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REVIEW ARTICLE

Crucial Triad in Pulp-Dentin Complex Regeneration: Dental Stem Cells, Scaffolds, and Signaling Molecules

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Abstract

BACKGROUND: Pulp damage can lead to dentinogenesis impairment, irreversible pulpitis, or pulp necrosis. Despite being the most used endodontic procedure to treat damaged pulp, root canal therapy only results in nonvital teeth which are prone to fractures and secondary infection. Pulp-dentin regeneration has a potential to regenerate structure similar to normal pulp-dentin complex, and can be achieved by combining dental stem cells, scaffold, and signaling molecules. This article reviews the role of various types of dental stem cells, scaffolds, signaling molecules, and their combinations in regenerating pulp-dentin complex.

CONTENT: Dental pulp stem cell (DPSC), stem cell from human exfoliated deciduous teeth (SHED), and dental follicle stem cell (DFSC) were reported to regenerate pulp-dentin complex *in situ*. SHED might be more promising than DPSCs and DFSCs for regenerating pulp-dentin complex, since SHED have a higher proliferation potential and higher expression levels of signaling molecules. Scaffolds have characteristics resembling extracellular matrix, thus providing a suitable microenvironment for transplanted dental stem cells. To accelerate the regeneration process, exogenous signaling molecules are often delivered together with dental stem cells. Scaffolds and signaling molecules have different regenerative potential, including induction of cell proliferation and migration, formation of pulp- and/or dentin-like tissue, as well as angiogenesis and neurogenesis promotion.

SUMMARY: Combinations of dental stem cells, scaffold, and signaling molecules are important to achieve the functional pulp-dentin complex formation. Current trends and future directions on regenerative endodontics should be explored. The right combination of dental stem cells, scaffold, and signaling molecules could be determined based on the patients' characteristics. Incomplete pulp-dentin regeneration could be overcome by applying dental stem cells, scaffold, and/or signaling molecules in multiple visits.

KEYWORDS: pulp-dentin regeneration, regenerative endodontics, dental stem cells, scaffold, signaling molecules

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Introduction

Dental pulp, the only soft tissue in the tooth, plays a critical role in sustaining tooth homeostasis. However, this tissue is vulnerable to various stimuli, including infections, iatrogenic causes, and trauma.(1) If not treated properly, pulp damage can lead to dentinogenesis impairment and irreversible pulpitis or even pulp necrosis, since this tissue has a limited self-repair capacity.(2)

Damaged pulp can be treated by several procedures. Root canal therapy, the most used endodontic procedure,

replaces inflamed or injured pulp with bioinert material fillings. However, this procedure results in nonvital teeth, which are prone to fractures and secondary infection. (3) Regenerative endodontic treatment or pulp-dentin regeneration is an alternative procedure based on the tissue engineering principle. Pulp-dentin regeneration is more holistic than other endodontic procedures since this procedure has a potential to regenerate structure similar to normal pulp-dentin complex. The main goals of pulp regeneration are pulp-dentin complex formation as well as angiogenesis and neurogenesis in the newly regenerated pulp.(4)

Tissue engineering combines dental stem cells, scaffold, and signaling molecules to mimic a suitable microenvironment for regenerating pulp-dentin complex. Numerous studies have been established to examine the effects of dental stem cells, scaffold, signaling molecules, and their combinations in pulp regeneration, providing a new insight in the field of regenerative dentistry and opening a great opportunity for further clinical applications. This article reviews the role of various types of dental stem cells, scaffolds, signaling molecules, and their combinations in regenerating pulp-dentin complex. The right combination of these components could increase pulp-dentin regeneration therapy efficiency.

Role of Dental Stem Cells in Regenerative Endodontics

Based on the locations, dental stem cells are classified as dental pulp stem cell (DPSC), stem cells from human exfoliated deciduous teeth (SHED), stem cells from the apical papilla (SCAP), dental follicle stem cell (DFSC), periodontal ligament stem cell (PDLSC).(5,6) DPSCs, SHED, and DFSCs were reported to have potential in regenerating pulp-dentin complex *in situ*, both in animal models (Table 1) and human subjects (Table 2).

Cell Number, Proliferation Rate, and Immunomodulatory Properties of DPSCs, SHED and DFSCs

DPSCs, SHED and DFSCs are different in several aspects, including the number of cells isolated from the tissues, proliferation rate, and immunomodulatory mechanisms. DPSCs and SHED have relatively high cell numbers in original cultures compared with DFSCs, because dental pulp, both in permanent and deciduous teeth, have relatively high amounts of stem cells compared with dental follicles of developing tooth germ. Since dental follicle tissues are smaller in size, located in sites that are relatively not easy to be accessed, and contain small amounts of cells, DFSCs are difficult to be obtained and distinguished from other types of dental stem cells.(4)

DPSCs have been reported to have a higher proliferation rate compared with bone marrow mesenchymal stem cell (BMMSC), while SHED have a higher proliferation rate than DPSCs.(33) It has been demonstrated that the proliferation rate of DFSCs is notably higher than DPSCs. (34) Moreover, in a recent study, DFSCs were shown to have a higher proliferation rate than SHED.(35) Thus, DFSCs might have the highest proliferation rate, followed by SHED and DPSCs. High proliferation of DFSCs implies that they are more immature, since this type of stem cells are isolated from developing tissues (36), and consequently they might be more plastic compared with other dental stem cells. In summary, DPSCs, SHED, and DFSCs vary in their proliferation rates, which could be determined by the developmental stages of the stem cell sources.

Mesenchymal stem cell (MSC), including DPSCs, SHED and DFSCs have been reported to modulate the immune system through several mechanisms.(37) DPSCs have been demonstrated to modulate the adaptive and innate immune responses through interaction with B cells, T cells, macrophages, dendritic cells (DCs), and natural killer (NK) cells. For instance, the production of B cell immunoglobulin and proliferation of T cell proliferation are inhibited in co-culture of peripheral blood mononuclear cells (PBMCs) and DPSCs. Transforming growth factor (TGF)-β secreted by DPSCs plays a crucial role in this inhibition and the addition of interferon (IFN)-γ to DPSCs culture enhances the inhibitory effects.(38) DPSCs markedly decrease $CD4^+$ and $CD8^+$ T cell proliferation, irrespective of hypoxia-inducible factor (HIF)-1α expression level in DPSCs. However, overexpression of HIF-1α increases the DPSCs inhibitory effect on DCs proliferation. Expression of HIF-1α by DPSCs also enhances the recruitment and differentiation of macrophages with M2 characteristics. Furthermore, NK cell-mediated cytotoxicity is suppressed in HIF-1α-overexpressed DPSCs.(39)

SHED have been shown to modulate T cells, macrophages and DCs. This type of stem cell restrains the differentiation of T helper (Th) 17 cells, and has greater immunomodulatory potential compared with BMMSCs. (40) SHED have been reported to promote phenotypic polarization of macrophage toward M2-like phenotype in transwell co-culture systems and increase the number of macrophages with M2-like phenotype in rat model of periodontitis.(41) A study demonstrates that SHED affect

Table 1. Regenerative potential of DPSCs, SHED, and DFSCs in animal model of pulp-dentin regeneration.

Type of Dental Stem Cells	Species	Regenerative Potential			Reference
		Pulp- and/or Dentin-like Tissue	Angiogenesis	Neurogenesis	
DPSC	Dog	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DSPP	Histology: Blood vessels in regenerated pulp	N/A	(7-10)
	Mini-pig	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DSP, DMP1, and BSP	Histology: Blood vessels in regenerated pulp	N/A	(11)
	Ferret	Histology: Formation of osteodentin mixed with loose connective tissue.	N/A	N/A	(12)
	Rat	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DMP1, DSPP, DSP, and OPN	Histology: Blood vessels in regenerated pulp Positive immunostaining: CD31	N/A	(13-15)
DPSC $CD31^-$	Dog	Histology: - Pulp tissue regeneration - Dentin formation Gene expression: *MMP20, syndecan 3, TRH-DE*	Positive immunostaining: BS-1 lectin	Positive immunostaining: PGP9.5	(16)
DPSC $CD105^+$	Dog	Histology: Pulp tissue regeneration	Histology: Blood vessels in regenerated pulp	N/A	(17)
Mobilized DPSC	Dog	Histology: - Pulp tissue regeneration - Dentin formation Gene expression: *tenascin C*, *syndecan 3*, *TRH-DE*, *MMP20*, *DSPP* Positive immunostaining: TRH-DE MRI: Signal intensity of transplanted teeth was similar compared with that in normal teeth.	Positive immunostaining: BS-1 lectin Laser Doppler flowmetry: Blood flow in regenerated pulp tissue is similar compared to that in normal pulp tissue.	Positive immunostaining: PGP9.5 Electric pulp test: Positive pulp sensibility response	(18-25)
hpDPSC	Dog	Histology: - Pulp tissue regeneration - Dentin formation	Positive immunostaining: BS-1 lectin	Positive immunostaining: PGP9.5	(25,26)
hpDPSC from deciduous teeth	Dog	Histology: - Pulp tissue regeneration - Dentin formation	Positive immunostaining: BS-1 lectin	Positive immunostaining: PGP9.5	(26)
SHED	Mini-pig	Histology: - Pulp tissue regeneration - Dentin formation	Histology: Blood vessels in regenerated pulp Positive immunostaining: CD31	Positive immunostaining: NeuN, neurofilament, CGRP, and TRPV1	(27,28)
DFSC	Mini-pig	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DMP-1, DSPP, COL1, COL3	N/A	N/A	(29)

N/A: Not applicable; DSPP: Dentin sialophosphoprotein; DSP: Dentin sialoprotein; DMP1: Dentin matrix acidic phosphoprotein 1; BSP: Bone sialoprotein; OPN: Osteopontin; MMP20: Matrix metalloproteinase 20; Thyrotropin-releasing hormone-degrading enzyme: TRH-DE; BS-1 lectin: *Bandeiraea simplicifolia* lectin 1; PGP9.5: Protein gene product 9.5; NeuN: Neuronal nuclei; CGRP: Calcitonin gene-related peptide; TRPV1: Transient receptor potential cation channel subfamily V member 1; COL1: Collagen type I; COL3: Collagen type III.

Table 2. Regenerative potential of DPSCs, SHED, and DFSCs in case reports and clinical trials of pulp-dentin regeneration.

Type of Dental Stem Cells	Regenerative Potential			Reference
	Pulp- and/or Dentin-like Tissue	Angiogenesis	Neurogenesis	
DPSC	CBCT: - Formation of dentin bridge - Apical canal calcification	Laser Doppler flowmetry: Blood perfusion in the transplanted tooth with low mean perfusion unit.	N/A	(30)
Mobilized DPSC	MRI: Complete pulp regeneration CBCT: - Formation of lateral dentin - Decrease in dental pulp volume	N/A	Electric pulp test: Positive pulp sensibility response	(31)
hpDPSC	MRI: Complete pulp regeneration CBCT: - Formation of lateral dentin - Decrease in dental pulp volume	N/A	Electric pulp test: Positive pulp sensibility response	(32)
SHED	Histology: Regenerated pulp with odontoblast layer, connective tissue, and blood vessels. CBCT: Increase in dentin thickness	Laser Doppler flowmetry: An increase in vascular formation as indicated by high perfusion units.	Positive immunostaining: NeuN Electric pulp test: Positive pulp sensibility response	(27)

N/A: Not applicable; CBCT: Cone beam computed tomography; MRI: Magnetic resonance imaging; NeuN: Neuronal nuclei.

differentiation, maturation, and T cell activation ability of DCs. The same study also shows that SHED augment T regulatory (Treg) cell induction ability of DCs. SHED-treated DCs have a lower level of IFN-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-2, as well as higher level of IL-10.(42)

DFSCs have immunomodulatory properties toward T cells and macrophages. A study demonstrates that DFSCs increase the number of Treg cells as well as suppress $CD4^+$ T cell proliferation via TGF-β and indoleamine 2,3-dioxygenase (IDO) pathways.(43) In lipopolysaccharide (LPS)-induced macrophage, this type of stem cell is involved in phenotypic polarization to M2 by secreting thrombospondin-1 and TGF-β3.(44) Therefore, the immunomodulatory activities of DPSCs are exerted on B cells, T cells, macrophages, DCs, and NK cells. SHED regulates T cells, macrophages and DCs, while DFSCs show immunomodulatory activities toward T cells and macrophages.

DPSCs, SHED and DFSCs Play a Crucial Role in Regenerating Pulp-dentin Complex

Dental stem cells are involved in pulp-dentin complex formation *in situ*. When transplanted into an emptied root canal or a tooth construct, DPSCs, SHED, and DFSCs generate tissue that has characteristics resembling dental pulp. Several biomarkers have been used to detect the presence of the regenerated pulp, such as thyrotropin-releasing hormone-degrading enzyme (*TRH-DE*), *syndecan 3*, and *tenascin*. Furthermore, magnetic resonance imaging (MRI) can also be utilized to assess pulp regeneration by dental stem cells in the root canal (Table 1, Table 2). After pulpectomy, the signal intensity of MRI is relatively low compared with those in the normal teeth. The signal intensity in the pulpectomized tooth then increases several days after transplantation and keeps decreasing until it is similar to normal pulp, indicating complete pulp regeneration.(21)

Formation of dentin-like structure by DPSCs, SHED, and DFSCs has also been documented by the generation of dentin matrix deposition that causes dentin thickening and the presence of odontoblast-like cells on the canal dentinal walls which express both specific and non-specific odontoblast markers. Specific odontoblasts markers include dentin sialoprotein (DSP), dentin sialophosphoprotein (DSPP) and dentin matrix acidic phosphoprotein (DMP) 1 (13-15), while non-specific odontoblasts markers include bone sialoprotein (BSP) and osteopontin (OPN).(11,14) There are several viewpoints regarding the use of non-specific odontoblasts markers for detecting newly regenerated dentin. Some investigators consider that enhanced expression of these markers suggests greater dentin regeneration potential (8,10,14,15,28) since they are involved in dentin formation. (45) Other investigators consider these markers as

osteogenic markers instead of odontogenic markers.(29,46) The increase in the expression of these markers implies that the regenerated structure has similar characteristics with bone instead of dentin.(46) Therefore, these markers should not be more strongly expressed in dentin than the expression of odontoblast-specific markers.(11,14,29) Besides detection of odontoblast markers, cone beam computed tomography (CBCT) can be used to assess dentin formation (Table 2), which is demonstrated by a reduction in low-density areas, indicating a decrease in pulp volume and an increase in dentin thickness.(32) Studies that use ectopic and semiorthotopic pulp-dentin regeneration models are not included in Table 1, since these models do not provide similar conditions as the human oral cavity.(4)

The research that assesses the ability of DFSCs to form pulp-dentin complex is more limited than those conducted using DPSCs and SHED. DFSCs are usually used to simultaneously regenerate pulp-dentin and cementum-periodontal complexes.(29) This may be caused by the tendency of DFSCs to regenerate periodontal tissue and tooth root rather than pulp-dentin complex. Transplantation of treated dentin matrix that contains DFSCs regenerates periodontal-like tissue in subcutaneous space and cementum-like tissue in the outer surface of dentin.(47) Moreover, combination of DFSCs and treated dentin matrix which is transplanted to the alveolar fossa of rats has a potential to induce root formation.(48) Thus, DFSCs are better to use in periodontal tissue and root regeneration, although they might also have a potential to regenerate pulp-dentin complex. Despite the large number of studies that explore the regenerative potential of DPSCs, SHED might be more promising than DPSCs, since SHED have a higher proliferation potential (33) and higher expression levels of signaling molecules which may contribute to the pulp-dentin regeneration.(49)

DPSCs, SHED and DFSCs are Involved in Angiogenesis

Angiogenesis has been reported to occur in pulp-like tissue regenerated by DPSCs and SHED *in situ*. There are limited studies that demonstrate the involvement of DFSCs in the angiogenesis process in regenerated pulp tissue (Table 1). The angiogenic potential of DFSCs has been reported to be lower compared with DPSCs and SCAP. (50) The new vessels provide oxygen and nutrition to the newly regenerated pulp, thus supporting the survival of the transplanted stem cells and facilitating further regeneration process. Blood vessels in the regenerated pulp can be detected using immunostaining of *Griffonia* (*Bandeiraea*) *simplicifolia* lectin 1 (BS-1 lectin) and CD31 (Table 1).

In addition, laser Doppler flowmetry can be used to assess angiogenesis and analyze the blood flow in the regenerated pulp tissue, as demonstrated by several studies. Blood flow in the pulp tissue regenerated by DPSCs is not remarkably different compared with that in normal pulp tissue, implying complete functional angiogenesis. (18) Human tooth with symptomatic irreversible pulpitis which is treated with DPSCs and normal tooth have low mean perfusion units. Blood perfusion in both teeth is indicated by pulse characteristics.(30) In addition, SHED-transplanted teeth experience an increase in the average of vascular formation.(27)

DPSCs, SHED, and DFSCs are involved in angiogenesis through differentiation toward endothelial cells (28) or angiogenic factors secretion. Several angiogenic factors that are expressed by these stem cells includes vascular endothelial growth factor (*VEGF*) (16,28,29), *HIF1A* (28), granulocyte-monocyte colony-stimulating factor (*GM-CSF*), matrix metalloproteinase 3 (*MMP3*) (16), selectin E (*SELE*) (18), angiopoietin (*ANGPT*), and von Willebrand factor (*VWF*).(15) These factors stimulate vessel formation by modulating local endothelial cells in a paracrine manner. (16) Several subsets of DPSCs have been reported to secrete angiogenic factors but they do not incorporate to the newly formed blood vessels, such as dental pulp $CD31^-$ side population cells (16) and granulocyte colony-stimulating factor (G-CSF) mobilized DPSCs.(18,51)

Angiogenesis in pulp-like tissue can be induced further by culturing dental stem cells under hypoxic conditions. Hypoxia mimics conditions in the dental pulp cavity (52), which increases the expression of *HIF1A*. Upregulation of this transcriptional factor activates the expression of angiogenesis-related genes.(25) Hypoxia culture on nanofibrous spongy microspheres increases angiogenesis potential of human DPSCs (hDPSCs) as indicated by more CD31-stained blood vessels in the regenerated pulp-like tissues.(13) Another research demonstrates that the expression levels of *HIF1A* in hypoxia preconditioned DPSCs (hpDPSCs) are two times higher compared with those in mobilized DPSCs, while *VEGF* expression levels in both DPSCs are similar. hpDPSCs have been demonstrated to have a similar neovascularization potential compared to mobilized DPSCs.(25) DPSCs from permanent and deciduous teeth that are cultured under hypoxic conditions have similar expression levels of *VEGF* and *GM-CSF*, as well as *in situ* neovascularization potential.(26) Furthermore, co-culture of dental stem cells with endothelial cells has also been demonstrated to enhance angiogenesis. Crosstalk between transplanted stem cells with endothelial cells has

been shown to increase the expression of angiogenic factors in both cells by activating specific pathways, such as nuclear factor κB (NF-κB).(53)

DPSCs, SHED and DFSCs are Involved in Neurogenesis

DPSCs, SHED, and DFSCs have a potential to induce neurogenesis, as shown by the studies that reported the presence of nerve fibers in pulp-like tissue after stem cell transplantation. Newly formed nerve fibers in orthotopic pulp regeneration models are detected using immunostaining of protein gene product 9.5 (PGP9.5), neuronal nuclei (NeuN), neurofilament, calcitonin gene-related peptide (CGRP), and transient receptor potential cation channel subfamily V member 1 (TRPV1) (Table 1). The expression of other neurological markers, such as sodium voltage-gated channel alpha subunit 1 (*SCN1A*) and *neuromodulin* genes (16,18), as well as tubulin-βIII (TUBB3) (29), nestin, and transient receptor potential cation channel subfamily M member 8 (TRPM8) protein (27), has also been detected in cultured or subcutaneously implanted stem cells. Electric pulp test is another common technique utilized for detecting nerve fibers in regenerated pulp tissue (Table 1, Table 2).

Mechanisms of neurogenesis induction are similar to the angiogenesis induction by DPSCs, SHED, and DFSCs. These types of stem cells have been reported to differentiate toward neural cells.(54,55). In addition, various neurogenic factors are expressed by DPSCs and SHED, including nerve growth factor (*NGF*), glial cell-derived neurotrophic factor (*GDNF*), brain-derived neurotrophic factor (*BDNF*), neuropeptide Y (*NPY*), and neurotrophin 3 (*NTF3*).(16,56) Investigations on neurogenic factors secreted by DFSCs are still limited. Hypoxic conditions could enhance the expression of neurogenic factors in dental stem cells. *NGF* and *BDNF* expression levels are notably higher in hpDPSCs compared with those in mobilized DPSCs, but *GDNF* expression level is lower. It has been reported that hpDPSCs have a similar reinnervation potential compared to mobilized DPSCs.(25) A recent study revealed that DPSCs from deciduous teeth had a markedly higher mRNA expression of *BDNF* compared with those obtained from permanent teeth, but not *NGF* or *GDNF*. However, both of these stem cells had a similar BDNF protein expression level and reinnervation potential.(26)

Factors Affecting the Regenerative Potential of DPSCs, SHED and DFSCs in Pulp-Dentin Complex Regeneration

Several factors may affect the regenerative potential of DPSCs, SHED, and DFSCs. Aging has been reported to cause the reduction of DPSCs regenerative potential. An animal study demonstrates that about 60% of root canal area is covered by pulp-dentin complex after 120 days in teeth of aged dogs (5–6 years of age) transplanted with autologous mobilized DPSCs.(19) This percentage is much lower than that in young dogs (8-10 months of age), which shows regeneration volume of more than 90% after 60 days. (18) SHED, which are obtained from dental pulp of younger individuals, have a higher expression of neuronal markers when compared with adult DPSCs, suggesting lower neurogenic potential in DPSCs.(57) In dental follicle cells, cell senescence is correlated with a decrease in osteogenic potential and lower WNT5A expression, although the role of WNT5A may be less significant in regulating the expression of osteogenic markers.(58)

Dental diseases, such as caries, are reported to have no effect or even increase regenerative potential of dental stem cells. SHED obtained from carious deciduous teeth has a similar osteogenic potential compared to those that are obtained from sound deciduous teeth.(59) Meanwhile, DPSCs isolated from teeth with deep caries have greater proliferation and angiogenesis abilities, as well as higher expression of odontoblast differentiation markers.(60,61)

Dental stem cells can differentiate not only to odontoblasts and dental pulp cells, but also to other types of cells, since it has been reported that transplantation of DPSCs regenerates periodontal ligament-, bone-, and cementum-like tissues instead of pulp-like tissue. Signals sent from tissues surrounding the root canal, such as alveolar bone and periodontal ligament, might affect the fate of transplanted dental stem cells.(46) Taken together, the success of stem cells-mediated pulp-dentin complex regeneration may be affected by aging, dental diseases, and signals sent from the surrounding tissues.

