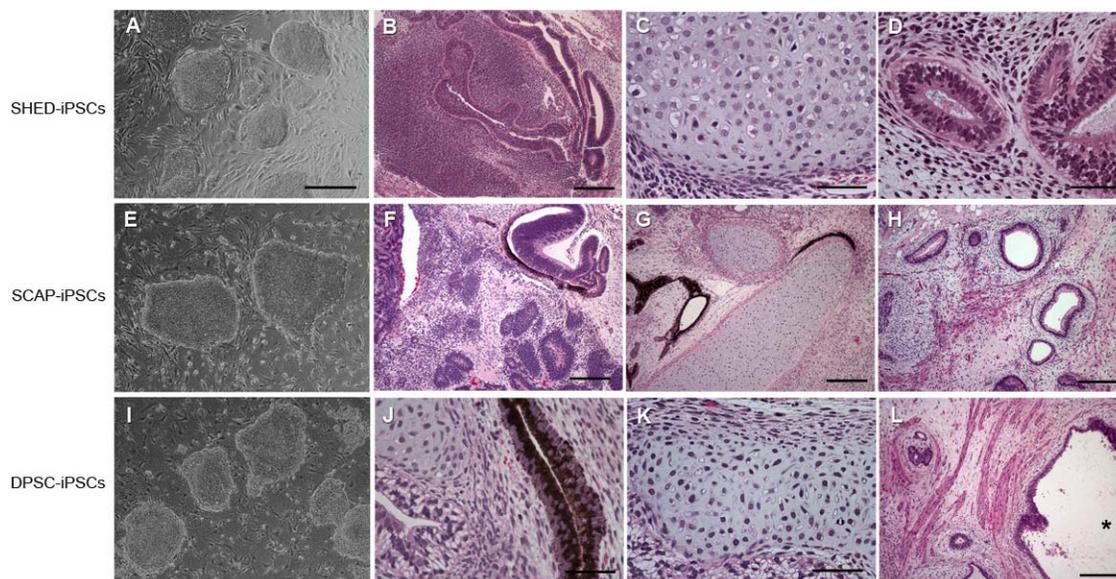
as vascular endothelial growth factor (VEGF) and/or platelet-derived growth factor (PDGF) should enhance and accelerate the pulp angiogenesis. Synthetic scaffolds such as PLG can be fabricated with impregnated these growth factors (54, 85-89). Alternatively, the insertion of engineered pulp tissue may have to be separated into multiple steps (17).

As long as good blood supply can be achieved, optimal cell density and the laid down of good quality extracellular matrix should occur. New odontoblast-like cells will form against the existing dentinal wall that has been chemically disinfected as evidenced by our recent report (4). Further, we have shown, as presented herein, new dentin-like tissue can be deposited onto the canal dentinal wall, although the nature of the new mineral tissue remains to be determined in terms of its mechanical and chemical properties.

With respect to innervation, it is likely that regenerated pulp contains ingrown nerve fibers from and adjacent natural tissues. DPSCs have been shown to either

produce neurotrophic factors or possess neural differentiation potential (57, 90). However, the specific innervation at the pulp-dentin complex makes this issue not quite straightforward. The reason why dentin is so sensitive to various irritations is the hydrodynamic activities of the dentinal tubules in association with the sensory A- $\delta$  fibers extending into the dentinal tubules in the predentin layer. Since the newly generated dentin does not appear to have well-organized dentinal tubules and is similar to reparative dentin, even if the A- $\delta$  fibers regenerate and reach the pulp-dentin junction, it may not cause the normal dentin sensitivity as the natural teeth.

Another issue on functional regeneration of pulp/dentin is the regeneration of enamel. Intact enamel overlaying dentin is needed to repair the damaged tooth. As noted before, enamel cannot self-regenerate; therefore, engineering approaches have to take place. Enamel regeneration has been tested by various acellular methods using recombinant enamel proteins amelogenin, surfactants, or using simple chemicals like calcium phosphate-containing solutions/paste to restore the enamel layer (91-93). However,



**Figure 8.** Induced pluripotent stem (iPS) cells derived from human dental stem cells. (A, E, I) ES cell-like colonies. SHED, SCAP, and DPSCs were reprogrammed into ES-cell like colonies with the 4 factors (Lin28, Nanog, Oct4 and Sox2). These iPS cells form teratomas in SCID mice containing tissues of all three germ layers. (B, F, J) Mainly primitive neural tissues, neural rosettes and retinal epithelium (ectoderm); (C, G, K) mainly cartilage (mesoderm); (D, H, L) mainly glandular tissue or respiratory epithelium (endoderm). Scale bars: (A, E, I) 500  $\mu$ m; (B, F-H, L), 200  $\mu$ m; (C, J, K), 50  $\mu$ m. \*, in (K) indicates the space of a cavity inside the teratoma. Adapted with permission from (96).

these approaches appear to be difficult to apply clinically or can only produce minimal amount of regenerated enamel on existing natural enamel. Thus, the final step of restoring tooth after pulp and dentin regeneration is likely to require the use of artificial materials.

As mentioned, the availability of cell source for cell-based tissue regeneration is an issue. Tooth and dental stem cell banking are essential infrastructure that has to be established in order to make cell-based therapy practical either for autologous or allogenic applications. Ultimately, the paucity of postnatal stem cells may be resolved by the generation of induced pluripotent (iPS) cells from somatic cells (94-95) or from dental stem cells (96). Our lab has recently established human iPS from dental stem cells that are capable of differentiating into cells of all three germ layers (Figure 8). These cells are potentially immortal and may serve as an unlimited cell source for regenerative medicine as well as for whole tooth and pulp/dentin regeneration.

## 7. CONCLUSION

Current clinical protocol of endodontic treatment sacrifices tissues in order to disinfect. After which, no regenerative process can take place as the lost tooth structure is replaced by artificial materials which does not strengthen the tooth. Regeneration of lost pulp and dentin tissues can reverse the deteriorated tooth, which is better than aggressive procedures that cause tooth structure loss. Dental stem cell-based approaches show capabilities of de novo regenerating pulp and new dentin. Further research is

needed to regenerate higher quality of pulp and dentin. With regard to cell source, establishing dental stem cell banking may be a necessary step and further progress on establishing individualized induced pluripotent stem cells for dental tissue regeneration is imminent.

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**Abbreviations.** PDL: periodontal ligament; MTA; mineral trioxide aggregate; PLG: poly(D, L-lactide-co-glycolide); PGA: polyglycolic acid; BMSSCs: bone marrow mesenchymal stromal cells; DPSCs: dental pulp stem cells; SHED: stem cells from human exfoliated deciduous teeth; PDLSCs: periodontal ligament stem cells; SCAP: stem cells from apical papilla; DFPCs: dental follicle progenitor cells; ABC: apical bud cell; MSCs: mesenchymal stem cells; SCID: severe combined immunodeficiency.

**Key Words:** Tissue Engineering, Regeneration, Enamel, Dental Pulp, Dentin, Stem Cells, Tooth Regeneration, Endodontics, Periodontal Ligament, Dental Pulp Stem Cells, Stem Cells From Apical Papilla, Scaffold, Review

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