Recent Advances on the Use of Dental Stem Cells in Regenerative Endodontics

Dental stem cells have been demonstrated to regenerate functional pulp-dentin complex in human subjects in several studies, most of them using autologous dental stem cells (Table 2). Combination of autologous mobilized DPSCs and good manufacturing practice (GMP)-grade G-CSF are transplanted into the teeth of five adult irreversible pulpitis patients.(31) Mobilized DPSCs are subsets of DPSCs isolated through G-CSF-induced cell mobilization.(51) Four weeks after transplantation, four patients show a positive electric pulp test result. Lateral dentin formation is observed in three patients as shown by CBCT imaging. Interestingly, all patients do not experience any adverse events or toxicity caused by mobilized DPSCs transplantation.(31)

Successful pulp regeneration using autologous DPSCs obtained from inflamed pulp has also been reported. DPSCs are obtained from the permanent tooth with symptomatic irreversible pulpitis. These stem cells are implanted with leukocyte platelet-rich fibrin (L-PRF) obtained from the patient's blood into the root canal of the same tooth. After 36 months, no tenderness to palpation or percussion, and no adverse events are observed. Laser Doppler flowmetry results demonstrate that both untreated and DPSCs-implanted teeth have pulse characteristics, implying blood perfusion in the teeth, although the mean perfusion units in those teeth are low.(30)

Transplantation of autologous hpDPSCs seeded on atelocollagen scaffold containing G-CSF in multirooted molars of two patients affected by symptomatic or asymptomatic irreversible pulpitis has been successfully demonstrated. No periapical radiolucency is observed by CBCT and radiographic examination after 48 weeks. Moreover, no adverse events or systemic toxicity are experienced by these patients as shown by the results of clinical and laboratory evaluation.(32)

SHED transplantation into injured human teeth markedly increases dentin thickness and root length, as well as reduces apical foramen width compared with the apexification procedure. An increase in vascular formation is observed in SHED transplantation group. In contrast, a decrease in vascular formation is observed in the apexification group. Teeth transplanted with SHED show a significantly higher mean decrease in sensation than those treated with apexification procedure. No adverse events are observed at 24 months after transplantation.(27)

Besides dental stem cells, induced pluripotent stem cell (iPSC), which is obtained by introducing reprogramming factors including octamer-binding transcription factor 4 (*Oct4*), Kruppel-like factor 4 (*Klf4*), sex determining region Y-box 2 (*Sox2*), *l-myc*, *c-myc*, and *Lin28* to somatic cells, can also be used in pulp-dentin regeneration.(62-65) Stem cells, such as DPSCs (63), and differentiated cells, such as fibroblasts (64) could be used to generate iPSCs. Generation of odontoblasts-like cells could be performed by directly inducing iPSCs.(63) In addition, iPSCs could be induced toward iPSCs-derived neural crest-like cells (iNCLCs), which in turn can be differentiated further into odontoblasts-like cells.(63,64) Differentiation to odontoblasts and generation of pulp-like tissue from iPSCs can be induced by transfection of specific genes (62), as well as addition of exogenous growth factors (63,64) and scaffolds (64).

Whole tooth regeneration is another promising advance in endodontic therapy. This method relies on the interaction between the dental mesenchyme and the dental epithelium to generate a bioengineered tooth bud.(66) Cells of the dental mesenchyme and the dental epithelium can be isolated from embryonic (67-69) or postnatal (67) dental tissues. Autologous (67), allogeneic (69), and xenogeneic (68) cells have been used in tooth bud production. Both types of cells are combined in collagen gel drop and cultured *in vitro* (67-69) or seeded in a scaffold (70). The bioengineered tooth bud is then transplanted to the jaw bone to regenerate the new tooth.

Role of Biomaterial Scaffolds in Regenerative Endodontic Therapy

Along with dental stem cells, the use of biomaterial scaffold (bioscaffold) also becomes a notable consideration in regenerative endodontics, especially for the formation of dental tissues. These biomaterials are expanded *in vitro* to environmentally mimic the *in vivo* condition.(71,72) Ideal scaffolds for regenerative endodontic therapy should resemble the extracellular matrix (ECM) of pulp-dentin complex in terms of dimensional stability, sufficient porosity with adequate particle size, similar biodegradability rate, as well as physical and mechanical strength (71,73,74), since biocompatibility is highly important to prevent adverse tissue reactions.(75)

Bioscaffold for regenerative endodontic therapy includes broad ranges of applications and sources. Based on the scaffold geometry, the existing biological constructs are porous scaffolds, fibrous scaffolds, microsphere/microparticle scaffolds, and solid free-form scaffolds.(76) Meanwhile, based on the material sources, bioscaffold can be classified into blood-derived scaffolds, natural-derived biomaterial scaffolds, and synthetic biomaterial scaffolds. Each scaffold has different regenerative properties and potential, including pulp and dentin regeneration, vascularization, as well as stem cell proliferation and differentiation (Table 3).

Blood-derived Scaffolds

Induction of bleeding and formation of intracanal blood-clot (BC) in the root canal is a well-known used method in regenerative endodontic therapy that applies the strategy of bioscaffold for pulp-dentin regeneration and dental tissue ingrowth.(78,106) BC is a gel-like lump obtained

Table 3. Regenerative potential of blood-derived, natural-derived polymer, and sythetic polymer bioscaffolds.

Types of Scaffolds	Regenerative Potential		References
	Pulp-dentin Regeneration	Vascularization	
Blood-derived			
BC	- Increasing root length and thickness - Increasing dental wall thickness - Improving bone density - Narrowing apical width - Healing the periapical lesion	- Improving vitality response (blood pump)	(77-86)
PRP	- Increasing root length and thickness - Increasing dental wall thickness - Improving bone density - Narrowing apical width - Healing the periapical lesion	- Improving vitality response (blood pump)	(77,78,80-83, 85-87)
PRF	- Increasing root length and thickness - Increasing dental wall thickness - Improving bone density - Narrowing apical width - Healing the periapical lesion	- Improving vitality response (blood pump)	(80,83,84,85,87)
Natural-derived polymers			
Collagen - BC	- Increasing root length - Enhancing mineralization of root canal - Increasing dental wall thickness - Narrowing apical width - Healing the periapical lesion - Increasing intracanal connective tissue formation	N/A	(88-92)
Gelatin - BC	- Increasing root lenght and thickness - Increasing root length - Increasing dental wall thickness - Narrowing apical width - Increasing intracanal connective tissue formation	N/A	(93,94)
Chitosan - BC - Sodium hyaluronate - Pectin	- Increasing root length and thickness - Increasing dental wall thickness - Enhancing mineralization of root canal - Narrowing apical width - Healing the periapical lesion - Increasing intracanal connective tissue formation	- Increasing vascularization	(95,96)
Fibrin	- Increasing root length and thickness - Enhancing mineralization of root canal - Narrowing apical width - Healing the periapical lesion	- Increasing vascularization	(94,97)
HA	- Increasing root length - Enhancing mineralization of root canal - Increasing dental wall thickness - Narrowing apical width - Healing the periapical lesion - Increasing intracanal connective tissue formation	- Increasing vascularization	(73,98)
Synthetic biomaterial			
PLLA - DPSC - Minced-pulp MSC	- Enhance tissue mineralization - Increase expression levels of *DMP1*, *DSPP*, *COL1*, and *OPN* genes	N/A	(99-101)
PLGA - DPSC - Magnesium	- Increase bone height and volume - Enhance bone mineralization - Enhance surface closing	- Initiate neurovascular regeneration	(102,103)
PCL - PDLSC - Fluorapatite	- Enhance bone formation in defect tissue - Improve periodontium neogenesis - Increase expression of *DMP1*, *DSPP*, *RUNX2*, *OCN*, *SPP1*, *COL1A1*, and *GDF5* genes	N/A	(104,105)

N/A: Not applicable; DMP1: Dentin matrix acidic phosphoprotein 1; DSPP: Dentin sialophosphoprotein; COL1: Collagen type I; OPN: Osteopontin; RUNX2: Runt-related transcription factor 2; OCN: Osteocalcin; SPP1: Secreted phosphoprotein 1; COL1A1: Collagen type I alpha 1; GDF5: Growth differentiation factor 5.

during the blood state alterations from liquid to solid. (74) This technique usually includes canal preparation and disinfection, followed by induction of BC from the periapical region.(107)

The practicality and success of regenerative endodontic therapy using BC, including in treating permanent or immature teeth with apical periodontitis and necrotic pulps, have been reported. In terms of pulp and dentin regeneration, BC bioscaffold therapy showed that it was able to give substantial results in increasing root length and thickness, thickening dental wall, improving bone density, providing apical closure, as well as periapical healing.(78-80,82,84,108) Immature symptomatic apical periodontitis teeth treated with BC scaffold showed a similar root morphology compared to other teeth that underwent normal development.(74,109)

Although has been performed a lot previously, yet the failure in inducing apical bleeding or in achieving adequate blood volume within the canal space remain as the common problems during the therapy with BC bioscaffold. The percentage of discoloration was also significantly greater in teeth with BC scaffold therapy compared with teeth with other platelets concentrates.(86) Hence, lately the use of autologous platelet concentrates, including platelet-rich plasma (PRP) and platelet-rich fibrin (PRF), have been explored as the possible scaffold source for regenerative endodontics therapy.(83,85)

PRP, an autologous first-generation platelet concentrate, is a high concentrate of autologous platelet obtained by centrifugation of autologous blood that may be source for several types of growth factors such as TGF-β, insulin growth factor (IGF), platelet-derived growth factor (PDGF), VEGF, as well as fibroblasts growth factor (FGF). (110,111) PRP preparation process consists of the removal of erythrocytes that would be expected to undergo necrosis shortly after clot formation. The PRP clot is composed of fibrin, fibronectin, and vitronectin, which are cell adhesion molecules required for cell migration.(78) PRP is an ideal scaffold regenerative endodontic treatment since it is comparably easy to prepare in a dental setting, rich in growth factors, and forms a 3D fibrin matrix that helps attract the growth factors.(77)

As a comparable autologous bioscaffold, PRP has been able to show results of further root development (including root lengthening and thickening), periapical lesion resolution, improvement of periapical bone density, and continued apical closure compared with BC in the regenerative treatment of teeth with necrotic pulps. (77,78,81,86) Most blood-derived bioscaffolds showed the ability to improve pulp vitality response. However, PRP was found to be more effective than BC in revascularization. Even though not significant PRP treatment showed highest vitality test response compared with BC treatment, which suggests the higher occurrence of pulp's blood supply.(83,85) PRP has also been proved to be successfully stimulating the collagen production, sustained release of growth factors, as well as enhanced recruitment, retention, and proliferation of undifferentiated mesenchymal and endothelial cells from periapical area.(77,82) At a certain concentration of range, PRP also may increase the proliferation of fibroblasts and osteoblasts.(111)

PRF, a second-generation platelet concentrate, is a non-thrombonized autologous fibrin mesh that responsible as a reservoir for the slow, continuous release of growth factors PRF is an unadulterated centrifuged blood which consists of autologous platelets and leukocytes present in a complex fibrin matrix, that is able to achieves polymerization naturally. PRF is composed of fibrin membranes enriched with platelets, growth factors, and cytokines.(86,112) The PRF clot is an autologous biomaterial and not an improved fibrin glue. Unlike the PRP, the strong fibrin matrix of PRF does not dissolve quickly after application, instead, it is formed slowly in a similar way to a natural BC.(80)

Although composed of almost similar fibrin membranes, PRF has lower risk than PRP during the application since there is no bovine thrombin and anticoagulants present. PRF also shows better potency in accelerating wound and tissue healing, as well as better efficiency for cell proliferation and migration than PRP. (113,114) PRF clots acted as successful scaffolds for the regeneration of dentin and pulpal contents in immature teeth with necrotic pulps because of its ability to increase root length, increase dental wall thickness, and healing the periapical lesion better than BC and PRP.(80,85) Meanwhile, in terms of clinical sign and symptom resolution, PRF achieved comparable outcomes to BC in regenerative endodontic therapy.(84) In the therapy of necrotic immature permanent teeth, revascularization/revitalization utilizing PRF also showed to be highly successful.(87)

When being combined with stem cells, PRP and PRF also show better regeneration potential. Human DPSCs was co-cultured with 10% of PRP showed higher expression levels of fetal liver kinase (Flk)-1, VEGF, PDGF, and stromal cell-derived factor 1 (SDF-1) mRNA compared with the combination of hDPSCs and fetal bovine serum (FBS). This suggests that PRP can promote vasculogenesis better than FBS in hDPSCs culture.(115) Both combinations of hDPSCs + PRP and hDPSCs + liquid-PRP showed

significant increase of cell migration, proliferation, and differentiation compared with hDPSCs only. Though in hDPSCs + liquid-PRF, the cell migration was observed faster than hDPSCs + PRP.(116)

Natural-derived Biomaterial Scaffolds

Natural-derived polymers are usually used as biomimetic materials for scaffold in regenerative endodontic therapy. Most of the natural polymers are bioactive, containing cellular binding motifs, thus promoting cell adhesion, and/or present soluble signaling factors that are capable in regulating cell behaviour. Hence they are also known to provide better biocompatibility compared with synthetic polymers.(96,117) Natural polymers are also known to be rapidly degradable compared with other types of scaffolds, thus allowing easier replacement with natural tissues after the degradation.(110,118) Natural polymers consist of natural polypeptides of the ECM, such as collagen, fibrin, gelatin and keratin, as well as polypeptides that are chemically similar to natural glycosaminoglycans, such as alginate, chitosan and hyaluronic acid (HA).(96)

For the regeneration of pulp and dentin-like tissue, polymers like collagen, gelatin, fibrin, chitosan, and HA have shown the ability to improve root development, including increase root length, root thickness, and enhance the mineralization of root canal.(73,89,91,93,94,96-98) While being used as a single scaffold, those natural polymers also showed better ability in increasing intracanal connective tissue formations and narrowing apical width compared with BC, healing the periapical lesion, increasing dental wall thickness, as well as resuming the maturation process for the immature teeth.(73,88-90,93,98)

Natural polymers are often combined and crosslinked with other bioscaffold or chemical agents to improve its potential in regenerative therapy.(119) Dental pulp regeneration through cell homing approaches can be improved by using the combination of HA hydrogel and BC, as well as combination of chitosan hydrogel and BC scaffolds.(73,120) Meanwhile, to fill root canal space with new vital tissue and to enhance the root canal mineralization, the combination of gelatin sponge and BC scaffold as well as collagen and BC scaffold can be used, and have shown better results compared with BC scaffold only.(92,93) To enhance scaffolds physical properties, the crosslinking between collagen hydrogel and cinnamaldehyde (CA) had shown to be successful. It resulted in the enhanced physical properties of collagen by CA, which upregulated the cellular adhesion compared with the collagen only. This means that this property was promoted in the presence of CA.(121)

In terms of its vascularization function, while being used as a single bioscaffold, both fibrin and HA have shown the potential of increasing vascularization better than the control.(73,97) On the other hand, chitosan, when being used alone, does not show vascularization potential, however when being combined with sodium hyaluronate or pectin, both combinations were able to increase vascularization of connective tissues.(95)

Besides its advantages in dental-pulp regeneration and vascularization, natural-derived bioscaffolds that are classified into moldable porous scaffold, such as chitosan and collagen as single scaffold, or even combination of gelatin/collagen hydrogens bioscaffold, also have the ability to promote cell adhesion, migration and proliferation.(96,110,119,122) And to induce hDPSCs cell migration, adhesion, and proliferation, which later followed by a culminated amount of mineralized matrix, scaffold from chitosan and collagen matrix can also be combined with calcium-aluminate.(123) In the combination with SCAP, cell viability promotion, mineralization, and odontoblastic-like differentiation can also be achieved by using HA-based injectable gel scaffold.(124)

Synthetic Biomaterial Scaffolds

While natural-derived polymers scaffolds offer good biocompatibility and bioactivity, synthetic polymers scaffolds offer more flexible and controllable physical and mechanical properties to fit for specific applications.(76,125) Polylactic acid (PLA) and polyglycolic acid (PGA), as well as their copolymers such as poly-L-lactic acid (PLLA), polylactic-polyglycolic acid (PLGA), and polycaprolactone (PCL) have been successfully reported as bioscaffold for regenerative endodontics therapy.(74)

Synthetic polymers scaffolds and its combination with other scaffold materials are able to induce pulp-dentin regeneration. The increase of mineralization, as well as tissue and bone formation, can be reached by using the combination of PLGA and magnesium scaffold, PLLA combined with DPSC or minced-pulp mesenchymal stem cell (MSC), as well as combination of PCL and PDLSC.(100,103,104) Other than that, culture of hDPSCs on either side of PLGA scaffold was also able to enhance surface closing in the opened side of scaffold. Meanwhile, in terms of pulp vascularization and neurogenesis, the enhancement of neurovascular regeneration through angiogenic and neurogenic paracrine secretion has been reported after the therapy with PLGA scaffold on hDPSCs culture.(102)

PLLA and PLGA scaffolds while being cultured in DPSC are able to improve DPSC differentiation and

proliferation, it also induces longer cell replicative lifespan.(99,100,102) PLLA scaffold was also used for human minced-pulp MSC, and the results found that the combination showed even better ability to increase cell differentiation and replication better than in DPSC.(100) Although not being used as scaffold as much as PLLA and PLGA, the use of PCL scaffolds in SCAP and hDPSCs seeding were also reported to be able to increase the cell proliferation and differentiation.(105,126)

A PLGA microsphere combined with hDPSCs, was able to increase hDPSCs proliferation and adhesion to the scaffold, as well as increase expression levels of *DMP1*, *DSPP*, *COL1*, and *OPN* genes.(101) Meanwhile, increased expression of *DMP1*, *DSPP*, runt-related transcription factor 2 (*RUNX2*), osteocalcin (*OCN*), secreted phosphoprotein 1 (*SPP1*), collagen type I alpha 1 (*COL1A1*), and growth differentiation factor 5 (*GDF5*) genes was obtained with the combination of PCL and fluorapatite.(105) In the construction of dental and periodontal pulp for the preservation of periodontal ligament fibroblasts (PDLF), the use of PLGA scaffold combined with PRF has shown the ability to sustain fibroblast viability.(74,127)

Role of Signaling Molecules in Regenerative Endodontics

Various signaling molecules, including growth factors and cytokines have been recognized to enhance the proliferation, migration and differentiation of dental stem cells. These molecules are naturally contained in the pulpal cells and dentin matrix, and involved in modulating dentin-pulp complex homeostasis.(128) In the pulp-dentin regeneration process, the remaining periapical and pulpal cells, adjacent dentin, or implanted platelet concentrates, blood clot scaffold, or stem cells are responsible for the release of signaling molecules. To accelerate the process, exogenous signaling molecules are often delivered together with dental stem cells in a scaffold. Addition of signaling molecules to transplanted dental stem cells is expected to mimic the signaling cascades that occur during the formation of pulp-dentin complex.(129)

Signaling Molecules Related to Cell Migration

Bone morphogenetic protein (BMP)-2, TGF-β1, basic FGF (bFGF), PDGF, VEGF, NGF, and BDNF have been reported to stimulate cell migration (Table 4). Induction of cell migration by these molecules is important, since cells must reach the damaged sites to regenerate the tissues. Several signaling pathways have been identified to be induced by these molecules in stimulating cell migration. For example, via PDGFR-β/Akt pathway, PDGF contributes in recruiting smooth muscle cells to blood vessels (168); BDNF accelerates DPSCs migration via extracellular signal-regulated kinase (Erk) phosphorylation (193); VEGF increases the migration of DPSCs through VEGF receptor (VEGFR) 2 activation and its downstream focal adhesion kinase (FAK) / phosphoinositide 3-kinase (PI3K) / Akt and p38 signaling.(181,182)

Signaling Molecules Related to Cell Proliferation

After reaching the damaged sites, cells must proliferate to increase the number of cells. BMP-2, TGF-β1, bFGF, PDGF and VEGF have been reported to increase proliferation (Table 4). However, the proliferation process is inhibited when cells start to enter the differentiation stage. Thus, signaling molecules which have proliferation-related functions may both inhibit proliferation and induce differentiation in a specific time point, as discussed in the subsequent sections. Several signaling pathways have been identified to be induced by these molecules in stimulating cell proliferation. BMP-2-induced cell proliferation involves BMP-2 receptor (BMP2R) activation as well as Erk1/2 and small mothers against decapentaplegic (Smad) 1/5 phosphorylation (131), while bFGF modulates the expression of cyclin B1 (CCNB1) and cell division control 2 (CDC2), which are related to cell-cycle regulation via mitogen-activated protein kinase kinase (MEK)/Erk pathway.(154) VEGF activates the Akt signaling pathway and increases cyclin D1 expression levels, which in turn promotes proliferation of DPSCs.(182)

Signaling Molecules Related to Dentinogenesis and Pulp Regeneration

BMP-2, TGF-β1, bFGF, PDGF, VEGF, and NGF have been reported to enhance dentinogenesis (Table 4). These molecules have been demonstrated to increase differentiation and mineralization of both dental pulp cells and dental stem cells as indicated by an increase in alkaline phosphatase (ALP) activity and mineralization, as well as upregulation of osteo-/odontogenic marker expression *in vitro*.(132,151,157,170,188) *In vivo*, these molecules are observed to stimulate dentin formation. (132,145,159,171,187)

TGF-β1 has been demonstrated to enhance ALP activity via activation of Smad2/3, TGF-β activated kinase 1 (TAK1), as well as Erk1/2 and p38.(148) BMP-2 has been known to induce phosphorylation of Erk1/2 and Smad1/5. (131) bFGF could induce mitogen-activated protein kinases

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Table 4. Regenerative potential of signaling molecules in pulp-dentin regeneration.

Signaling Molecule	Regenerative Potential					Reference
	Cell Migration	Cell Proliferation	Pulp- and/or Dentin-like Tissue	Angiogenesis	Neurogenesis	
BMP-2	Inducing migration of dental pulp cells	Increasing proliferation of dental pulp cells	• Increasing ALP activity and mineralization • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *ALP*, *RUNX2*, *COL1*, *DSPP*, *DMP1*, *DSP*, *MMP20*, *BSP*, *OCN*, and *OSX* - Protein expression: RUNX2, DSPP, DMP1, BSP, and OCN	N/A	N/A	(130-141)
TGF-β1	Inducing migration of dental pulp cells	Increasing proliferation of DPSCs and dental pulp cells	• Increasing ALP activity, mineralization, and collagen content • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *DSPP*, *DSP*, *MMP20*, *RUNX2*, *DMP1*, *COL1A1*, and *BSP* - Protein expression: N-cadherin, TIMP1, COL1A1, DMP1, and BSP • Downregulating protein expression: MMP3	• Inducing smooth muscle cell differentation • Maintaining blood vessels stability • Upregulating differentiation markers - Gene expression: *αSMA, SM22α, CALP, SMTN*, and *MYH11* - Protein expression: αSMA, SM22α, CALP, SMTN, ANGPT1, Tie2, and MYH11	N/A	(137,142-151)
bFGF	Inducing migration of SCAP, mobilized DPSCs, BMMSCs, periodontal ligament fibroblasts, and endothelial cells	Increasing proliferation of SHED, DPSCs, mobilized DPSCs, BMMSCs, dental pulp cells, periodontal ligament fibroblasts, and endothelial cells	• Increasing ALP activity and mineralization • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *DSPP*, *MMP20*, *TRH-DE*, *ALP*, *TIMP1*, *DMP1*, *COL1A2*, *OPN*, and *OCN* - Protein expression: DSPP, DMP1, TIMP1, and COL1	• Enhancing blood vessel formation • Upregulating differentiation markers - Gene expression: *VEGFR2*, *Tie2*, *ANGPT1*, *VWF*, *VE-cadherin*, and *CD31* - Protein expression: VEGFR2, Tie2, ANGPT1, VWF, VE-cadherin, and CD31	• Inducing neuronal and glial differentation • Promoting axonal sprouting and growth • Upregulating differentiation markers - Gene expression: *Nestin*, *TUBB3*, *Sox2*, *VIM*, *NEFM*, *MAP2*, *NEFH*, *GFAP*, and *S100B* - Protein expression: Nestin, NEFM, TUBB3, NeuN, GFAP, S100B, and MAP2	(152-168)
PDGF	Inducing migration of DPSCs, SHED, dental pulp cells, and smooth muscle cells	Increasing proliferation of DPSCs	• Increasing ALP activity and mineralization • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *DMP1*, *DSPP*, and *OCN* - Protein expression: DMP1 and DSPP	• Inducing smooth muscle and endothelial cell differentation • Enhancing blood vessel formation • Promoting blood vessel stabilization • Upregulating differentiation markers - Gene expression: *αSMA*, *SM22α*, *CALP*, *SMTN*, and *MYH11* - Protein expression: αSMA, SM22α, CALP, SMTN, VEGFR2, Tie2, CD31, and VE-cadherin	N/A	(142,150, 168-174)

Table 4. Regenerative potential of signaling molecules in pulp-dentin regeneration (cont).

Signaling Molecule	Regenerative Potential					Reference
	Cell Migration	Cell Proliferation	Pulp- and/or Dentin-like Tissue	Angiogenesis	Neurogenesis	
VEGF	Inducing migration of DPSCs and endothelial cells	Increasing proliferation of DPSCs and dental pulp cells	• Increasing ALP activity and mineralization • Upregulating odontoblast markers - Gene expression: *ALP*, *OCN*, *OSX*, *DSPP*, *RUNX2*, *DMP1*, *COL1A2*, *BSP*, *TGFB1*, and *OPN* - Protein expression: DMP1, DSPP, and OSX	• Inducing endothelial cell differentation • Enhancing blood vessel formation • Promoting blood vessel anastomosis • Upregulating differentiation markers - Gene expression: *VWF*, *VEGFR2*, *VE-cadherin*, *CD31*, *VEGFR1*, *EphrinB2*, Tie2, and *ANGPT* - Protein expression: VWF, VEGFR2, VE-cadherin, CD31, Tie2, F8	N/A	(130,136,157, 162,175-188)
NGF	Inducing migration of glial cells	N/A	• Improving pulpal architecture and cell organization • Upregulating gene expressions of differentiation markers: *DSPP*, *DMP1*, and *TGFB1*	N/A	• Inducing neuronal and glial differentation • Promoting axonal sprouting and growth • Upregulating differentiation markers - Gene expression: *Nestin* - Protein expression: S100, neurofilament, and p75NTR	(156,188-191)
BDNF	Increasing migration of DPSCs	N/A	N/A	N/A	• Inducing neuronal and glial differentation • Upregulating protein expressions of differentiation markers: DCX, NeuN, S100B and p75NTR.	(192,193)

N/A: Not applicable; ALP: Alkaline phosphatase; RUNX2: Runt-related transcription factor 2; COL1: Collagen type I; DSPP: Dentin sialophosphoprotein; DMP1: Dentin matrix acidic phosphoprotein 1; DSP: Dentin sialoprotein; MMP: Matrix metalloproteinase; BSP: Bone sialoprotein; OCN: Osteocalcin; OSX: Osterix; COL1A1: Collagen type I alpha 1; TIMP1: Tissue inhibitor of metalloproteinase 1; αSMA: Alpha smooth muscle actin, SM22α: Smooth muscle protein 22 alpha, CALP: Calponin, SMTN: Smoothelin, ANGPT: Angiopoietin, MYH11: Myosin heavy chain 11; TRH-DE: thyrotropin-releasing hormone-degrading enzyme; OPN: Osteopontin; VEGFR: vascular endothelial growth factor receptor; VWF: von Willebrand factor; TUBB3: tubulin beta III ; Sox2: sex determining region Y-box 2; VIM: Vimentin; NEFM: Neurofilament medium chain; MAP2: Microtubule associated protein 2; NEFH: Neurofilament heavy chain; GFAP: Glial fibrillary acidic protein; S100: S100 calcium binding protein; NeuN: Neuronal nuclei; TGFB1: Transforming growth factor beta 1; F8: Coagulation factor VIII; p75NTR: p75 neurotrophin receptor; DCX: Doublecortin.

(MAPKs) (p38, JNK, and Erk), PI3K/Akt, protein kinase C (PKC), and NF-κB (194), BMP or Wnt signaling.(195) Meanwhile, VEGF has been known to activate Akt, MAPKs (p38, JNK, and Erk), and NF-κB.(157)

Intriguingly, induction of differentiation and mineralization by TGF-β1 and BMP-2 is often associated with a decrease in cell proliferation (136,151). In addition, TGF-β1 increases the expression of early marker genes of odonto-/osteo-genic differentiation and decreases the expression of late-stage mineralization genes.(151) VEGF might not be able to trigger full osteo-odontogenic differentiation, and facilitate only the early stage of cell differentiation.(187) VEGF potential in inducing mineralization is lower compared with bFGF (157) and NGF.(188) The potential of PDGF in enhancing hard tissue formation has been shown to be lower than other materials, such as enamel matrix derivative (EMD) and mineral trioxide aggregate (MTA).(196) Furthermore, PDGF-BB has been reported to inhibit the formation of mineral nodules.(14) Therefore, PDGF should be used in combination with other materials to increase the mineralization potential.(171,172) However, studies regarding signaling pathways that are involved in PDGF and NGF-induced dentin formation are limited.

bFGF, TGF-β1, and NGF are known to contribute to pulp regeneration (Table 4). bFGF regulates growth of dental pulp cells, upregulates the expression of CDC2, CCNB1, and tissue inhibitor of metalloproteinase 1 (TIMP1), as well as inhibits ALP activity and collagen I production through activation of FGF receptor (FGFR) and MEK/Erk signaling.(154) Meanwhile, TGF-β1 has been demonstrated to increase TIMP1 production, collagen content, and procollagen I, but slightly attenuate MMP3 production, which are related to the activation of activin receptor-like kinase-5(ALK5)/Smad2/3, TAK1, MEK/Erk, and p38 signaling.(143,148) NGF has been reported to upregulate the expression of healing and repair-related genes (188), as well as improve pulp cell organization and pulpal architecture.(189) Thus, bFGF, TGF-β1 and NGF are involved in pulp regeneration by altering matrix turnover and dental pulp cell proliferation, as well as modulating pulp repair-related gene expression.

Signaling Molecules Related to Angiogenesis

VEGF, PDGF, bFGF, and TGF-β1 have been reported to induce angiogenesis (Table 4) by promoting differentiation of dental stem cells toward endothelial (162,175) or smooth muscle cells (149,150), as shown by upregulation of several differentiation genes.(144,150,162) These signaling molecules also induce the formation of capillary-like structures, both *in vitro* (162,170,175) and *in vivo*.(170,176) VEGF has been demonstrated to accelerate angiogenesis, since angiogenesis could occur even in the absence of this molecule.(176) This molecule induces angiogenesis by inducing VEGFR phosphorylation and activating downstream Akt, MAPKs (p38, JNK, and Erk), NF-κB.(157) Besides formation of new blood vessels, VEGF has been reported to induce anastomosis of DPSCs-derived blood vessels by increasing vascular endothelial (VE)-cadherin expression through the activation of MEK1/Erk, which in turn causes E-26 transformation-specific-related gene (ERG) transcription factor binds to *VE-cadherin* promoter.(184) VEGF-induced angiogenesis could be enhanced by inhibiting specific pathways or combining it with other molecules. Combination of VEGF with SB-431542, an inhibitor of TGF-β1 signaling, has been shown to markedly promote SHED differentiation toward endothelial cells, since Smad1/2 inhibition is correlated with VEGFR2 activation.(175) IGF-1 (182) and SDF-1α (179) were also reported to have a synergistic effect in enhancing angiogenesis when combined with VEGF.

PDGF-BB alone induces capillary sprouting, and this phenomenon could be enhanced by bFGF.(168) bFGF alone could induce angiogenesis, but its angiogenic potential is lower than VEGF.(157) PDGF-BB has been reported to promote blood vessels maturation by regulating the investment of smooth muscle cells to DPSCs-derived capillaries through PDGFRβ and Akt phosphorylation in both types of cells.(168) In addition, DPSCs-derived smooth muscle cells that are produced after TGF-β1 treatment have been reported to stabilize blood vessels through ANGPT1/Tie2 and VEGF/VEGFR2 signaling.(149) Combination of PDGF-BB and TGF-β1 induces the expression of smooth muscle-specific early, mid, and late markers, as well as enhances contraction ability in DPSCs, although the cells do not undergo morphological alterations toward smooth muscle-specific cell shapes.(150)

Signaling Molecules Related to Neurogenesis

NGF, BDNF and bFGF have been reported to induce neurogenesis (Table 4). In several neurogenesis induction studies, NGF and BDNF are combined with other neurotrophin and non-neurotrophin signaling molecules.(156,188,193) Meanwhile, bFGF is usually combined with epidermal growth factor (EGF) for neural induction.(167) Addition of these molecules increases the expression levels of neural markers and promotes morphological alterations of the treated cells toward neuronal and glial cells.

(156,192,197) These molecules have also been reported to induce axonal sprouting and promote axonal growth. (167,191)

NGF and BDNF induce neurogenesis via non-specific activation of p75 neurotrophin receptor (p75NTR). In addition, NGF specifically activates tropomyosin-related kinase A (TrkA), while BDNF specifically activates TrkB.(198) Meanwhile, bFGF induces neurogenesis via activation of FGFR (199). Activation of these receptors have been reported to induce the phospholipase C (PLC)-γ pathway, which in turn promotes neuronal differentiation. (198,199) Besides, combination of bFGF and NGF also stimulates neuronal differentiation via PI3K/Akt and Erk pathways.(156)

Future Perspectives on the Use of Dental Stem Cells, Scaffold, and Signaling Molecules Combination in Regenerative Endodontics

Numerous studies have reported successful pulp-dentin complex regeneration using specific combinations of dental stem cells, scaffold, and signaling molecules. Despite most of the ongoing regenerative endodontics studies using these combinations are conducted in animal models (23,200), these combinations are also reported to induce pulp-dentin regeneration in human subjects. Several examples of dental stem cells, scaffold, and signaling molecules combination that have been known to regenerate human pulp-dentin complex are combination of hpDPSCs, G-CSF, and atelocollagen scaffold (31,32), as well as combination of DPSCs and L-PRF (30), which acts as scaffold and contains PDGF and TGF-β.(201) Indeed, the regenerative endodontics field is constantly growing. There will be new findings and innovation regarding dental stem cell biology, the development of new types of scaffolds, and the best way to deliver stem cells and signaling molecules to the root canal, which open a new perspective on a new era of endodontic therapy. Thus, current trends and future directions on regenerative endodontics should be further explored.

In most pulp-dentin regeneration studies using human subjects, a scaffold that already contains dental stem cells and immobilized signaling molecules is directly transplanted to the root canal in a single appointment.(30-32) Despite the success of this current protocol in regenerating functional pulp-dentin complex, the current procedure might not be similar to the natural process of pulp-dentin regeneration, which involves specific cellular processes. Additionally, regeneration of the pulp-dentin complex may be incomplete in some patients due to differences in pulp-dentin damage severity. To achieve complete pulp-dentin regeneration, additional dental stem cells and/or signaling molecules could be applied in the several next appointments. Since scaffolds have different physical characteristics and biocompatibility, different types of scaffolds could be used to facilitate pulp-dentin regeneration in different parts of teeth. Different types of dental stem cells, signaling molecules, and scaffolds could also be combined with other endodontic procedures, such as apexification and pulp revascularization (202) to enhance the regeneration process in different parts of teeth. Therefore, dental stem cell, scaffold, and/or signaling molecules application could be performed in multiple appointments to mimic the cellular processes that are involved in the regeneration process. Hence gradual pulp-dentin regeneration could be achieved.

Although studies regarding tissue engineering-based pulp-dentin regeneration show promising results, there are several challenges for its future clinical translation that need to be addressed. Regenerated pulp-dentin complex should have a precise and highly ordered histological structure as compared to that in normal teeth.(4) Besides, different oral diseases, such as irreversible pulpitis and necrotic pulp, as well as the presence of residual bacteria and lipopolysaccharide may affect the root canal microenvironment, which in turn alter the fate of transplanted dental stem cells.(203,204) Other factors, including age and the presence of systemic diseases might also affect regeneration potential of stem cells.(4,205) Since each type of dental stem cell, scaffold, and signaling molecule has unique characteristics and functions, they can be utilized to address these challenges by combining these components together to achieve successful regeneration. Thus, the right combination of dental stem cells, scaffolds, and signaling molecules is needed to enhance the pulp-dentin regeneration process.

Conclusion

Combinations of dental stem cells, scaffold, and signaling molecules mimic the cellular microenvironment that is suitable for regeneration. Hence, they are important to achieve the functional pulp-dentin complex formation. Since regenerative endodontics is a constantly growing field, current trends and future directions in this field are still needed to be further explored. The right combination of dental stem cells, scaffolds, and signaling molecules

could be determined based on the patients' characteristics. Incomplete pulp-dentin regeneration, which may occur in some cases, could be overcome by applying dental stem cells, scaffolds, and/or signaling molecules in multiple appointments to achieve gradual pulp-dentin regeneration.

Authors Contribution

FS, AS, WA, WW proposed the manuscript topic. All Authors were involved in the drafting and manuscript writing process. FS, MC, NMD, SJAI were involved in the manuscript revisions. FS supervised the manuscript. All authors finalized the last version of the manuscript.

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REVIEW ARTICLE

Crucial Triad in Pulp-Dentin Complex Regeneration: Dental Stem Cells, Scaffolds, and Signaling Molecules

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Abstract

BACKGROUND: Pulp damage can lead to dentinogenesis impairment, irreversible pulpitis, or pulp necrosis. Despite being the most used endodontic procedure to treat damaged pulp, root canal therapy only results in nonvital teeth which are prone to fractures and secondary infection. Pulp-dentin regeneration has a potential to regenerate structure similar to normal pulp-dentin complex, and can be achieved by combining dental stem cells, scaffold, and signaling molecules. This article reviews the role of various types of dental stem cells, scaffolds, signaling molecules, and their combinations in regenerating pulp-dentin complex.

CONTENT: Dental pulp stem cell (DPSC), stem cell from human exfoliated deciduous teeth (SHED), and dental follicle stem cell (DFSC) were reported to regenerate pulp-dentin complex *in situ*. SHED might be more promising than DPSCs and DFSCs for regenerating pulp-dentin complex, since SHED have a higher proliferation potential and higher expression levels of signaling molecules. Scaffolds have characteristics resembling extracellular matrix, thus providing a suitable microenvironment for transplanted dental stem cells. To accelerate the regeneration process, exogenous signaling molecules are often delivered together with dental stem cells. Scaffolds and signaling molecules have different regenerative potential, including induction of cell proliferation and migration, formation of pulp- and/or dentin-like tissue, as well as angiogenesis and neurogenesis promotion.

SUMMARY: Combinations of dental stem cells, scaffold, and signaling molecules are important to achieve the functional pulp-dentin complex formation. Current trends and future directions on regenerative endodontics should be explored. The right combination of dental stem cells, scaffold, and signaling molecules could be determined based on the patients' characteristics. Incomplete pulp-dentin regeneration could be overcome by applying dental stem cells, scaffold, and/or signaling molecules in multiple visits.

KEYWORDS: pulp-dentin regeneration, regenerative endodontics, dental stem cells, scaffold, signaling molecules

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Introduction

Dental pulp, the only soft tissue in the tooth, plays a critical role in sustaining tooth homeostasis. However, this tissue is vulnerable to various stimuli, including infections, iatrogenic causes, and trauma.(1) If not treated properly, pulp damage can lead to dentinogenesis impairment and irreversible pulpitis or even pulp necrosis, since this tissue has a limited self-repair capacity.(2)

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REVIEW ARTICLE

Crucial Triad in Pulp-Dentin Complex Regeneration: Dental Stem Cells, Scaffolds, and Signaling Molecules

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Dental pulp, the only soft tissue in the tooth, plays a critical role in sustaining tooth homeostasis. However, this tissue is vulnerable to various stimuli, including infections, iatrogenic causes, and trauma.(1) If not treated properly, pulp damage can lead to dentinogenesis impairment and irreversible pulpitis or even pulp necrosis, since this tissue has a limited self-repair capacity.(2)

Damaged pulp can be treated by several procedures. Root canal therapy, the most used endodontic procedure,

replaces inflamed or injured pulp with bioinert material fillings. However, this procedure results in nonvital teeth, which are prone to fractures and secondary infection. (3) Regenerative endodontic treatment or pulp-dentin regeneration is an alternative procedure based on the tissue engineering principle. Pulp-dentin regeneration is more holistic than other endodontic procedures since this procedure has a potential to regenerate structure similar to normal pulp-dentin complex. The main goals of pulp regeneration are pulp-dentin complex formation as well as angiogenesis and neurogenesis in the newly regenerated pulp.(4)

Tissue engineering combines dental stem cells, scaffold, and signaling molecules to mimic a suitable microenvironment for regenerating pulp-dentin complex. Numerous studies have been established to examine the effects of dental stem cells, scaffold, signaling molecules, and their combinations in pulp regeneration, providing a new insight in the field of regenerative dentistry and opening a great opportunity for further clinical applications. This article reviews the role of various types of dental stem cells, scaffolds, signaling molecules, and their combinations in regenerating pulp-dentin complex. The right combination of these components could increase pulp-dentin regeneration therapy efficiency.

Role of Dental Stem Cells in Regenerative Endodontics

Based on the locations, dental stem cells are classified as dental pulp stem cell (DPSC), stem cells from human exfoliated deciduous teeth (SHED), stem cells from the apical papilla (SCAP), dental follicle stem cell (DFSC), periodontal ligament stem cell (PDLSC).(5,6) DPSCs, SHED, and DFSCs were reported to have potential in regenerating pulp-dentin complex *in situ*, both in animal models (Table 1) and human subjects (Table 2).

Cell Number, Proliferation Rate, and Immunomodulatory Properties of DPSCs, SHED and DFSCs

DPSCs, SHED and DFSCs are different in several aspects, including the number of cells isolated from the tissues, proliferation rate, and immunomodulatory mechanisms. DPSCs and SHED have relatively high cell numbers in original cultures compared with DFSCs, because dental pulp, both in permanent and deciduous teeth, have relatively high amounts of stem cells compared with dental follicles of developing tooth germ. Since dental follicle tissues are smaller in size, located in sites that are relatively not easy to be accessed, and contain small amounts of cells, DFSCs are difficult to be obtained and distinguished from other types of dental stem cells.(4)

DPSCs have been reported to have a higher proliferation rate compared with bone marrow mesenchymal stem cell (BMMSC), while SHED have a higher proliferation rate than DPSCs.(33) It has been demonstrated that the proliferation rate of DFSCs is notably higher than DPSCs. (34) Moreover, in a recent study, DFSCs were shown to have a higher proliferation rate than SHED.(35) Thus, DFSCs might have the highest proliferation rate, followed by SHED and DPSCs. High proliferation of DFSCs implies that they are more immature, since this type of stem cells are isolated from developing tissues (36), and consequently they might be more plastic compared with other dental stem cells. In summary, DPSCs, SHED, and DFSCs vary in their proliferation rates, which could be determined by the developmental stages of the stem cell sources.

Mesenchymal stem cell (MSC), including DPSCs, SHED and DFSCs have been reported to modulate the immune system through several mechanisms.(37) DPSCs have been demonstrated to modulate the adaptive and innate immune responses through interaction with B cells, T cells, macrophages, dendritic cells (DCs), and natural killer (NK) cells. For instance, the production of B cell immunoglobulin and proliferation of T cell proliferation are inhibited in co-culture of peripheral blood mononuclear cells (PBMCs) and DPSCs. Transforming growth factor (TGF)-β secreted by DPSCs plays a crucial role in this inhibition and the addition of interferon (IFN)-γ to DPSCs culture enhances the inhibitory effects.(38) DPSCs markedly decrease $CD4^+$ and $CD8^+$ T cell proliferation, irrespective of hypoxia-inducible factor (HIF)-1α expression level in DPSCs. However, overexpression of HIF-1α increases the DPSCs inhibitory effect on DCs proliferation. Expression of HIF-1α by DPSCs also enhances the recruitment and differentiation of macrophages with M2 characteristics. Furthermore, NK cell-mediated cytotoxicity is suppressed in HIF-1α-overexpressed DPSCs.(39)

SHED have been shown to modulate T cell, macrophages and DCs. This type of stem cell restrains the differentiation of T helper (Th) 17 cells, and has greater immunomodulatory potential compared with BMMSCs. (40) SHED have been reported to promote phenotypic polarization of macrophage toward M2-like phenotype in transwell co-culture systems and increase the number of macrophages with M2-like phenotype in rat model of periodontitis.(41) A study demonstrates that SHED affect

Table 1. Regenerative potential of DPSCs, SHED, and DFSCs in animal model of pulp-dentin regeneration.

Type of Dental Stem Cells	Species	Regenerative Potential			Reference
		Pulp- and/or Dentin-like Tissue	Angiogenesis	Neurogenesis	
DPSC	Dog	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DSPP	Histology: Blood vessels in regenerated pulp	N/A	(7-10)
	Mini-pig	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DSP, DMP1, and BSP	Histology: Blood vessels in regenerated pulp	N/A	(11)
	Ferret	Histology: Formation of osteodentin mixed with loose connective tissue.	N/A	N/A	(12)
	Rat	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DMP1, DSPP, DSP, and OPN	Histology: Blood vessels in regenerated pulp Positive immunostaining: CD31	N/A	(13-15)
DPSC $CD31^+$	Dog	Histology: - Pulp tissue regeneration - Dentin formation Gene expression: *MMP20, syndecan 3, TRH-DE*	Positive immunostaining: BS-1 lectin	Positive immunostaining: PGP9.5	(16)
DPSC $CD105^+$	Dog	Histology: Pulp tissue regeneration	Histology: Blood vessels in regenerated pulp	N/A	(17)
Mobilized DPSC	Dog	Histology: - Pulp tissue regeneration - Dentin formation Gene expression: *tenascin C*, *syndecan 3*, *TRH-DE*, *MMP20*, *DSPP* Positive immunostaining: TRH-DE MRI: Signal intensity of transplanted teeth was similar compared with that in normal teeth.	Positive immunostaining: BS-1 lectin Laser Doppler flowmetry: Blood flow in regenerated pulp tissue is similar compared to that in normal pulp tissue.	Positive immunostaining: PGP9.5 Electric pulp test: Positive pulp sensibility response	(18-25)
hpDPSC	Dog	Histology: - Pulp tissue regeneration - Dentin formation	Positive immunostaining: BS-1 lectin	Positive immunostaining: PGP9.5	(25,26)
hpDPSC from deciduous teeth	Dog	Histology: - Pulp tissue regeneration - Dentin formation	Positive immunostaining: BS-1 lectin	Positive immunostaining: PGP9.5	(26)
SHED	Mini-pig	Histology: - Pulp tissue regeneration - Dentin formation	Histology: Blood vessels in regenerated pulp Positive immunostaining: CD31	Positive immunostaining: NeuN, neurofilament, CGRP, and TRPV1	(27,28)
DFSC	Mini-pig	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DMP-1, DSPP, COL1, COL3	N/A	N/A	(29)

N/A: Not applicable; DSPP: Dentin sialophosphoprotein; DSP: Dentin sialoprotein; DMP1: Dentin matrix acidic phosphoprotein 1; BSP: Bone sialoprotein; OPN: Osteopontin; MMP20: Matrix metalloproteinase 20; Thyrotropin-releasing hormone-degrading enzyme: TRH-DE; BS-1 lectin: *Bandeiraea simplicifolia* lectin 1; PGP9.5: Protein gene product 9.5; NeuN: Neuronal nuclei; CGRP: Calcitonin gene-related peptide; TRPV1: Transient receptor potential cation channel subfamily V member 1; COL1: Collagen type I; COL3: Collagen type III.

Table 2. Regenerative potential of DPSCs, SHED, and DFSCs in case reports and clinical trials of pulp-dentin regeneration.

Type of Dental Stem Cells	Regenerative Potential			Reference
	Pulp- and/or Dentin-like Tissue	Angiogenesis	Neurogenesis	
DPSC	CBCT: - Formation of dentin bridge - Apical canal calcification	Laser Doppler flowmetry: Blood perfusion in the transplanted tooth with low mean perfusion unit.	N/A	(30)
Mobilized DPSC	MRI: Complete pulp regeneration CBCT: - Formation of lateral dentin - Decrease in dental pulp volume	N/A	Electric pulp test: Positive pulp sensibility response	(31)
hpDPSC	MRI: Complete pulp regeneration CBCT: - Formation of lateral dentin - Decrease in dental pulp volume	N/A	Electric pulp test: Positive pulp sensibility response	(32)
SHED	Histology: Regenerated pulp with odontoblast layer, connective tissue, and blood vessels. CBCT: Increase in dentin thickness	Laser Doppler flowmetry: An increase in vascular formation as indicated by high perfusion units.	Positive immunostaining: NeuN Electric pulp test: Positive pulp sensibility response	(27)

N/A: Not applicable; CBCT: Cone beam computed tomography; MRI: Magnetic resonance imaging; NeuN: Neuronal nuclei.

differentiation, maturation, and T cell activation ability of DCs. The same study also shows that SHED augment T regulatory (Treg) cell induction ability of DCs. SHED-treated DCs have a lower level of IFN-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-2, as well as higher level of IL-10.(42)

DFSCs have immunomodulatory properties toward T cells and macrophages. A study demonstrates that DFSCs increase the number of Treg cells as well as suppress $CD4^+$ T cell proliferation via TGF-β and indoleamine 2,3-dioxygenase (IDO) pathways.(43) In lipopolysaccharide (LPS)-induced macrophage, this type of stem cell is involved in phenotypic polarization to M2 by secreting thrombospondin-1 and TGF-β3.(44) Therefore, the immunomodulatory activities of DPSCs are exerted on B cells, T cells, macrophages, DCs, and NK cells. SHED regulates T cells, macrophages and DCs, while DFSCs show immunomodulatory activities toward T cells and macrophages.

DPSCs, SHED and DFSCs Play a Crucial Role in Regenerating Pulp-dentin Complex

Dental stem cells are involved in pulp-dentin complex formation *in situ*. When transplanted into an emptied root canal or a tooth construct, DPSCs, SHED, and DFSCs generate tissue that has characteristics resembling dental pulp. Several biomarkers have been used to detect the presence of the regenerated pulp, such as thyrotropin-releasing hormone-degrading enzyme (*TRH-DE*), *syndecan 3*, and *tenascin*. Furthermore, magnetic resonance imaging (MRI) can also be utilized to assess pulp regeneration by dental stem cells in the root canal (Table 1, Table 2). After pulpectomy, the signal intensity of MRI is relatively low compared with those in the normal teeth. The signal intensity in the pulpectomized tooth then increases several days after transplantation and keeps decreasing until it is similar to normal pulp, indicating complete pulp regeneration.(21)

Formation of dentin-like structure by DPSCs, SHED, and DFSCs has also been documented by the generation of dentin matrix deposition that causes dentin thickening and the presence of odontoblast-like cells on the canal dentinal walls which express both specific and non-specific odontoblast markers. Specific odontoblasts markers include dentin sialoprotein (DSP), dentin sialophosphoprotein (DSPP) and dentin matrix acidic phosphoprotein (DMP) 1 (13-15), while non-specific odontoblasts markers include bone sialoprotein (BSP) and osteopontin (OPN).(11,14) There are several viewpoints regarding the use of non-specific odontoblasts markers for detecting newly regenerated dentin. Some investigators consider that enhanced expression of these markers suggests greater dentin regeneration potential (8,10,14,15,28) since they are involved in dentin formation. (45) Other investigators consider these markers as

osteogenic markers instead of odontogenic markers.(29,46) The increase in the expression of these markers implies that the regenerated structure has similar characteristics with bone instead of dentin.(46) Therefore, these markers should not be more strongly expressed in dentin than the expression of odontoblast-specific markers.(11,14,29) Besides detection of odontoblast markers, cone beam computed tomography (CBCT) can be used to assess dentin formation (Table 2), which is demonstrated by a reduction in low-density areas, indicating a decrease in pulp volume and an increase in dentin thickness.(32) Studies that use ectopic and semiorthotopic pulp-dentin regeneration models are not included in Table 1, since these models do not provide similar conditions as the human oral cavity.(4)

The research that assesses the ability of DFSCs to form pulp-dentin complex is more limited than those conducted using DPSCs and SHED. DFSCs are usually used to simultaneously regenerate pulp-dentin and cementum-periodontal complexes.(29) This may be caused by the tendency of DFSCs to regenerate periodontal tissue and tooth root rather than pulp-dentin complex. Transplantation of treated dentin matrix that contains DFSCs regenerates periodontal-like tissue in subcutaneous space and cementum-like tissue in the outer surface of dentin.(47) Moreover, combination of DFSCs and treated dentin matrix which is transplanted to the alveolar fossa of rats has a potential to induce root formation.(48) Thus, DFSCs are better to use in periodontal tissue and root regeneration, although they might also have a potential to regenerate pulp-dentin complex. Despite the large number of studies that explore the regenerative potential of DPSCs, SHED might be more promising than DPSCs, since SHED have a higher proliferation potential (33) and higher expression levels of signaling molecules which may contribute to the pulp-dentin regeneration.(49)

DPSCs, SHED and DFSCs are Involved in Angiogenesis

Angiogenesis has been reported to occur in pulp-like tissue regenerated by DPSCs and SHED *in situ*. There are limited studies that demonstrate the involvement of DFSCs in the angiogenesis process in regenerated pulp tissue (Table 1). The angiogenic potential of DFSCs has been reported to be lower compared with DPSCs and SCAP. (50) The new vessels provide oxygen and nutrition to the newly regenerated pulp, thus supporting the survival of the transplanted stem cells and facilitating further regeneration process. Blood vessels in the regenerated pulp can be detected using immunostaining of *Griffonia* (*Bandeiraea*) *simplicifolia* lectin 1 (BS-1 lectin) and CD31 (Table 1).

In addition, laser Doppler flowmetry can be used to assess angiogenesis and analyze the blood flow in the regenerated pulp tissue, as demonstrated by several studies. Blood flow in the pulp tissue regenerated by DPSCs is not remarkably different compared with that in normal pulp tissue, implying complete functional angiogenesis. (18) Human tooth with symptomatic irreversible pulpitis which is treated with DPSCs and normal tooth have low mean perfusion units. Blood perfusion in both teeth is indicated by pulse characteristics.(30) In addition, SHED-transplanted teeth experience an increase in the average of vascular formation.(27)

DPSCs, SHED, and DFSCs are involved in angiogenesis through differentiation toward endothelial cells (28) or angiogenic factors secretion. Several angiogenic factors that are expressed by these stem cells includes vascular endothelial growth factor (*VEGF*) (16,28,29), *HIF1A* (28), granulocyte-monocyte colony-stimulating factor (*GM-CSF*), matrix metalloproteinase 3 (*MMP3*) (16), selectin E (*SELE*) (18), angiopoietin (*ANGPT*), and von Willebrand factor (*VWF*).(15) These factors stimulate vessel formation by modulating local endothelial cells in a paracrine manner. (16) Several subsets of DPSCs have been reported to secrete angiogenic factors but they do not incorporate to the newly formed blood vessels, such as dental pulp $CD31^-$ side population cells (16) and granulocyte colony-stimulating factor (G-CSF) mobilized DPSCs.(18,51)

Angiogenesis in pulp-like tissue can be induced further by culturing dental stem cells under hypoxic conditions. Hypoxia mimics conditions in the dental pulp cavity (52), which increases the expression of *HIF1A*. Upregulation of this transcriptional factor activates the expression of angiogenesis-related genes.(25) Hypoxia culture on nanofibrous spongy microspheres increases angiogenesis potential of human DPSCs (hDPSCs) as indicated by more CD31-stained blood vessels in the regenerated pulp-like tissues.(13) Another research demonstrates that the expression levels of *HIF1A* in hypoxia preconditioned DPSCs (hpDPSCs) are two times higher compared with those in mobilized DPSCs, while *VEGF* expression levels in both DPSCs are similar. hpDPSCs have been demonstrated to have a similar neovascularization potential compared to mobilized DPSCs.(25) DPSCs from permanent and deciduous teeth that are cultured under hypoxic conditions have similar expression levels of *VEGF* and *GM-CSF*, as well as *in situ* neovascularization potential.(26) Furthermore, co-culture of dental stem cells with endothelial cells has also been demonstrated to enhance angiogenesis. Crosstalk between transplanted stem cells with endothelial cells has

been shown to increase the expression of angiogenic factors in both cells by activating specific pathways, such as nuclear factor κB (NF-κB).(53)

DPSCs, SHED and DFSCs are Involved in Neurogenesis

DPSCs, SHED, and DFSCs have a potential to induce neurogenesis, as shown by the studies that reported the presence of nerve fibers in pulp-like tissue after stem cell transplantation. Newly formed nerve fibers in orthotopic pulp regeneration models are detected using immunostaining of protein gene product 9.5 (PGP9.5), neuronal nuclei (NeuN), neurofilament, calcitonin gene-related peptide (CGRP), and transient receptor potential cation channel subfamily V member 1 (TRPV1) (Table 1). The expression of other neurological markers, such as sodium voltage-gated channel alpha subunit 1 (*SCN1A*) and *neuromodulin* genes (16,18), as well as tubulin-βIII (TUBB3) (29), nestin, and transient receptor potential cation channel subfamily M member 8 (TRPM8) protein (27), has also been detected in cultured or subcutaneously implanted stem cells. Electric pulp test is another common technique utilized for detecting nerve fibers in regenerated pulp tissue (Table 1, Table 2).

Mechanisms of neurogenesis induction are similar to the angiogenesis induction by DPSCs, SHED, and DFSCs. These types of stem cells have been reported to differentiate toward neural cells.(54,55). In addition, various neurogenic factors are expressed by DPSCs and SHED, including nerve growth factor (*NGF*), glial cell-derived neurotrophic factor (*GDNF*), brain-derived neurotrophic factor (*BDNF*), neuropeptide Y (*NPY*), and neurotrophin 3 (*NTF3*).(16,56) Investigations on neurogenic factors secreted by DFSCs are still limited. Hypoxic conditions could enhance the expression of neurogenic factors in dental stem cells. *NGF* and *BDNF* expression levels are notably higher in hpDPSCs compared with those in mobilized DPSCs, but *GDNF* expression level is lower. It has been reported that hpDPSCs have a similar reinnervation potential compared to mobilized DPSCs.(25) A recent study revealed that DPSCs from deciduous teeth had a markedly higher mRNA expression of *BDNF* compared with those obtained from permanent teeth, but not *NGF* or *GDNF*. However, both of these stem cells had a similar BDNF protein expression level and reinnervation potential.(26)

Factors Affecting the Regenerative Potential of DPSCs, SHED and DFSCs in Pulp-Dentin Complex Regeneration

Several factors may affect the regenerative potential of DPSCs, SHED, and DFSCs. Aging has been reported to cause the reduction of DPSCs regenerative potential. An animal study demonstrates that about 60% of root canal area is covered by pulp-dentin complex after 120 days in teeth of aged dogs (5–6 years of age) transplanted with autologous mobilized DPSCs.(19) This percentage is much lower than that in young dogs (8-10 months of age), which shows regeneration volume of more than 90% after 60 days. (18) SHED, which are obtained from dental pulp of younger individuals, have a higher expression of neuronal markers when compared with adult DPSCs, suggesting lower neurogenic potential in DPSCs.(57) In dental follicle cells, cell senescence is correlated with a decrease in osteogenic potential and lower WNT5A expression, although the role of WNT5A may be less significant in regulating the expression of osteogenic markers.(58)

Dental diseases, such as caries, are reported to have no effect or even increase regenerative potential of dental stem cells. SHED obtained from carious deciduous teeth has a similar osteogenic potential compared to those that are obtained from sound deciduous teeth.(59) Meanwhile, DPSCs isolated from teeth with deep caries have greater proliferation and angiogenesis abilities, as well as higher expression of odontoblast differentiation markers.(60,61)

Dental stem cells can differentiate not only to odontoblasts and dental pulp cells, but also to other types of cells, since it has been reported that transplantation of DPSCs regenerates periodontal ligament-, bone-, and cementum-like tissues instead of pulp-like tissue. Signals sent from tissues surrounding the root canal, such as alveolar bone and periodontal ligament, might affect the fate of transplanted dental stem cells.(46) Taken together, the success of stem cells-mediated pulp-dentin complex regeneration may be affected by aging, dental diseases, and signals sent from the surrounding tissues.

Recent Advances on the Use of Dental Stem Cells in Regenerative Endodontics

Dental stem cells have been demonstrated to regenerate functional pulp-dentin complex in human subjects in several studies, most of them using autologous dental stem cells (Table 2). Combination of autologous mobilized DPSCs and good manufacturing practice (GMP)-grade G-CSF are transplanted into the teeth of five adult irreversible pulpitis patients.(31) Mobilized DPSCs are subsets of DPSCs isolated through G-CSF-induced cell mobilization.(51) Four weeks after transplantation, four patients show a positive electric pulp test result. Lateral dentin formation is observed in three patients as shown by CBCT imaging. Interestingly, all patients do not experience any adverse events or toxicity caused by mobilized DPSCs transplantation.(31)

Successful pulp regeneration using autologous DPSCs obtained from inflamed pulp has also been reported. DPSCs are obtained from the permanent tooth with symptomatic irreversible pulpitis. These stem cells are implanted with leukocyte platelet-rich fibrin (L-PRF) obtained from the patient's blood into the root canal of the same tooth. After 36 months, no tenderness to palpation or percussion, and no adverse events are observed. Laser Doppler flowmetry results demonstrate that both untreated and DPSCs-implanted teeth have pulse characteristics, implying blood perfusion in the teeth, although the mean perfusion units in those teeth are low.(30)

Transplantation of autologous hpDPSCs seeded on atelocollagen scaffold containing G-CSF in multirooted molars of two patients affected by symptomatic or asymptomatic irreversible pulpitis has been successfully demonstrated. No periapical radiolucency is observed by CBCT and radiographic examination after 48 weeks. Moreover, no adverse events or systemic toxicity are experienced by these patients as shown by the results of clinical and laboratory evaluation.(32)

SHED transplantation into injured human teeth markedly increases dentin thickness and root length, as well as reduces apical foramen width compared with the apexification procedure. An increase in vascular formation is observed in SHED transplantation group. In contrast, a decrease in vascular formation is observed in the apexification group. Teeth transplanted with SHED show a significantly higher mean decrease in sensation than those treated with apexification procedure. No adverse events are observed at 24 months after transplantation.(27)

Besides dental stem cells, induced pluripotent stem cell (iPSC), which is obtained by introducing reprogramming factors including octamer-binding transcription factor 4 (*Oct4*), Kruppel-like factor 4 (*Klf4*), sex determining region Y-box 2 (*Sox2*), *l-myc*, *c-myc*, and *Lin28* to somatic cells, can also be used in pulp-dentin regeneration.(62-65) Stem cells, such as DPSCs (63), and differentiated cells, such as fibroblasts (64) could be used to generate iPSCs. Generation of odontoblasts-like cells could be performed by directly inducing iPSCs.(63) In addition, iPSCs could be induced toward iPSCs-derived neural crest-like cells (iNCLCs), which in turn can be differentiated further into odontoblasts-like cells.(63,64) Differentiation to odontoblasts and generation of pulp-like tissue from iPSCs can be induced by transfection of specific genes (62), as well as addition of exogenous growth factors (63,64) and scaffolds (64).

Whole tooth regeneration is another promising advance in endodontic therapy. This method relies on the interaction between the dental mesenchyme and the dental epithelium to generate a bioengineered tooth bud.(66) Cells of the dental mesenchyme and the dental epithelium can be isolated from embryonic (67-69) or postnatal (67) dental tissues. Autologous (67), allogeneic (69), and xenogeneic (68) cells have been used in tooth bud production. Both types of cells are combined in collagen gel drop and cultured *in vitro* (67-69) or seeded in a scaffold (70). The bioengineered tooth bud is then transplanted to the jaw bone to regenerate the new tooth.

Role of Biomaterial Scaffolds in Regenerative Endodontic Therapy

Along with dental stem cells, the use of biomaterial scaffold (bioscaffold) also becomes a notable consideration in regenerative endodontics, especially for the formation of dental tissues. These biomaterials are expanded *in vitro* to environmentally mimic the *in vivo* condition.(71,72) Ideal scaffolds for regenerative endodontic therapy should resemble the extracellular matrix (ECM) of pulp-dentin complex in terms of dimensional stability, sufficient porosity with adequate particle size, similar biodegradability rate, as well as physical and mechanical strength (71,73,74), since biocompatibility is highly important to prevent adverse tissue reactions.(75)

Bioscaffold for regenerative endodontic therapy includes broad ranges of applications and sources. Based on the scaffold geometry, the existing biological constructs are porous scaffolds, fibrous scaffolds, microsphere/microparticle scaffolds, and solid free-form scaffolds.(76) Meanwhile, based on the material sources, bioscaffold can be classified into blood-derived scaffolds, natural-derived biomaterial scaffolds, and synthetic biomaterial scaffolds. Each scaffold has different regenerative properties and potential, including pulp and dentin regeneration, vascularization, as well as stem cell proliferation and differentiation (Table 3).

Blood-derived scaffolds

Induction of bleeding and formation of intracanal blood-clot (BC) in the root canal is a well-known used method in regenerative endodontic therapy that applies the strategy of bioscaffold for pulp-dentin regeneration and dental tissue ingrowth.(78,106) BC is a gel-like lump obtained

Table 3. Regenerative potential of blood-derived, natural-derived polymer, and sythetic polymer bioscaffolds.

Types of Scaffolds	Regenerative Potential		References
	Pulp-dentin Regeneration	Vascularization	
Blood-derived			
BC	- Increasing root length and thickness - Increasing dental wall thickness - Improving bone density - Narrowing apical width - Healing the periapical lesion	- Improving vitality response (blood pump)	(77-86)
PRP	- Increasing root length and thickness - Increasing dental wall thickness - Improving bone density - Narrowing apical width - Healing the periapical lesion	- Improving vitality response (blood pump)	(77,78,80-83, 85-87)
PRF	- Increasing root length and thickness - Increasing dental wall thickness - Improving bone density - Narrowing apical width - Healing the periapical lesion	- Improving vitality response (blood pump)	(80,83,84,85,87)
Natural-derived polymers			
Collagen - BC	- Increasing root length - Enhancing mineralization of root canal - Increasing dental wall thickness - Narrowing apical width - Healing the periapical lesion - Increasing intracanal connective tissue formation	N/A	(88-92)
Gelatin - BC	- Increasing root lenght and thickness - Increasing root length - Increasing dental wall thickness - Narrowing apical width - Increasing intracanal connective tissue formation	N/A	(93,94)
Chitosan - BC - Sodium hyaluronate - Pectin	- Increasing root length and thickness - Increasing dental wall thickness - Enhancing mineralization of root canal - Narrowing apical width - Healing the periapical lesion - Increasing intracanal connective tissue formation	- Increasing vascularization	(95,96)
Fibrin	- Increasing root length and thickness - Enhancing mineralization of root canal - Narrowing apical width - Healing the periapical lesion	- Increasing vascularization	(94,97)
HA	- Increasing root length - Enhancing mineralization of root canal - Increasing dental wall thickness - Narrowing apical width - Healing the periapical lesion - Increasing intracanal connective tissue formation	- Increasing vascularization	(73,98)
Synthetic biomaterial			
PLLA - DPSC - Minced-pulp MSC	- Enhance tissue mineralization - Increase expression levels of *DMP1, DSPP, COL1*, and *OPN* genes	N/A	(99-101)
PLGA - DPSC - Magnesium	- Increase bone height and volume - Enhance bone mineralization - Enhance surface closing	- Initiate neurovascular regeneration	(102,103)
PCL - PDLSC - Fluorapatite	- Enhance bone formation in defect tissue - Improve periodontium neogenesis - Increase expression of *DMP1, DSPP, RUNX2, OCN, SPP1, COL1A1*, and *GDF5* genes	N/A	(104,105)

N/A: Not applicable; DMP1: Dentin matrix acidic phosphoprotein 1; DSPP: Dentin sialophosphoprotein; COL1: Collagen type I; OPN: Osteopontin; RUNX2: Runt-related transcription factor 2; OCN: Osteocalcin; SPP1: Secreted phosphoprotein 1; COL1A1: Collagen type I alpha 1; GDF5: Growth differentiation factor 5.

during the blood state alterations from liquid to solid. (74) This technique usually includes canal preparation and disinfection, followed by induction of BC from the periapical region.(107)

The practicality and success of regenerative endodontic therapy using BC, including in treating permanent or immature teeth with apical periodontitis and necrotic pulps, have been reported. In terms of pulp and dentin regeneration, BC bioscaffold therapy showed that it was able to give substantial results in increasing root length and thickness, thickening dental wall, improving bone density, providing apical closure, as well as periapical healing.(78-80,82,84,108) Immature symptomatic apical periodontitis teeth treated with BC scaffold showed a similar root morphology compared to other teeth that underwent normal development.(74,109)

Although has been performed a lot previously, yet the failure in inducing apical bleeding or in achieving adequate blood volume within the canal space remain as the common problems during the therapy with BC bioscaffold. The percentage of discoloration was also significantly greater in teeth with BC scaffold therapy compared with teeth with other platelets concentrates.(86) Hence, lately the use of autologous platelet concentrates, including platelet-rich plasma (PRP) and platelet-rich fibrin (PRF), have been explored as the possible scaffold source for regenerative endodontics therapy.(83,85)

PRP, an autologous first-generation platelet concentrate, is a high concentrate of autologous platelet obtained by centrifugation of autologous blood that may be source for several types of growth factors such as TGF-β, insulin growth factor (IGF), platelet-derived growth factor (PDGF), VEGF, as well as fibroblasts growth factor (FGF). (110,111) PRP preparation process consists of the removal of erythrocytes that would be expected to undergo necrosis shortly after clot formation. The PRP clot is composed of fibrin, fibronectin, and vitronectin, which are cell adhesion molecules required for cell migration.(78) PRP is an ideal scaffold regenerative endodontic treatment since it is comparably easy to prepare in a dental setting, rich in growth factors, and forms a 3D fibrin matrix that helps attract the growth factors.(77)

As a comparable autologous bioscaffold, PRP has been able to show results of further root development (including root lengthening and thickening), periapical lesion resolution, improvement of periapical bone density, and continued apical closure compared with BC in the regenerative treatment of teeth with necrotic pulps. (77,78,81,86) Most blood-derived bioscaffolds showed the ability to improve pulp vitality response. However, PRP was found to be more effective than BC in revascularization. Even though not significant PRP treatment showed highest vitality test response compared with BC treatment, which suggests the higher occurrence of pulp's blood supply.(83,85) PRP has also been proved to be successfully stimulating the collagen production, sustained release of growth factors, as well as enhanced recruitment, retention, and proliferation of undifferentiated mesenchymal and endothelial cells from periapical area.(77,82) At a certain concentration of range, PRP also may increase the proliferation of fibroblasts and osteoblasts.(111)

PRF, a second-generation platelet concentrate, is a non-thrombonized autologous fibrin mesh that responsible as a reservoir for the slow, continuous release of growth factors. PRF is an unadulterated centrifuged blood which consists of autologous platelets and leukocytes present in a complex fibrin matrix, that is able to achieves polymerization naturally. PRF is composed of fibrin membranes enriched with platelets, growth factors, and cytokines.(86,112) The PRF clot is an autologous biomaterial and not an improved fibrin glue. Unlike the PRP, the strong fibrin matrix of PRF does not dissolve quickly after application, instead, it is formed slowly in a similar way to a natural BC.(80)

Although composed of almost similar fibrin membranes, PRF has lower risk than PRP during the application since there is no bovine thrombin and anticoagulants present. PRF also shows better potency in accelerating wound and tissue healing, as well as better efficiency for cell proliferation and migration than PRP. (113,114) PRF clots acted as successful scaffolds for the regeneration of dentin and pulpal contents in immature teeth with necrotic pulps because of its ability to increase root length, increase dental wall thickness, and healing the periapical lesion better than BC and PRP.(80,85) Meanwhile, in terms of clinical sign and symptom resolution, PRF achieved comparable outcomes to BC in regenerative endodontic therapy.(84) In the therapy of necrotic immature permanent teeth, revascularization/revitalization utilizing PRF also showed to be highly successful.(87)

When being combined with stem cells, PRP and PRF also show better regeneration potential. Human DPSCs was co-cultured with 10% of PRP showed higher expression levels of fetal liver kinase (Flk)-1, VEGF, PDGF, and stromal cell-derived factor 1 (SDF-1) mRNA compared with the combination of hDPSCs and fetal bovine serum (FBS). This suggests that PRP can promote vasculogenesis better than FBS in hDPSCs culture.(115) Both combinations of hDPSCs + PRP and hDPSCs + liquid-PRP showed

significant increase of cell migration, proliferation, and differentiation compared with hDPSCs only. Though in hDPSCs + liquid-PRF, the cell migration was observed faster than hDPSCs + PRP.(116)

Natural-derived Biomaterial Scaffolds

Natural-derived polymers are usually used as biomimetic materials for scaffold in regenerative endodontic therapy. Most of the natural polymers are bioactive, containing cellular binding motifs, thus promoting cell adhesion, and/or present soluble signaling factors that are capable in regulating cell behaviour. Hence they are also known to provide better biocompatibility compared with synthetic polymers.(96,117) Natural polymers are also known to be rapidly degradable compared with other types of scaffolds, thus allowing easier replacement with natural tissues after the degradation.(110,118) Natural polymers consist of natural polypeptides of the ECM, such as collagen, fibrin, gelatin and keratin, as well as polypeptides that are chemically similar to natural glycosaminoglycans, such as alginate, chitosan and hyaluronic acid (HA).(96)

For the regeneration of pulp and dentin-like tissue, polymers like collagen, gelatin, fibrin, chitosan, and HA have shown the ability to improve root development, including increase root length, root thickness, and enhance the mineralization of root canal.(73,89,91,93,94,96-98) While being used as a single scaffold, those natural polymers also showed better ability in increasing intracanal connective tissue formations and narrowing apical width compared with BC, healing the periapical lesion, increasing dental wall thickness, as well as resuming the maturation process for the immature teeth.(73,88-90,93,98)

Natural polymers are often combined and crosslinked with other bioscaffold or chemical agents to improve its potential in regenerative therapy.(119) Dental pulp regeneration through cell homing approaches can be improved by using the combination of HA hydrogel and BC, as well as combination of chitosan hydrogel and BC scaffolds.(73,120) Meanwhile, to fill root canal space with new vital tissue and to enhance the root canal mineralization, the combination of gelatin sponge and BC scaffold as well as collagen and BC scaffold can be used, and have shown better results compared with BC scaffold only.(92,93) To enhance scaffolds physical properties, the crosslinking between collagen hydrogel and cinnamaldehyde (CA) had shown to be successful. It resulted in the enhanced physical properties of collagen by CA, which upregulated the cellular adhesion compared with the collagen only. This means that this property was promoted in the presence of CA.(121)

In terms of its vascularization function, while being used as a single bioscaffold, both fibrin and HA have shown the potential of increasing vascularization better than the control.(73,97) On the other hand, chitosan, when being used alone, does not show vascularization potential, however when being combined with sodium hyaluronate or pectin, both combinations were able to increase vascularization of connective tissues.(95)

Besides its advantages in dental-pulp regeneration and vascularization, natural-derived bioscaffolds that are classified into moldable porous scaffold, such as chitosan and collagen as single scaffold, or even combination of gelatin/collagen hydrogens bioscaffold, also have the ability to promote cell adhesion, migration and proliferation.(96,110,119,122) And to induce hDPSCs cell migration, adhesion, and proliferation, which later followed by a culminated amount of mineralized matrix, scaffold from chitosan and collagen matrix can also be combined with calcium-aluminate.(123) In the combination with SCAP, cell viability promotion, mineralization, and odontoblastic-like differentiation can also be achieved by using HA-based injectable gel scaffold.(124)

Synthetic Biomaterial Scaffolds

While natural-derived polymers scaffolds offer good biocompatibility and bioactivity, synthetic polymers scaffolds offer more flexible and controllable physical and mechanical properties to fit for specific applications.(76,125) Polylactic acid (PLA) and polyglycolic acid (PGA), as well as their copolymers such as poly-L-lactic acid (PLLA), polylactic-polyglycolic acid (PLGA), and polycaprolactone (PCL) have been successfully reported as bioscaffold for regenerative endodontics therapy.(74)

Synthetic polymers scaffolds and its combination with other scaffold materials are able to induce pulp-dentin regeneration. The increase of mineralization, as well as tissue and bone formation, can be reached by using the combination of PLGA and magnesium scaffold, PLLA combined with DPSC or minced-pulp mesenchymal stem cell (MSC), as well as combination of PCL and PDLSC.(100,103,104) Other than that, culture of hDPSCs on either side of PLGA scaffold was also able to enhance surface closing in the opened side of scaffold. Meanwhile, in terms of pulp vascularization and neurogenesis, the enhancement of neurovascular regeneration through angiogenic and neurogenic paracrine secretion has been reported after the therapy with PLGA scaffold on hDPSCs culture.(102)

PLLA and PLGA scaffolds while being cultured in DPSC are able to improve DPSC differentiation and

proliferation, it also induces longer cell replicative lifespan.(99,100,102) PLLA scaffold was also used for human minced-pulp MSC, and the results found that the combination showed even better ability to increase cell differentiation and replication better than in DPSC.(100) Although not being used as scaffold as much as PLLA and PLGA, the use of PCL scaffolds in SCAP and hDPSCs seeding were also reported to be able to increase the cell proliferation and differentiation.(105,126)

A PLGA microsphere combined with hDPSCs, was able to increase hDPSCs proliferation and adhesion to the scaffold, as well as increase expression levels of *DMP1*, *DSPP*, *COL1*, and *OPN* genes.(101) Meanwhile, increased expression of *DMP1*, *DSPP*, runt-related transcription factor 2 (*RUNX2*), osteocalcin (*OCN*), secreted phosphoprotein 1 (*SPP1*), collagen type I alpha 1 (*COL1A1*), and growth differentiation factor 5 (*GDF5*) genes was obtained with the combination of PCL and fluorapatite.(105) In the construction of dental and periodontal pulp for the preservation of periodontal ligament fibroblasts (PDLF), the use of PLGA scaffold combined with PRF has shown the ability to sustain fibroblast viability.(74,127)

Role of Signaling Molecules in Regenerative Endodontics

Various signaling molecules, including growth factors and cytokines have been recognized to enhance the proliferation, migration and differentiation of dental stem cells. These molecules are naturally contained in the pulpal cells and dentin matrix, and involved in modulating dentin-pulp complex homeostasis.(128) In the pulp-dentin regeneration process, the remaining periapical and pulpal cells, adjacent dentin, or implanted platelet concentrates, blood clot scaffold, or stem cells are responsible for the release of signaling molecules. To accelerate the process, exogenous signaling molecules are often delivered together with dental stem cells in a scaffold. Addition of signaling molecules to transplanted dental stem cells is expected to mimic the signaling cascades that occur during the formation of pulp-dentin complex.(129)

Signaling Molecules Related to Cell Migration

Bone morphogenetic protein (BMP)-2, TGF-β1, basic FGF (bFGF), PDGF, VEGF, NGF, and BDNF have been reported to stimulate cell migration (Table 4). Induction of cell migration by these molecules is important, since cells must reach the damaged sites to regenerate the tissues. Several signaling pathways have been identified to be induced by these molecules in stimulating cell migration. For example, via PDGFR-β/Akt pathway, PDGF contributes in recruiting smooth muscle cells to blood vessels (168); BDNF accelerates DPSCs migration via extracellular signal-regulated kinase (Erk) phosphorylation (193); VEGF increases the migration of DPSCs through VEGF receptor (VEGFR) 2 activation and its downstream focal adhesion kinase (FAK) / phosphoinositide 3-kinase (PI3K) / Akt and p38 signaling.(181,182)

Signaling Molecules Related to Cell Proliferation

After reaching the damaged sites, cells must proliferate to increase the number of cells. BMP-2, TGF-β1, bFGF, PDGF and VEGF have been reported to increase proliferation (Table 4). However, the proliferation process is inhibited when cells start to enter the differentiation stage. Thus, signaling molecules which have proliferation-related functions may both inhibit proliferation and induce differentiation in a specific time point, as discussed in the subsequent sections. Several signaling pathways have been identified to be induced by these molecules in stimulating cell proliferation. BMP-2-induced cell proliferation involves BMP-2 receptor (BMP2R) activation as well as Erk1/2 and small mothers against decapentaplegic (Smad) 1/5 phosphorylation (131), while bFGF modulates the expression of cyclin B1 (CCNB1) and cell division control 2 (CDC2), which are related to cell-cycle regulation via mitogen-activated protein kinase kinase (MEK)/Erk pathway.(154) VEGF activates the Akt signaling pathway and increases cyclin D1 expression levels, which in turn promotes proliferation of DPSCs.(182)

Signaling Molecules Related to Dentinogenesis and Pulp Regeneration

BMP-2, TGF-β1, bFGF, PDGF, VEGF, and NGF have been reported to enhance dentinogenesis (Table 4). These molecules have been demonstrated to increase differentiation and mineralization of both dental pulp cells and dental stem cells as indicated by an increase in alkaline phosphatase (ALP) activity and mineralization, as well as upregulation of osteo-/odontogenic marker expression *in vitro*.(132,151,157,170,188) *In vivo*, these molecules are observed to stimulate dentin formation. (132,145,159,171,187)

TGF-β1 has been demonstrated to enhance ALP activity via activation of Smad2/3, TGF-β activated kinase 1 (TAK1), as well as Erk1/2 and p38.(148) BMP-2 has been known to induce phosphorylation of Erk1/2 and Smad1/5. (131) bFGF could induce mitogen-activated protein kinases

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Table 4. Regenerative potential of signaling molecules in pulp-dentin regeneration.

Signaling Molecule	Regenerative Potential					Reference
	Cell Migration	Cell Proliferation	Pulp- and/or Dentin-like Tissue	Angiogenesis	Neurogenesis	
BMP-2	Inducing migration of dental pulp cells	Increasing proliferation of dental pulp cells	• Increasing ALP activity and mineralization • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *ALP*, *RUNX2*, *COL1*, *DSPP*, *DMP1*, *DSP*, *MMP20*, *BSP*, *OCN*, and *OSX* - Protein expression: RUNX2, DSPP, DMP1, BSP, and OCN	N/A	N/A	(130-141)
TGF-β1	Inducing migration of dental pulp cells	Increasing proliferation of DPSCs and dental pulp cells	• Increasing ALP activity, mineralization, and collagen content • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *DSPP*, *DSP*, *MMP20*, *RUNX2*, *DMP1*, *COL1A1*, and *BSP* - Protein expression: N-cadherin, TIMP1, COL1A1, DMP1, and BSP • Downregulating protein expression: MMP3	• Inducing smooth muscle cell differentation • Maintaining blood vessels stability • Upregulating differentiation markers - Gene expression: *αSMA, SM22α, CALP, SMTN*, and *MYH11* - Protein expression: αSMA, SM22α, CALP, SMTN, ANGPT1, Tie2, and MYH11	N/A	(137,142-151)
bFGF	Inducing migration of SCAP, mobilized DPSCs, BMMSCs, periodontal ligament fibroblasts, and endothelial cells	Increasing proliferation of SHED, DPSCs, mobilized DPSCs, BMMSCs, dental pulp cells, periodontal ligament fibroblasts, and endothelial cells	• Increasing ALP activity and mineralization • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *DSPP*, *MMP20*, *TRH-DE*, *ALP*, *TIMP1*, *DMP1*, *COL1A2*, *OPN*, and *OCN* - Protein expression: DSPP, DMP1, TIMP1, and COL1	• Enhancing blood vessel formation • Upregulating differentiation markers - Gene expression: *VEGFR2*, *Tie2*, *ANGPT1*, *VWF*, *VE-cadherin*, and *CD31* - Protein expression: VEGFR2, Tie2, ANGPT1, VWF, VE-cadherin, and CD31	• Inducing neuronal and glial differentation • Promoting axonal sprouting and growth • Upregulating differentiation markers - Gene expression: *Nestin*, *TUBB3*, *Sox2*, *VIM*, *NEFM*, *MAP2*, *NEFH*, *GFAP*, and *S100B* - Protein expression: Nestin, NEFM, TUBB3, NeuN, GFAP, S100B, and MAP2	(152-168)
PDGF	Inducing migration of DPSCs, SHED, dental pulp cells, and smooth muscle cells	Increasing proliferation of DPSCs	• Increasing ALP activity and mineralization • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *DMP1*, *DSPP*, and *OCN* - Protein expression: DMP1 and DSPP	• Inducing smooth muscle and endothelial cell differentation • Enhancing blood vessel formation • Promoting blood vessel stabilization • Upregulating differentiation markers - Gene expression: *αSMA*, *SM22α*, *CALP*, *SMTN*, and *MYH11* - Protein expression: αSMA, SM22α, CALP, SMTN, VEGFR2, Tie2, CD31, and VE-cadherin	N/A	(142,150, 168-174)

Table 4. Regenerative potential of signaling molecules in pulp-dentin regeneration (cont).

Signaling Molecule	Regenerative Potential					Reference
	Cell Migration	Cell Proliferation	Pulp- and/or Dentin-like Tissue	Angiogenesis	Neurogenesis	
VEGF	Inducing migration of DPSCs and endothelial cells	Increasing proliferation of DPSCs and dental pulp cells	• Increasing ALP activity and mineralization • Upregulating odontoblast markers - Gene expression: *ALP*, *OCN*, *OSX*, *DSPP*, *RUNX2*, *DMP1*, *COL1A2*, *BSP*, *TGFB1*, and *OPN* - Protein expression: DMP1, DSPP, and OSX	• Inducing endothelial cell differentation • Enhancing blood vessel formation • Promoting blood vessel anastomosis • Upregulating differentiation markers - Gene expression: *VWF*, *VEGFR2*, *VE-cadherin*, *CD31*, *VEGFR1*, *EphrinB2*, Tie2, and *ANGPT* - Protein expression: VWF, VEGFR2, VE-cadherin, CD31, Tie2, F8	N/A	(130,136,157, 162,175-188)
NGF	Inducing migration of glial cells	N/A	• Improving pulpal architecture and cell organization • Upregulating gene expressions of differentiation markers: *DSPP*, *DMP1*, and *TGFB1*	N/A	• Inducing neuronal and glial differentation • Promoting axonal sprouting and growth • Upregulating differentiation markers - Gene expression: *Nestin* - Protein expression: S100, neurofilament, and p75NTR	(156,188-191)
BDNF	Increasing migration of DPSCs	N/A	N/A	N/A	• Inducing neuronal and glial differentation • Upregulating protein expressions of differentiation markers: DCX, NeuN, S100B and p75NTR.	(192,193)

N/A: Not applicable; ALP: Alkaline phosphatase; RUNX2: Runt-related transcription factor 2; COL1: Collagen type I; DSPP: Dentin sialophosphoprotein; DMP1: Dentin matrix acidic phosphoprotein 1; DSP: Dentin sialoprotein; MMP: Matrix metalloproteinase; BSP: Bone sialoprotein; OCN: Osteocalcin; OSX: Osterix; COL1A1: Collagen type I alpha 1; TIMP1: Tissue inhibitor of metalloproteinase 1; αSMA: Alpha smooth muscle actin, SM22α: Smooth muscle protein 22 alpha, CALP: Calponin, SMTN: Smoothelin, ANGPT: Angiopoietin, MYH11: Myosin heavy chain 11; TRH-DE: thyrotropin-releasing hormone-degrading enzyme; OPN: Osteopontin; VEGFR: vascular endothelial growth factor receptor; VWF: von Willebrand factor; TUBB3: tubulin beta III ; Sox2: sex determining region Y-box 2; VIM: Vimentin; NEFM: Neurofilament medium chain; MAP2: Microtubule associated protein 2; NEFH: Neurofilament heavy chain; GFAP: Glial fibrillary acidic protein; S100: S100 calcium binding protein; NeuN: Neuronal nuclei; TGFB1: Transforming growth factor beta 1; F8: Coagulation factor VIII; p75NTR: p75 neurotrophin receptor; DCX: Doublecortin.

(MAPKs) (p38, JNK, and Erk), PI3K/Akt, protein kinase C (PKC), and NF-κB (194), BMP or Wnt signaling.(195) Meanwhile, VEGF has been known to activate Akt, MAPKs (p38, JNK, and Erk), and NF-κB.(157)

Intriguingly, induction of differentiation and mineralization by TGF-β1 and BMP-2 is often associated with a decrease in cell proliferation (136,151). In addition, TGF-β1 increases the expression of early marker genes of odonto-/osteo-genic differentiation and decreases the expression of late-stage mineralization genes.(151) VEGF might not be able to trigger full osteo-odontogenic differentiation, and facilitate only the early stage of cell differentiation.(187) VEGF potential in inducing mineralization is lower compared with bFGF (157) and NGF.(188) The potential of PDGF in enhancing hard tissue formation has been shown to be lower than other materials, such as enamel matrix derivative (EMD) and mineral trioxide aggregate (MTA).(196) Furthermore, PDGF-BB has been reported to inhibit the formation of mineral nodules.(14) Therefore, PDGF should be used in combination with other materials to increase the mineralization potential.(171,172) However, studies regarding signaling pathways that are involved in PDGF and NGF-induced dentin formation are limited.

bFGF, TGF-β1, and NGF are known to contribute to pulp regeneration (Table 4). bFGF regulates growth of dental pulp cells, upregulates the expression of CDC2, CCNB1, and tissue inhibitor of metalloproteinase 1 (TIMP1), as well as inhibits ALP activity and collagen I production through activation of FGF receptor (FGFR) and MEK/Erk signaling.(154) Meanwhile, TGF-β1 has been demonstrated to increase TIMP1 production, collagen content, and procollagen I, but slightly attenuate MMP3 production, which are related to the activation of activin receptor-like kinase-5(ALK5)/Smad2/3, TAK1, MEK/Erk, and p38 signaling.(143,148) NGF has been reported to upregulate the expression of healing and repair-related genes (188), as well as improve pulp cell organization and pulpal architecture.(189) Thus, bFGF, TGF-β1 and NGF are involved in pulp regeneration by altering matrix turnover and dental pulp cell proliferation, as well as modulating pulp repair-related gene expression.

Signaling Molecules Related to Angiogenesis

VEGF, PDGF, bFGF, and TGF-β1 have been reported to induce angiogenesis (Table 4) by promoting differentiation of dental stem cells toward endothelial (162,175) or smooth muscle cells (149,150), as shown by upregulation of several differentiation genes.(144,150,162) These signaling molecules also induce the formation of capillary-like structures, both *in vitro* (162,170,175) and *in vivo*.(170,176) VEGF has been demonstrated to accelerate angiogenesis, since angiogenesis could occur even in the absence of this molecule.(176) This molecule induces angiogenesis by inducing VEGFR phosphorylation and activating downstream Akt, MAPKs (p38, JNK, and Erk), NF-κB. (157) Besides formation of new blood vessels, VEGF has been reported to induce anastomosis of DPSCs-derived blood vessels by increasing vascular endothelial (VE)-cadherin expression through the activation of MEK1/Erk, which in turn causes E-26 transformation-specific-related gene (ERG) transcription factor binds to *VE-cadherin* promoter.(184) VEGF-induced angiogenesis could be enhanced by inhibiting specific pathways or combining it with other molecules. Combination of VEGF with SB-431542, an inhibitor of TGF-β1 signaling, has been shown to markedly promote SHED differentiation toward endothelial cells, since Smad1/2 inhibition is correlated with VEGFR2 activation.(175) IGF-1 (182) and SDF-1α (179) were also reported to have a synergistic effect in enhancing angiogenesis when combined with VEGF.

PDGF-BB alone induces capillary sprouting, and this phenomenon could be enhanced by bFGF.(168) bFGF alone could induce angiogenesis, but its angiogenic potential is lower than VEGF.(157) PDGF-BB has been reported to promote blood vessels maturation by regulating the investment of smooth muscle cells to DPSCs-derived capillaries through PDGFRβ and Akt phosphorylation in both types of cells.(168) In addition, DPSCs-derived smooth muscle cells that are produced after TGF-β1 treatment have been reported to stabilize blood vessels through ANGPT1/Tie2 and VEGF/VEGFR2 signaling.(149) Combination of PDGF-BB and TGF-β1 induces the expression of smooth muscle-specific early, mid, and late markers, as well as enhances contraction ability in DPSCs, although the cells do not undergo morphological alterations toward smooth muscle-specific cell shapes.(150)

Signaling Molecules Related to Neurogenesis

NGF, BDNF and bFGF have been reported to induce neurogenesis (Table 4). In several neurogenesis induction studies, NGF and BDNF are combined with other neurotrophin and non-neurotrophin signaling molecules. (156,188,193) Meanwhile, bFGF is usually combined with epidermal growth factor (EGF) for neural induction.(167) Addition of these molecules increases the expression levels of neural markers and promotes morphological alterations of the treated cells toward neuronal and glial cells.

(156,192,197) These molecules have also been reported to induce axonal sprouting and promote axonal growth. (167,191)

NGF and BDNF induce neurogenesis via non-specific activation of p75 neurotrophin receptor (p75NTR). In addition, NGF specifically activates tropomyosin-related kinase A (TrkA), while BDNF specifically activates TrkB.(198) Meanwhile, bFGF induces neurogenesis via activation of FGFR (199). Activation of these receptors have been reported to induce the phospholipase C (PLC)-γ pathway, which in turn promotes neuronal differentiation. (198,199) Besides, combination of bFGF and NGF also stimulates neuronal differentiation via PI3K/Akt and Erk pathways.(156)

Future Perspectives on the Use of Dental Stem Cells, Scaffold, and Signaling Molecules Combination in Regenerative Endodontics

Numerous studies have reported successful pulp-dentin complex regeneration using specific combinations of dental stem cells, scaffold, and signaling molecules. Despite most of the ongoing regenerative endodontics studies using these combinations are conducted in animal models (23,200), these combinations are also reported to induce pulp-dentin regeneration in human subjects. Several examples of dental stem cells, scaffold, and signaling molecules combination that have been known to regenerate human pulp-dentin complex are combination of hpDPSCs, G-CSF, and atelocollagen scaffold (31,32), as well as combination of DPSCs and L-PRF (30), which acts as scaffold and contains PDGF and TGF-β.(201) Indeed, the regenerative endodontics field is constantly growing. There will be new findings and innovation regarding dental stem cell biology, the development of new types of scaffolds, and the best way to deliver stem cells and signaling molecules to the root canal, which open a new perspective on a new era of endodontic therapy. Thus, current trends and future directions on regenerative endodontics should be further explored.

In most pulp-dentin regeneration studies using human subjects, a scaffold that already contains dental stem cells and immobilized signaling molecules is directly transplanted to the root canal in a single appointment.(30-32) Despite the success of this current protocol in regenerating functional pulp-dentin complex, the current procedure might not be similar to the natural process of pulp-dentin regeneration, which involves specific cellular processes. Additionally, regeneration of the pulp-dentin complex may be incomplete in some patients due to differences in pulp-dentin damage severity. To achieve complete pulp-dentin regeneration, additional dental stem cells and/or signaling molecules could be applied in the several next appointments. Since scaffolds have different physical characteristics and biocompatibility, different types of scaffolds could be used to facilitate pulp-dentin regeneration in different parts of teeth. Different types of dental stem cells, signaling molecules, and scaffolds could also be combined with other endodontic procedures, such as apexification and pulp revascularization (202) to enhance the regeneration process in different parts of teeth. Therefore, dental stem cell, scaffold, and/or signaling molecules application could be performed in multiple appointments to mimic the cellular processes that are involved in the regeneration process. Hence gradual pulp-dentin regeneration could be achieved.

Although studies regarding tissue engineering-based pulp-dentin regeneration show promising results, there are several challenges for its future clinical translation that need to be addressed. Regenerated pulp-dentin complex should have a precise and highly ordered histological structure as compared to that in normal teeth.(4) Besides, different oral diseases, such as irreversible pulpitis and necrotic pulp, as well as the presence of residual bacteria and lipopolysaccharide may affect the root canal microenvironment, which in turn alter the fate of transplanted dental stem cells.(203,204) Other factors, including age and the presence of systemic diseases might also affect regeneration potential of stem cells.(4,205) Since each type of dental stem cell, scaffold, and signaling molecule has unique characteristics and functions, they can be utilized to address these challenges by combining these components together to achieve successful regeneration. Thus, the right combination of dental stem cells, scaffolds, and signaling molecules is needed to enhance the pulp-dentin regeneration process.

Conclusion

Combinations of dental stem cells, scaffold, and signaling molecules mimic the cellular microenvironment that is suitable for regeneration. Hence, they are important to achieve the functional pulp-dentin complex formation. Since regenerative endodontics is a constantly growing field, current trends and future directions in this field are still needed to be further explored. The right combination of dental stem cells, scaffolds, and signaling molecules

could be determined based on the patients' characteristics. Incomplete pulp-dentin regeneration, which may occur in some cases, could be overcome by applying dental stem cells, scaffolds, and/or signaling molecules in multiple appointments to achieve gradual pulp-dentin regeneration.

Authors Contribution

FS, AS, WA, WW proposed the manuscript topic. All Authors were involved in the drafting and manuscript writing process. FS, MC, NMD, SJAI were involved in the manuscript revisions. FS supervised the manuscript. All authors finalized the last version of the manuscript.

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Combining Dental Stem Cells, Scaffold, and Signaling Molecules for Pulp-Dentin Complex Regeneration

Abstract

Background: Pulp damage can lead to dentinogenesis impairment, irreversible pulpitis, or pulp necrosis. Despite being the most used endodontic procedure to treat damaged pulp, root canal therapy only results in nonvital teeth which are prone to fractures and secondary infection. Pulp-dentin regeneration has a potential to regenerate structure similar to normal pulp-dentin complex, and can be achieved by combining dental stem cells, scaffold, and signaling molecules. This article reviews the role of various types of dental stem cells, scaffolds, signaling molecules, and their combinations in regenerating pulp-dentin complex.

Content: Dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHED), and dental follicle stem cells (DFSCs) were reported to regenerate pulp-dentin complex *in situ*. SHED might be more promising than DPSCs and DFSCs for regenerating pulp-dentin complex, since SHED have a higher proliferation potential and higher expression levels of signaling molecules. Scaffolds have characteristics resembling extracellular matrix, hence providing a suitable microenvironment for transplanted dental stem cells. To accelerate the regeneration process, exogenous signaling molecules are often delivered together with dental stem cells. Scaffolds and signaling molecules have different regenerative potential, including induction of cell proliferation and migration, formation of pulp- and/or dentin-like tissue, as well as angiogenesis and neurogenesis promotion.

Summary: Combinations of dental stem cells, scaffold, and signaling molecules are important to achieve the functional pulp-dentin complex formation. Current trends and future directions on

regenerative endodontics should be explored. The right combination of dental stem cells, scaffold, and signaling molecules could be determined based on the patients' characteristics. Incomplete pulp-dentin regeneration could be overcome by applying dental stem cells, scaffold, and/or signaling molecules in multiple visits.

Keywords: pulp-dentin regeneration, regenerative endodontics, dental stem cells, scaffold, signaling molecules

Introduction

Dental pulp, the only soft tissue in the tooth, plays a critical role in sustaining tooth homeostasis. However, this tissue is vulnerable to various stimuli, including infections, iatrogenic causes, and trauma.(1) If not treated properly, pulp damage can lead to dentinogenesis impairment and irreversible pulpitis or even pulp necrosis, since this tissue has a limited self-repair capacity.(2)

Damaged pulp can be treated by several procedures. Root canal therapy, the most used endodontic procedure, is based on the use of inert materials to fill the pulp chamber after pulpectomy. However, this procedure results in nonvital teeth, which are prone to fractures and secondary infection.(3) Regenerative endodontic treatment or pulp-dentin regeneration is an alternative procedure based on the tissue engineering principle. Pulp-dentin regeneration is more promising than other endodontic procedures since this procedure has a potential to regenerate structure similar to normal pulp-dentin complex. The main goals of pulp regeneration are pulp-dentin complex formation as well as angiogenesis and neurogenesis in the newly regenerated pulp.(4)

Comment [I1]: sentences need to be rephrase

Comment [I2]: promising for what?

Tissue engineering combines dental stem cells, scaffold, and signaling molecules to mimic a suitable microenvironment for regenerating pulp-dentin complex. Numerous studies have been established to examine the effects of dental stem cells, scaffold, signaling molecules, and their combinations in pulp regeneration, providing a new insight in the field of regenerative dentistry and opening a great opportunity for further clinical applications. This article reviews the role of various types of dental stem cells, scaffolds, signaling molecules, and their combinations in regenerating pulp-dentin complex. The right combination of these components could increase pulp-dentin regeneration therapy efficiency.

Comment [I3]: stem cell to mimic or the signaling molecules?

Role of Dental Stem Cells in Regenerative Endodontics

Based on the locations, dental stem cells are classified as dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHED), stem cells from the apical papilla (SCAP), dental follicle stem cells (DFSCs), periodontal ligament stem cells (PDLSCs). DPSCs, SHED, and DFSCs were reported to have potential in regenerating pulp-dentin complex *in situ*, both in animal models (Table 1) and human subjects (Table 2).

Cell Number, Proliferation Rate, and Immunomodulatory Properties of DPSCs, SHED and DFSCs

DPSCs, SHED and DFSCs are different in several aspects, including the number of cells isolated from the tissues, proliferation rate, and immunomodulatory mechanisms. DPSCs and SHED have relatively high cell numbers in original cultures compared to DFSCs, because dental pulp, both in permanent and deciduous teeth, have relatively high amounts of stem cells compared to dental follicles of developing tooth germ. Since dental follicles only contain small

Comment [I4]: number of cell isolated will be depend on the size or quality of the tissue

Comment [I5]: immunomodulatory secretions??

amounts of cells and located in sites that are relatively not easy to be accessed, DFSCs are difficult to be obtained and distinguished from other types of dental stem cells.(4)

Comment [I6]: is there a parameter to equalized the sample used in isolation??

DPSCs have been reported to have a higher proliferation rate compared with bone marrow mesenchymal stem cells (BMMSCs), while SHED have a higher proliferation rate than DPSCs.(31) It has been demonstrated that the proliferation rate of DFSCs is notably higher than DPSCs.(32) Moreover, in a recent study, DFSCs were shown to have a higher proliferation rate than SHED.(33) Thus, DFSCs might have the highest proliferation rate, followed by SHED and DPSCs. High proliferation of DFSCs implies that they are more immature, since this type of stem cells are isolated from developing tissues (34), and consequently they might be more plastic compared with other dental stem cells. In summary, DPSCs, SHED, and DFSCs vary in their proliferation rates, which could be determined by the developmental stages of the stem cell sources.

DPSCs, SHED and DFSCs have also been reported to modulate the immune system through several mechanisms. DPSCs have been demonstrated to modulate the adaptive and innate immune responses through interaction with B cells, T cells, macrophages, dendritic cells (DCs), and natural killer (NK) cells. For instance, the production of B cell immunoglobulin and proliferation of T cell proliferation are inhibited in co-culture of peripheral blood mononuclear cells (PBMCs) and DPSCs. Transforming growth factor (TGF)-β secreted by DPSCs plays a crucial role in this inhibition and the addition of interferon (IFN)-γ to DPSCs culture enhances the inhibitory effects.(35) DPSCs markedly decrease $CD4^+$ and $CD8^+$ T cell proliferation, irrespective of hypoxia-inducible factor (HIF)-1α expression level in DPSCs. However, overexpression of HIF-1α increases the DPSCs inhibitory effect on DCs proliferation. Expression of HIF-1α by DPSCs also enhances the recruitment and differentiation of

macrophages with M2 characteristics. Furthermore, NK cell-mediated cytotoxicity is suppressed in HIF-1α-overexpressed DPSCs.(36)

SHED have been shown to modulate T cells, macrophages and DCs. This type of stem cell restrains the differentiation of T helper (Th) 17 cells, and has greater immunomodulatory potential compared with BMMSCs.(37) SHED have been reported to promote phenotypic polarization of macrophage toward M2-like phenotype in transwell co-culture systems and increase the number of macrophages with M2-like phenotype in rat model of periodontitis.(38) A study demonstrates that SHED affect differentiation, maturation, and T cell activation ability of DCs. The same study also shows that SHED augment T regulatory (Treg) cell induction ability of DCs. SHED-treated DCs have a lower level of IFN-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-2, as well as higher level of IL-10.(39)

Meanwhile, DFSCs have immunomodulatory properties toward T cells and macrophages. A study demonstrates that DFSCs increase the number of Treg cells as well as suppress $CD4^+$ T cell proliferation via TGF-β and indoleamine 2,3-dioxygenase (IDO) pathways.(40) In lipopolysaccharide (LPS)-induced macrophage, this type of stem cell is involved in phenotypic polarization to M2 by secreting thrombospondin-1 and TGF-β3.(41) Therefore, the immunomodulatory activities of DPSCs are exerted on B cells, T cells, macrophages, DCs, and NK cells. SHED regulates T cells, macrophages and DCs, while DFSCs show immunomodulatory activities toward T cells and macrophages.

DPSCs, SHED and DFSCs Play a Crucial Role in Regenerating Pulp-dentin Complex

Dental stem cells are involved in pulp-dentin complex formation *in situ*. When transplanted into an emptied root canal or a tooth construct, DPSCs, SHED, and DFSCs generate

tissue that has characteristics resembling dental pulp. Several biomarkers have been used to detect the presence of the regenerated pulp, such as thyrotropin-releasing hormone-degrading enzyme (*TRH-DE*), *syndecan 3*, and *tenascin*. Furthermore, magnetic resonance imaging (MRI) can also be utilized to assess pulp regeneration by dental stem cells in the root canal (Table 1, Table 2). After pulpectomy, the signal intensity of MRI is relatively low compared with those in the normal teeth. The signal intensity in the pulpectomized tooth then increases several days after transplantation and keeps decreasing until it is similar to normal pulp, indicating complete pulp regeneration.(19)

Formation of dentin-like structure by DPSCs, SHED, and DFSCs has also been documented by the generation of dentin matrix deposition that causes dentin thickening and the presence of odontoblast-like cells on the canal dentinal walls which express both specific and non-specific odontoblast markers. Specific odontoblasts markers include enamelysin/matrix metalloproteinase (MMP) 20, dentin sialoprotein (DSP), dentin sialo phosphoprotein (DSPP) and dentin matrix acidic phosphoprotein (DMP) 1 (9,16), while non-specific odontoblasts markers include bone sialoprotein (BSP), alkaline phosphatase (ALP), osteopontin (OPN), osteocalcin (OCN), osterix (OSX), and Runt-related transcription factor 2 (RUNX2).(12,27) There are several viewpoints regarding the use of non-specific odontoblasts markers for detecting newly regenerated dentin. Some investigators consider that enhanced expression of these markers suggest greater dentin regeneration potential (6,8,12,13,26) since they involve in dentin formation.(42) Other investigators consider these markers as osteogenic markers instead of odontogenic markers.(27,43) The increase in the expression of these markers implies that the regenerated structure has similar characteristics with bone instead of dentin.(43). Therefore, these markers should not be more strongly expressed in dentin than the expression of

odontoblast-specific markers.(9,12,27) Besides detection of odontoblast markers, cone beam computed tomography (CBCT) can be used to assess dentin formation (Table 2), which is demonstrated by a reduction in low-density areas, indicating a decrease in pulp volume and an increase in dentin thickness.(30) Studies that use ectopic and semi orthotopic pulp-dentin regeneration models are not included in Table 1, since these models do not provide similar conditions as the human oral cavity.(4)

Additionally, the research that assesses the ability of DFSCs to form pulp-dentin complex is more limited than those conducted using DPSCs and SHED. DFSCs are usually used to simultaneously regenerate pulp-dentin and cementum-periodontal complexes.(27) This may be caused by the tendency of DFSCs to regenerate periodontal tissue and tooth root rather than pulp-dentin complex. Transplantation of treated dentin matrix that contains DFSCs regenerates periodontal-like tissue in subcutaneous space and cementum-like tissue in the outer surface of dentin.(44) Moreover, combination of DFSCs and treated dentin matrix which is transplanted to the alveolar fossa of rats have a potential to induce root formation.(45) Thus, DFSCs are better to use in periodontal tissue and root regeneration, although they might also have a potential to regenerate pulp-dentin complex. Despite the large number of studies that explore the regenerative potential of DPSCs, SHED might be more promising than DPSCs, since SHED have a higher proliferation potential (31) and higher expression levels of signaling molecules which may contribute to the pulp-dentin regeneration.(46)

DPSCs, SHED and DFSCs are Involved in Angiogenesis

Angiogenesis has been reported to occur in pulp-like tissue regenerated by DPSCs and SHED *in situ*. There are limited studies that demonstrate the involvement of DFSCs in the

angiogenesis process in regenerated pulp tissue (Table 1). The angiogenic potential of DFSCs has been reported to be lower compared to DPSCs and SCAP.(47) The new vessels provide oxygen and nutrition to the newly regenerated pulp, hence support the survival of the transplanted stem cells, and facilitate further regeneration process. Blood vessels in the regenerated pulp can be detected using immunostaining of *Griffonia* (*Bandeiraea*) *simplicifolia* lectin 1 (BS-1 lectin) and CD31 (Table 1).

In addition, laser Doppler flowmetry can be used to assess angiogenesis and analyze the blood flow in the regenerated pulp tissue, as demonstrated by several studies. Blood flow in the pulp tissue regenerated by DPSCs is not remarkably different compared to that in normal pulp tissue, implying complete functional angiogenesis.(16) Human tooth with symptomatic irreversible pulpitis which is treated with DPSCs and normal tooth have low mean perfusion units. Blood perfusion in both teeth is indicated by pulse characteristics.(28) In addition, SHED-transplanted teeth experience an increase in the average of vascular formation.(25)

DPSCs, SHED, and DFSCs are involved in angiogenesis through differentiation toward endothelial cells (26) or angiogenic factors secretion. Several angiogenic factors that are produced by these stem cells includes vascular endothelial growth factor (VEGF) (14,26,27), HIF-1α (26,30), granulocyte-monocyte colony-stimulating factor (GM-CSF), MMP3 (14), E-selectin (16), angiopoietin (ANGPT), and von Willebrand factor (vWF).(13) These factors stimulate vessel formation by modulating local endothelial cells in a paracrine manner.(14) Several subsets of DPSCs have been reported to secrete angiogenic factors but they do not incorporate to the newly formed blood vessels, such as dental pulp $CD31^-$ side population cells (14) and granulocyte colony-stimulating factor (G-CSF) mobilized DPSCs.(16,48)

Angiogenesis in pulp-like tissue can be induced further by culturing dental stem cells under hypoxic conditions. Hypoxia mimics conditions in the dental pulp cavity (49), which increases the expression of HIF-1α. Upregulation of this transcriptional factor activates the expression of angiogenesis-related genes.(11,23) Hypoxia culture on nanofibrous spongy microspheres increases angiogenesis potential of human DPSCs (hDPSCs) as indicated by more CD31-stained blood vessels in the regenerated pulp-like tissues.(11) Another research demonstrates that the mRNA expression levels of HIF-1α in hypoxia preconditioned DPSCs (hpDPSCs) are two times higher compared to those in mobilized DPSCs, while VEGF expression levels in both DPSCs are similar. hpDPSCs have been demonstrated to have a similar neovascularization potential compared to mobilized DPSCs.(23) DPSCs from permanent and deciduous teeth that are cultured under hypoxic conditions have similar expression levels of VEGF and GM-CSF, as well as *in situ* neovascularization potential.(24) Furthermore, co-culture of dental stem cells with endothelial cells has also been demonstrated to enhance angiogenesis. Crosstalk between transplanted stem cells with endothelial cells has been shown to increase the expression of angiogenic factors in both cells by activating specific pathways, such as nuclear factor κB (NF-κB).(50)

DPSCs, SHED and DFSCs are Involved in Neurogenesis

DPSCs, SHED, and DFSCs have a potential to induce neurogenesis, as shown by the studies that reported the presence of nerve fibers in pulp-like tissue after stem cell transplantation. Newly formed nerve fibers in orthotopic pulp regeneration models are detected using immunostaining of protein gene product 9.5 (PGP9.5), neuronal nuclear (NeuN), neurofilament (NF), calcitonin gene-related peptide (CGRP), and transient receptor potential

cation channel subfamily V member 1 (TRPV1) (Table 1). The expression of other neurological markers, such as sodium voltage-gated channel alpha subunit 1 (*SCN1A*) and *neuromodulin* genes (14,16), as well as tubulin-βIII (27), nestin, and transient receptor potential cation channel subfamily M member 8 (TRPM8) protein (25), has also been detected in cultured or subcutaneously implanted stem cells. Electric pulp test is another common technique utilized for detecting nerve fibers in regenerated pulp tissue (Table 1, Table 2).

Mechanisms of neurogenesis induction are similar to the angiogenesis induction by DPSCs, SHED, and DFSCs. These types of stem cells have been reported to differentiate toward neural cells.(51,52). In addition, various neurogenic factors are produced by DPSCs and SHED, including nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), neuropeptide Y, and neurotrophin 3 (NT3).(14) Investigations on neurogenic factors secreted by DFSCs are still limited. Hypoxic conditions could enhance the expression of neurogenic factors in dental pulp stem cells. NGF and BDNF expression levels are notably higher in hpDPSCs compared to those in mobilized DPSCs, but GDNF expression level is lower. It has been reported that hpDPSCs have a similar reinnervation potential compared to mobilized DPSCs.(23) A recent study revealed that DPSCs from deciduous teeth had a markedly higher expression of BDNF compared to those obtained from permanent teeth, but not NGF or GDNF. However, both of these stem cells had a similar reinnervation potential.(24)

Factors Affecting the Regenerative Potential of DPSCs, SHED and DFSCs in Pulp-Dentin Complex Regeneration

Several factors may affect the regenerative potential of DPSCs, SHED, and DFSCs. Aging has been reported to cause the reduction of DPSCs regenerative potential. An animal study demonstrated that about 60% of root canal area is covered by pulp-dentin complex after 120 days in teeth of aged dogs transplanted with autologous mobilized DPSCs.(17) This percentage is much lower than that in young dogs, which shows regeneration volume of more than 90% after 60 days.(16) SHED, which are obtained from dental pulp of younger individuals, have a higher expression of neuronal markers when compared to adult DPSCs, suggesting lower neurogenic potential in DPSCs.(53) In dental follicle cells, cell senescence is correlated with a decrease in osteogenic potential and lower WNT5A expression, although the role of WNT5A may be less significant in regulating the expression of osteogenic markers.(54)

Comment [I7]: what is the range of age young and aged dog??

Dental diseases, such as caries, are reported to have no effect or even increase regenerative potential of dental stem cells. SHED obtained from carious deciduous teeth has a similar osteogenic potential compared to those that were obtained from sound deciduous teeth.(55) Meanwhile, DPSCs isolated from teeth with deep caries have greater proliferation and angiogenesis abilities, as well as higher expression of odontoblast differentiation markers.(56,57)

Dental stem cells can differentiate not only to odontoblasts and dental pulp cells, but also to other types of cells, since it has been reported that transplantation of DPSCs regenerates periodontal ligament-, bone-, and cementum-like tissues instead of pulp-like tissue. Signals sent from tissues surrounding the root canal, such as alveolar bone and periodontal ligament, might affect the fate of transplanted dental stem cells.(43) Taken together, the success of stem cells-mediated pulp-dentin complex regeneration may be affected by aging, dental diseases, and signals sent from the surrounding tissues.

Recent Advances on the Use of Dental Stem Cells in Regenerative Endodontics

Dental stem cells have been demonstrated to regenerate functional pulp-dentin complex in human subjects in several studies, most of them using autologous dental stem cells (Table 2). Combination of autologous mobilized DPSCs and good manufacturing practice (GMP)-grade G-CSF are transplanted into the teeth of five adult irreversible pulpitis patients.(29) Mobilized DPSCs are subsets of DPSCs isolated through G-CSF-induced cell mobilization.(48) Four weeks after transplantation, four patients show a positive electric pulp test result. Lateral dentin formation is observed in three patients as shown by CBCT imaging. Interestingly, all patients do not experience any adverse effects or toxicity caused by mobilized DPSCs transplantation.(29)

Comment [I8]: event??

Successful pulp regeneration using autologous DPSCs obtained from inflamed pulp has also been reported. DPSCs are obtained from the permanent tooth with symptomatic irreversible pulpitis. These stem cells are implanted with leukocyte platelet-rich fibrin (L-PRF) obtained from the patient's blood into the root canal of the same tooth. After 36 months, no tenderness to palpation or percussion, and no adverse effects are observed. Laser Doppler flowmetry results demonstrate that both untreated and DPSCs-implanted teeth have pulse characteristics, implying blood perfusion in the teeth, although the mean perfusion units in those teeth are low.(28)

Transplantation of autologous hpDPSCs seeded on atelocollagen scaffold containing G-CSF in multirooted molars of two patients affected by symptomatic or asymptomatic irreversible pulpitis has been successfully demonstrated. No periapical radiolucency is observed by CBCT and radiographic examination after 48 weeks. Moreover, no adverse events or systemic toxicity are experienced by these patients as shown by the results of clinical and laboratory evaluation.(30)

SHED transplantation into injured human teeth markedly increases dentin thickness and root length, as well as reduces apical foramen width compared to the apexification procedure. An increase in vascular formation is observed in SHED transplantation group. In contrast, a decrease in vascular formation is observed in the apexification group. Teeth transplanted with SHED show a significantly higher mean decrease in sensation than those treated with apexification procedure. No adverse events were observed at 24 months after transplantation.(25)

Besides dental stem cells, induced pluripotent stem cells (iPSCs), which are obtained by introducing reprogramming factors including octamer-binding transcription factor 4 (*Oct4*), Kruppel-like factor 4 (*Klf4*), sex determining region Y-box 2 (*Sox2*), *l-myc*, *c-myc*, and *Lin28* to somatic cells, can also be used in pulp-dentin regeneration.(58-60) Stem cells, such as DPSCs (59), and differentiated cells, such as fibroblasts (60) could be used to generate iPSCs. Generation of odontoblasts-like cells could be performed by directly inducing iPSCs.(59) In addition, iPSCs could be induced toward iPSCs-derived neural crest-like cells (iNCLCs), which in turn can be differentiated further into odontoblasts-like cells.(59,60) Differentiation to odontoblasts and generation of pulp-like tissue from iPSCs can be induced by transfection of specific genes (58), as well as addition of exogenous growth factors (59,60) and scaffold.(60)

Whole tooth regeneration is another promising advance in endodontic therapy. This method relies on the interaction between the dental mesenchyme and the dental epithelium to generate a bioengineered tooth bud.(61) Cells of the dental mesenchyme and the dental epithelium can be isolated from embryonic (62-64) or postnatal (62) dental tissues. Autologous (62), allogeneic (64), and xenogeneic (63) cells have been used in tooth bud production. Both types of cells are combined in collagen gel drop and cultured *in vitro* (62-64) or seeded in a

Comment [I9]: mesenchymal?

scaffold.(65) The bioengineered tooth bud is then transplanted to the jaw bone to regenerate the new tooth.

Role of Biomaterial Scaffolds in Regenerative Endodontic Therapy

Along with dental stem cells, the use of biomaterial scaffold (bioscaffold) also becomes a notable consideration in regenerative endodontics, especially for the formation of dental tissues. These biomaterials are expanded *in vitro* to environmentally mimic the *in vivo* condition.(66) Ideal scaffolds for regenerative endodontic therapy should resemble the extracellular matrix (ECM) of pulp-dentin complex in terms of dimensional stability, sufficient porosity with adequate particle size, similar biodegradability rate, as well as physical and mechanical strength (66-68), since biocompatibility is highly important to prevent adverse tissue reactions.(69)

Bioscaffold for regenerative endodontic therapy includes broad ranges of applications and sources. Based on the scaffold geometry, the existing biological constructs are porous scaffolds, fibrous scaffolds, microsphere/microparticle scaffolds, and solid free-form scaffolds.(70) Meanwhile, based on the material sources, bioscaffold can be classified into blood-derived scaffolds, natural-derived biomaterial scaffolds, and synthetic biomaterial scaffolds. Each scaffold has different regenerative properties and potential, including pulp and dentin regeneration, vascularization, as well as stem cell proliferation and differentiation (Table 3).

Blood-derived Scaffolds

Induction of bleeding and formation of intracanal blood-clot (BC) in the root canal is a well-known used method in regenerative endodontic therapy that applies the strategy of

bioscaffold for pulp-dentin regeneration and dental tissue ingrowth.(72,100) BC is a gel-like lump obtained during the blood state alterations from liquid to solid.(68) This technique usually includes canal preparation and disinfection, followed by induction of BC from the periapical region.(101)

The practicality and success of regenerative endodontic therapy using BC, including in treating permanent or immature teeth with apical periodontitis and necrotic pulps, have been reported. In terms of pulp and dentin regeneration, BC bioscaffold therapy showed that it was able to give substantial results in increasing root length and thickness, thickening dental wall, improving bone density, providing apical closure, as well as periapical healing.(72-74,76,78,102) Immature symptomatic apical periodontitis teeth treated with BC scaffold showed a similar root morphology compared to other teeth that underwent normal development.(68,103)

Although has been performed a lot previously, yet the failure in inducing apical bleeding or in achieving adequate blood volume within the canal space remain as the common problems during the therapy with BC bioscaffold. The percentage of discoloration was also significantly greater in teeth with BC scaffold therapy compared to teeth with other platelets concentrates.(80) Hence, lately the use of autologous platelet concentrates, including platelet-rich plasma (PRP) and platelet-rich fibrin (PRF), have been explored as the possible scaffold source for regenerative endodontics therapy.(77,79)

PRP, an autologous first-generation platelet concentrate, is a high concentrate of autologous platelet obtained by centrifugation of autologous blood that may be source for several types of growth factors such as TGF-β, insulin growth factor (IGF), platelet-derived growth factor (PDGF), VEGF, as well as fibroblasts growth factor (FGF).(104,105) PRP preparation process consists of the removal of erythrocytes that would be expected to undergo necrosis

shortly after clot formation. The PRP clot is composed of fibrin, fibronectin, and vitronectin, which are cell adhesion molecules required for cell migration.(72) PRP is an ideal scaffold regenerative endodontic treatment since it is comparably easy to prepare in a dental setting, rich in growth factors, and forms a 3D fibrin matrix that helps attract the growth factors.(71)

As a comparable autologous bioscaffold, PRP has been able to show results of further root development (including root lengthening and thickening), periapical lesion resolution, improvement of periapical bone density, and continued apical closure compared to BC in the regenerative treatment of teeth with necrotic pulps.(71,72,75,80) Most blood-derived bioscaffolds showed the ability to improve pulp vitality response. However, PRP was found to be more effective than BC in revascularization. Even though not significant PRP treatment showed highest vitality test response compared to BC treatment, which suggests the higher occurrence of pulp's blood supply.(77,79) PRP has also been proved to be successfully stimulating the collagen production, sustained release of growth factors, as well as enhanced recruitment, retention, and proliferation of undifferentiated mesenchymal and endothelial cells from periapical area.(71,76) At a certain concentration of range, PRP also may increase the proliferation of fibroblasts and osteoblasts.(105)

PRF, a second-generation platelet concentrate, is a non-thrombonized autologous fibrin mesh that responsible as a reservoir for the slow, continuous release of growth factors PRF is an unadulterated centrifuged blood which consists of autologous platelets and leukocytes present in a complex fibrin matrix, that is able to achieves polymerization naturally. PRF is composed of fibrin membranes enriched with platelets, growth factors, and cytokines (80,106). The PRF clot is an autologous biomaterial and not an improved fibrin glue. Unlike the PRP, the strong fibrin

matrix of PRF does not dissolve quickly after application, instead, it is formed slowly in a similar way to a natural BC.(74)

Although composed of almost similar fibrin membranes, PRF has lower risk than PRP during the application since there is no bovine thrombin and anticoagulants present. PRF also shows better potency in accelerating wound and tissue healing, as well as better efficiency for cell proliferation and migration than PRP (107,108). PRF clots acted as successful scaffolds for the regeneration of dentin and pulpal contents in immature teeth with necrotic pulps because of its ability to increase root length, increase dental wall thickness, and healing the periapical lesion better than BC and PRP.(74,79) Meanwhile, in terms of clinical sign and symptom resolution, PRF achieved comparable outcomes to BC in regenerative endodontic therapy.(78) In the therapy of necrotic immature permanent teeth, revascularization/revitalization utilizing PRF also showed to be highly successful.(81)

When being combined with stem cells, PRP and PRF also show better regeneration potential. Human DPSCs was co-cultured with 10% of PRP showed higher expression levels of fetal liver kinase (Flk)-1, VEGF, PDGF, and stromal cell-derived factor 1 (SDF-1) mRNA compared to the combination of hDPSCs and fetal bovine serum (FBS). This suggests that PRP can promote vasculogenesis better than FBS in hDPSCs culture.(109) Both combinations of hDPSCs + PRP and hDPSCs + liquid-PRP showed significant increase of cell migration, proliferation, and differentiation compared to hDPSCs only. Though in hDPSCs + liquid-PRF, the cell migration was observed faster than hDPSCs + PRP.(110)

Natural-derived Biomaterial Scaffolds

Natural-derived polymers are usually used as biomimetic materials for scaffold in regenerative endodontic therapy. Most of the natural polymers are bioactive, containing cellular binding motifs, thus promoting cell adhesion, and/or present soluble signaling factors that are capable in regulating cell behaviour, hence it is also known to provide better biocompatibility compared to synthetic polymers.(90,111) Natural polymers are also known to be rapidly degradable compared to other types of scaffolds, hence allowing easier replacement with natural tissues after the degradation.(104,112) Natural polymers consist of natural polypeptides of the ECM, such as collagen, fibrin, gelatine and keratin, as well as polypeptides that are chemically similar to natural glycosaminoglycans, such as alginate, chitosan and hyaluronic acid (HA).(90)

For the regeneration of pulp and dentin-like tissue, polymers like collagen, gelatine, fibrin, chitosan, and HA have shown the ability to improve root development, including increase root length, root thickness, and enhance the mineralization of root canal (67,83,85,88,87,90-92). While being used as a single scaffold, those natural polymers also showed better ability in increasing intracanal connective tissue formations and narrowing apical width compared with BC, healing the periapical lesion, increasing dental wall thickness, as well as resuming the maturation process for the immature teeth.(67,82-84,87,92)

Natural polymers are often combined and crosslinked with other bioscaffold or chemical agents to improve its potential in regenerative therapy.(113) Dental pulp regeneration through cell homing approaches can be improved by using the combination of HA hydrogel and BC, as well as combination of chitosan hydrogel and BC scaffolds.(67,114) Meanwhile, to fill root canal space with new vital tissue and to enhance the root canal mineralization, the combination of gelatine sponge and BC scaffold as well as collagen and BC scaffold can be used, and have shown better results compared to BC scaffold only.(86,87) To enhance scaffolds physical

properties, the crosslinking between collagen hydrogel and cinnamaldehyde (CA) had shown to be successful. It resulted in the enhanced physical properties of collagen by CA, which upregulated the cellular adhesion compared to the collagen only. This means that this property was promoted in the presence of CA.(115)

In terms of its vascularization function, while being used as a single bioscaffold, both fibrin and HA have shown the potential of increasing vascularization better than the control.(67,91) On the other hand, chitosan, when being used alone, does not show vascularization potential, however when being combined with sodium hyaluronate or pectin, both combinations were able to increase vascularization of connective tissues.(89)

Besides its advantages in dental-pulp regeneration and vascularization, natural-derived bioscaffolds that are classified into moldable porous scaffold, such as chitosan and collagen as single scaffold, or even combination of gelatine/collagen hydrogens bioscaffold, also have the ability to promote cell adhesion, migration and proliferation (90,104,113,116). And to induce hDPSCs cell migration, adhesion, and proliferation, which later followed by a culminated amount of mineralized matrix, scaffold from chitosan and collagen matrix can also be combined with calcium-aluminate.(117) In the combination with SCAP, cell viability promotion, mineralization, and odontoblastic-like differentiation can also be achieved by using HA-based injectable gel scaffold.(118)

Synthetic Biomaterial Scaffolds

While natural-derived polymers scaffolds offer good biocompatibility and bioactivity, synthetic polymers scaffolds offer more flexible and controllable physical and mechanical properties to fit for specific applications.(70,119) Polylactic acid (PLA) and polyglycolic acid

(PGA), as well as their copolymers such as poly-L-lactic acid (PLLA), polylactic-polyglycolic acid (PLGA), and polycaprolactone (PCL) have been successfully reported to be used as bioscaffold for regenerative endodontics therapy.(68)

Synthetic polymers scaffolds and its combination with other scaffold materials are able to induce pulp-dentin regeneration. The increase of mineralization, as well as tissue and bone formation, can be reached by using the combination of PLGA and magnesium scaffold, PLLA combined with DPSC or minced-pulp mesenchymal stem cell (MSC), as well as combination of PCL and PDLSC.(94,97,98) Other than that, culture of hDPSCs on either side of PLGA scaffold was also able to enhance surface closing in the opened side of scaffold. Meanwhile, in terms of pulp vascularization and neurogenesis, the enhancement of neurovascular regeneration through angiogenic and neurogenic paracrine secretion has been reported after the therapy with PLGA scaffold on hDPSCs culture.(96)

PLLA and PLGA scaffolds while being cultured in DPSC are able to improve DPSC differentiation and proliferation, it also induces longer cell replicative lifespan (93,94,96). PLLA scaffold was also used for human minced-pulp MSC, and the results found that the combination showed even better ability to increase cell differentiation and replication better than in DPSC (Liang, et al., 2017). Although not being used as scaffold as much as PLLA and PLGA, the use of PCL scaffolds in SCAP and hDPSCs seeding were also reported to be able to increase the cell proliferation and differentiation.(99,120)

A PLGA microsphere combined with hDPSCs, was able to increase hDPSCs proliferation and adhesion to the scaffold, as well as increase expression levels of *DMP1*, *DSPP*, *COL1*, and *OPN* genes.(95) While increased expression of *DMP1, DSPP, RUNX2, OCN, SPP1, COL1a1*, and *GDF5* genes was obtained with the combination of PCL and fluorapatite.(99) In

the construction of dental and periodontal pulp for the preservation of periodontal ligament fibroblasts (PDLF), the use of PLGA scaffold combined with PRF has shown the ability to sustain fibroblast viability.(68,121)

Role of Signaling Molecules in Regenerative Endodontics

Various signaling molecules, including growth factors and cytokines have been recognized to enhance the proliferation, migration and differentiation of dental stem cells. These molecules are naturally contained in the pulpal cells and dentin matrix, and involved in modulating dentin-pulp complex homeostasis.(122) In the pulp-dentin regeneration process, the remaining periapical and pulpal cells, adjacent dentin, or implanted platelet concentrates, blood clot scaffold, or stem cells are responsible for the release of signaling molecules. To accelerate the process, exogenous signaling molecules are often delivered together with dental stem cells in a scaffold. Addition of signaling molecules to transplanted dental stem cells is expected to mimic the signaling cascades that occur during the formation of pulp-dentin complex.(123)

Signaling Molecules Related to Cell Migration

Bone morphogenetic protein (BMP)-2, TGF-β1, basic FGF (bFGF), PDGF, VEGF, NGF, and BDNF have been reported to stimulate cell migration (Table 4). Induction of cell migration by these molecules is important, since cells must reach the damaged sites to regenerate the tissues. Several signaling pathways have been identified to be induced by these molecules in stimulating cell migration. For example, via PDGFR-β/Akt pathway, PDGF contributes in recruiting smooth muscle cells to blood vessels (162); BDNF accelerates DPSCs migration via extracellular signal-regulated kinase (Erk) phosphorylation (187); VEGF increases the migration

of DPSCs through VEGF receptor (VEGFR) 2 activation and its downstream focal adhesion kinase (FAK) / phosphoinositide 3-kinase (PI3K) / Akt and p38 signaling.(175,176)

Signaling Molecules Related to Cell Proliferation

After reaching the damaged sites, cells must proliferate to increase the number of cells. BMP-2, TGF-β1, bFGF, PDGF and VEGF have been reported to increase proliferation (Table 4). However, the proliferation process is inhibited when cells start to enter the differentiation stage. Thus, signaling molecules which have proliferation-related functions may both inhibit proliferation and induce differentiation in a specific time point, as discussed in the subsequent sections. Several signaling pathways have been identified to be induced by these molecules in stimulating cell proliferation. BMP-2-induced cell proliferation involves BMP-2 receptor (BMP2R) activation as well as Erk1/2 and small mothers against decapentaplegic (Smad) 1/5 phosphorylation (Huang 2018), while bFGF modulates the expression of Cyclin B1 and cell division control 2 (cdc2), which are related to cell-cycle regulation via mitogen-activated protein kinase kinase (MEK)/Erk pathway.(148) VEGF activates the Akt signaling pathway and increases cyclin D1 expression levels, which in turn promotes proliferation of DPSCs.(176)

Signaling Molecules Related to Dentinogenesis and Pulp Regeneration

BMP-2, TGF-β1, bFGF, PDGF, VEGF, and NGF have been reported to enhance dentinogenesis (Table 4). These molecules have been demonstrated to increase differentiation and mineralization of both dental pulp cells and dental stem cells as indicated by an increase in ALP activity and mineralization, as well as upregulation of osteo-/odontogenic marker

expression *in vitro*.(126,145,150,151,182) *In vivo*, these molecules are observed to stimulate dentin formation.(126,139,153,165,181)

TGF-β1 has been demonstrated to enhance ALP activity via activation of Smad2/3, TGF-β activated kinase 1 (TAK1), as well as Erk1/2 and p38. (142) BMP-2 has been known to induce phosphorylation of Erk1/2 and Smad1/5.(125) bFGF could induce mitogen-activated protein kinases (MAPKs) (p38, JNK, and Erk), PI3K/Akt, protein kinase C (PKC), and nuclear factor κB (NF-κB) (188), BMP or Wnt signaling.(189) Meanwhile, VEGF has been known to activate Akt, MAPKs (p38, JNK, and Erk), and NF-κB.(151)

Intriguingly, induction of differentiation and mineralization by TGF-β1 and BMP-2 is often associated with a decrease in cell proliferation (130,145). In addition, TGF-β1 increases the expression of early marker genes of odonto-/osteo-genic differentiation and decreases the expression of late-stage mineralization genes.(145) VEGF might not be able to trigger full osteo-odontogenic differentiation, and facilitate only the early stage of cell differentiation.(181) VEGF potential in inducing mineralization is lower compared with bFGF (151) and NGF.(182) The potential of PDGF in enhancing hard tissue formation has been shown to be lower than other materials, such as enamel matrix derivative (EMD) and mineral trioxide aggregate (MTA).(190) Furthermore, PDGF-BB has been reported to inhibit the formation of mineral nodules.(12) Therefore, PDGF should be used in combination with other materials to increase the mineralization potential.(165,166) However, studies regarding signaling pathways that are involved in PDGF and NGF-induced dentin formation are limited.

bFGF, TGF-β1, and NGF are known to contribute to pulp regeneration (Table 4). bFGF regulates growth of dental pulp cells, upregulates the expression of cdc2, cyclin B1, and tissue inhibitor of metalloproteinase-1 (TIMP-1), as well as inhibits ALP activity and collagen I

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production through activation of FGF receptors (FGFRs) and MEK/Erk signaling.(148) Meanwhile, TGF-β1 has been demonstrated to increase TIMP-1 production, collagen content, and procollagen I, but slightly attenuate MMP-3 production, which are related to the activation of activin receptor-like kinase-5(ALK5)/Smad2/3, TAK1, MEK/Erk, and p38 signaling.(137,142) NGF has been reported to upregulate the expression of healing and repair-related genes (182), as well as improve pulp cell organization and pulpal architecture.(183) Thus, bFGF, TGF-β1 and NGF are involved in pulp regeneration by altering matrix turnover and dental pulp cell proliferation, as well as modulating pulp repair-related gene expression.

Signaling Molecules Related to Angiogenesis

VEGF, PDGF, bFGF, and TGF-β1 have been reported to induce angiogenesis (Table 4) by promoting differentiation of dental stem cells toward endothelial (156,169) or smooth muscle cells (143,144), as shown by upregulation of several differentiation genes (138,144,156). These signaling molecules also induce the formation of capillary-like structures, both *in vitro* (156,164,169) and *in vivo* (164,170).

VEGF has been demonstrated to accelerate angiogenesis, since angiogenesis could occur even in the absence of this molecule (170). This molecule induces angiogenesis by inducing VEGFR phosphorylation and activating downstream Akt, MAPKs (p38, JNK, and Erk), NF-κB (151). Besides formation of new blood vessels, VEGF has been reported to induce anastomosis of DPSCs-derived blood vessels by increasing vascular endothelial (VE)-cadherin expression through the activation of MEK1/Erk, which in turn causes E-26 transformation-specific-related gene (ERG) transcription factor binds to *VE-cadherin* promoter (178). VEGF-induced angiogenesis could be enhanced by inhibiting specific pathways or combining it with other

molecules. Combination of VEGF with SB-431542, an inhibitor of TGF-β1 signaling, has been shown to markedly promote SHED differentiation toward endothelial cells, since Smad1/2 inhibition is correlated with VEGFR2 activation (169). IGF-1 (169) and SDF-1α (173) were also reported to have a synergistic effect in enhancing angiogenesis when combined with VEGF.

PDGF-BB alone induces capillary sprouting, and this phenomenon could be enhanced by bFGF (162). bFGF alone could induce angiogenesis, but its angiogenic potential is lower than VEGF (151). PDGF-BB has been reported to promote blood vessels maturation by regulating the investment of smooth muscle cells to DPSCs-derived capillaries through PDGFRβ and Akt phosphorylation in both types of cells (162). In addition, DPSCs-derived smooth muscle cells that are produced after TGF-β1 treatment have been reported to stabilize blood vessels through ANGPT1/Tie2 and VEGF/VEGFR2 signaling (143). Combination of PDGF-BB and TGF-β1 induces the expression of smooth muscle-specific early, mid, and late markers, as well as enhances contraction ability in DPSCs, although the cells do not undergo morphological alterations toward smooth muscle-specific cell shapes (144).

Signaling Molecules Related to Neurogenesis

NGF, BDNF and bFGF have been reported to induce neurogenesis (Table 4). In several neurogenesis induction studies, NGF and BDNF are combined with other neurotrophin and non-neurotrophin signaling molecules (150,182,187). Meanwhile, bFGF is usually combined with epidermal growth factor (EGF) for neural induction (161). Addition of these molecules increases the expression levels of neural markers and promotes morphological alterations of the treated cells toward neuronal and glial cells (150,186). These molecules have also been reported to induce axonal sprouting and promote axonal growth (161,185).

NGF and BDNF induce neurogenesis via non-specific activation of p75 neurotrophin receptor (p75NTR). In addition, NGF specifically activates tropomyosin-related kinase A (TrkA), while BDNF specifically activates TrkB (191). Meanwhile, bFGF induces neurogenesis via activation of FGFR (192). Activation of these receptors have been reported to induce the phospholipase C (PLC)-γ pathway, which in turn promotes neuronal differentiation (191,192). Besides, combination of bFGF and NGF also stimulates neuronal differentiation via PI3K/Akt and Erk pathways (150).

Future Perspectives on the Use of Dental Stem Cells, Scaffold, and Signaling Molecules Combination in Regenerative Endodontics

Numerous studies have reported successful pulp-dentin complex regeneration using specific combinations of dental stem cells, scaffold, and signaling molecules. Despite most of the ongoing regenerative endodontics studies using these combinations are conducted in animal models (21,193), these combinations were also reported to induce pulp-dentin regeneration in human subjects. Several examples of dental stem cells, scaffold, and signaling molecules combination that have been known to regenerate human pulp-dentin complex are combination of hpDPSCs, G-CSF, and atelocollagen scaffold (29,30), as well as combination of DPSCs and L-PRF (28), which acts as scaffold and contains PDGF and TGF-β (194). Indeed, the regenerative endodontics field is constantly growing. There will be new findings and innovation regarding dental stem cell biology, the development of new types of scaffolds, and the best way to deliver stem cells and signaling molecules to the root canal, which open a new perspective on a new era of endodontic therapy. Thus, current trends and future directions on regenerative endodontics should be further explored.

In most pulp-dentin regeneration studies using human subjects, a scaffold that already contains dental stem cells and immobilized signaling molecules is directly transplanted to the root canal in a single appointment (28-30). Despite the success of this current protocol in regenerating functional pulp-dentin complex, the current procedure might not be similar to the natural process of pulp-dentin regeneration, which involves specific cellular processes. Additionally, regeneration of the pulp-dentin complex may be incomplete in some patients due to differences in pulp-dentin damage severity. To achieve complete pulp-dentin regeneration, additional dental stem cells and/or signaling molecules could be applied in the several next appointments. Since scaffolds have different physical characteristics and biocompatibility, different types of scaffolds could be used to facilitate pulp-dentin regeneration in different parts of teeth. Different types of dental stem cells, signaling molecules, and scaffolds could also be combined with other endodontic procedures, such as apexification and pulp revascularization (195) to enhance the regeneration process in different parts of teeth. Therefore, dental stem cell, scaffold, and/or signaling molecules application could be performed in multiple appointments to mimic the cellular processes that are involved in the regeneration process, hence gradual pulp-dentin regeneration could be achieved.

Although studies regarding tissue engineering-based pulp-dentin regeneration show promising results, there are several challenges for its future clinical translation that need to be addressed. Regenerated pulp-dentin complex should have a precise and highly ordered histological structure as compared to that in normal teeth (4). Besides, different oral diseases, such as irreversible pulpitis and necrotic pulp, as well as the presence of residual bacteria and lipopolysaccharide may affect the root canal microenvironment, which in turn alter the fate of transplanted dental stem cells (196,197). Other factors, including age and the presence of

systemic diseases might also affect the regeneration potential of dental stem cells (4). Since each type of dental stem cell, scaffold, and signaling molecule has unique characteristics and functions, they can be utilized to address these challenges by combining these components together to achieve successful regeneration. Thus, the right combination of dental stem cells, scaffold, and signaling molecules is needed to enhance the pulp-dentin regeneration process.

Conclusion

Combinations of dental stem cells, scaffold, and signaling molecules mimic the cellular microenvironment that is suitable for regeneration, hence they are important to achieve the functional pulp-dentin complex formation. Since regenerative endodontics is a constantly growing field, current trends and future directions in this field are still needed to be further explored. The right combination of dental stem cells, scaffold, and signaling molecules could be determined based on the patients' characteristics. Incomplete pulp-dentin regeneration, which may occur in some cases, could be overcome by applying dental stem cells, scaffold, and/or signaling molecules in multiple appointments to achieve gradual pulp-dentin regeneration.

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Table 1. Regenerative potential of DPSCs, SHED, and DFSCs in animal model of pulp-dentin regeneration.

Type of Dental Stem Cells	Species	Regenerative Potential			Reference
		Pulp- and/or Dentin-like Tissue	Angiogenesis	Neurogenesis	
DPSCs	Dog	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DSPP	Histology: Blood vessels in regenerated pulp	N/A	(5-8)
	Mini-pig	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DSP, DMP-1, and BSP	Histology: Blood vessels in regenerated pulp	N/A	(9)
	Ferret	Histology: Formation of osteodentin mixed with loose connective tissue.	N/A	N/A	(10)
	Rat	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DMP1, DSPP, DSP, and OPN	Histology: Blood vessels in regenerated pulp Positive immunostaining: CD31	N/A	(11-13)

DPSCs $CD31^-$	Dog	Histology: - Pulp tissue regeneration - Dentin formation Gene expression: *MMP20, syndecan 3, TRH-DE*	Positive immunostaining: BS-1 lectin	Positive immunostaining: PGP9.5	(14)
DPSCs $CD105^+$	Dog	Histology: Pulp tissue regeneration	Histology: Blood vessels in regenerated pulp	N/A	(15)
Mobilized DPSCs	Dog	Histology: - Pulp tissue regeneration - Dentin formation Gene expression: *tenascin C, syndecan 3, TRH-DE, MMP20, DSPP* Positive immunostaining: TRH-DE MRI: Signal intensity of transplanted teeth was similar compared with that in normal teeth.	Positive immunostaining: BS-1 lectin Laser Doppler flowmetry: Blood flow in regenerated pulp tissue is similar compared to that in normal pulp tissue.	Positive immunostaining: PGP9.5 Electric pulp test: Positive pulp sensibility response	(16-23)
hpDPSCs	Dog	Histology: - Pulp tissue regeneration - Dentin formation	Positive immunostaining: BS-1 lectin	Positive immunostaining: PGP9.5	(23,24)

hpDPSCs from deciduous teeth	Dog	Histology: - Pulp tissue regeneration - Dentin formation	Positive immunostaining: BS-1 lectin	Positive immunostaining: PGP9.5	(24)
SHED	Mini-pig	Histology: - Pulp tissue regeneration - Dentin formation	Histology: Blood vessels in regenerated pulp Positive immunostaining: CD31	Positive immunostaining: NeuN, NF, CGRP, and TRPV1	(25-26)
DFSC	Mini-pig	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DMP-1, DSPP, COL1, COL3	N/A	N/A	(27)

Table 2. Regenerative potential of DPSCs, SHED, and DFSCs in case reports and clinical trials of pulp-dentin regeneration.

Type of Dental Stem Cells	Regenerative Potential			Reference
	Pulp- and/or Dentin-like Tissue	**Angiogenesis**	**Neurogenesis**	
DPSCs	CBCT: - Formation of dentin bridge - Apical canal calcification	Laser Doppler flowmetry: Blood perfusion in the transplanted tooth with low mean perfusion unit.	N/A	(28)
Mobilized DPSCs	MRI: Complete pulp regeneration CBCT: - Formation of lateral dentin - Decrease in dental pulp volume	N/A	Electric pulp test: Positive pulp sensibility response	(29)
hpDPSCs	MRI: Complete pulp regeneration CBCT: - Formation of lateral dentin - Decrease in dental pulp volume	N/A	Electric pulp test: Positive pulp sensibility response	(30)
SHED	Histology: Regenerated pulp with odontoblast layer, connective tissue, and blood vessels. CBCT: Increase in dentin thickness	Laser Doppler flowmetry: An increase in vascular formation as indicated by high perfusion units.	Positive immunostaining: NeuN Electric pulp test: Positive pulp sensibility response	(25)

Table 3. Regenerative potential of blood-derived, natural-derived polymer, and sythetic polymer bioscaffolds.

Types of Scaffolds		Regenerative Potential		References
		Pulp-dentin Regeneration	Vascularization	
Blood-derived				
	BC	- Increasing root length and thickness - Increasing dental wall thickness - Improving bone density - Narrowing apical width - Healing the periapical lesion	- Improving vitality response (blood pump)	(71-80)
	PRP	- Increasing root length and thickness - Increasing dental wall thickness - Improving bone density - Narrowing apical width - Healing the periapical lesion	- Improving vitality response (blood pump)	(71,72,74-77,79-81)
	PRF	- Increasing root length and thickness - Increasing dental wall thickness - Improving bone density - Narrowing apical width - Healing the periapical lesion	- Improving vitality response (blood pump)	(74,77,78,79,81)
Natural-derived polymers				

	Collagen - BC	- Increasing root length - Enhancing mineralization of root canal - Increasing dental wall thickness - Narrowing apical width - Healing the periapical lesion - Increasing intracanal connective tissue formation	N/A	(82-86)
	Gelatin - BC	- Increasing root lenght and thickness - Increasing root length - Increasing dental wall thickness - Narrowing apical width - Increasing intracanal connective tissue formation	N/A	(87,88)
	Chitosan - BC - Sodium hyaluronate - Pectin	- Increasing root length and thickness - Increasing dental wall thickness - Enhancing mineralization of root canal - Narrowing apical width - Healing the periapical lesion - Increasing intracanal connective tissue formation	- Increasing vascularization	(89,90)
	Fibrin	- Increasing root length and thickness - Enhancing mineralization of root canal - Narrowing apical width - Healing the periapical lesion	- Increasing vascularization	(88,91)

	HA	- Increasing root length - Enhancing mineralization of root canal - Increasing dental wall thickness - Narrowing apical width - Healing the periapical lesion - Increasing intracanal connective tissue formation	- Increasing vascularization	(67,92)
Synthetic biomaterial				
	PLLA - DPSC - Minced-pulp MSC	- Enhance tissue mineralization - Increase expression levels of *DMP1, DSPP, COL1*, and *OPN* genes	N/A	(93-95)
	PLGA - DPSC - Magnesium	- Increase bone height and volume - Enhance bone mineralization - Enhance surface closing	- Initiate neurovascular regeneration	(96,97)
	PCL - PDLSC - Fluorapatite	- Enhance bone formation in defect tissue - Improve periodontium neogenesis - Increase expression of *DMP1, DSPP, RUNX2, OCN, SPP1, COL1a1*, and *GDF5* genes	N/A	(98,99)

Table 4. Regenerative potential of signaling molecules in pulp-dentin regeneration.

Signaling Molecule	Regenerative Potential					Reference
	Cell Migration	**Cell Proliferation**	**Pulp- and/or Dentin-like Tissue**	**Angiogenesis**	**Neurogenesis**	
BMP-2	Inducing migration of dental pulp cells	Increasing proliferation of dental pulp cells	• Increasing ALP activity and mineralization • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *ALP*, *RUNX2*, *COL1*, *DSPP*, *DMP1*, *DSP*, *MMP20*, *BSP*, *OCN*, and *OSX* - Protein expression: RUNX2, DSPP, DMP1, BSP, and OCN	N/A	N/A	(124-135)

TGF-β1	Inducing migration of dental pulp cells	Increasing proliferation of DPSCs and dental pulp cells	• Increasing ALP activity, mineralization, and collagen content • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *DSPP*, *DSP*, *MMP20*, *RUNX2*, *DMP1*, *COL1A1*, and *BSP* - Protein expression: N-cadherin, TIMP1, COL1A1, DMP1, and BSP • Downregulating protein expression: MMP-3	• Inducing smooth muscle cell differentation • Maintaining blood vessels stability • Upregulating differentiation markers - Gene expression: *αSMA, SM22α, CALP*, *SMTN*, and *MYH11* - Protein expression: αSMA, SM22α, CALP, SMTN, ANGPT1, Tie2, and SM-MHC	N/A	(131,136-145)

bFGF	Inducing migration of SCAP, mobilized DPSCs, BMMSCs, periodontal ligament fibroblasts, and endothelial cells	Increasing proliferation of SHED, DPSCs, mobilized DPSCs, BMMSCs, dental pulp cells, periodontal ligament fibroblasts, and endothelial cells	• Increasing ALP activity and mineralization • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *DSPP*, *MMP20*, *TRH-DE*, *ALP*, *TIMP1*, *DMP1*, *COL1A2*, *OPN*, and *OCN* - Protein expression: DSPP, DMP1, TIMP1, and COL1	• Enhancing blood vessel formation • Upregulating differentiation markers - Gene expression: *VEGFR2*, *Tie2*, *ANGPT1*, *VWF*, *VE-cadherin*, and *CD31* - Protein expression: VEGFR2, Tie2, ANGPT1, vWF, VE-cadherin, and CD31	• Inducing neuronal and glial differentation • Promoting axonal sprouting and growth • Upregulating differentiation markers - Gene expression: *Nestin*, *TUBB3*, *SOX2*, *VIM*, *NEFM*, *MAP2*, *NEFH*, *GFAP*, and *S100B* - Protein expression: Nestin, NEFM, TUBB3, NeuN, GFAP, S100B, and MAP2	(146-162)

PDGF	Inducing migration of DPSCs, SHED, dental pulp cells, and smooth muscle cells	Increasing proliferation of DPSCs	• Increasing ALP activity and mineralization • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *DMP1*, *DSPP*, and *OCN* - Protein expression: DMP1 and DSPP	• Inducing smooth muscle and endothelial cell differentation • Enhancing blood vessel formation • Promoting blood vessel stabilization • Upregulating differentiation markers - Gene expression: *αSMA*, *SM22α*, *CALP*, *SMTN*, and *MYH11* - Protein expression: α-SMA, SM22-α, CALP, SMTN, VEGFR2, Tie-2, CD31, and VE-cadherin	N/A	(136,143,144,163-168)

VEGF	Inducing migration of DPSCs and endothelial cells	Increasing proliferation of DPSCs and dental pulp cells	• Increasing ALP activity and mineralization • Upregulating odontoblast markers - Gene expression: *ALP*, *OCN*, *OSX*, *DSPP*, *RUNX2*, *DMP1*, *COL1A2*, *BSP*, *TGFB1*, and *OPN* - Protein expression: DMP1, DSPP, and OSX	• Inducing endothelial cell differentation • Enhancing blood vessel formation • Promoting blood vessel anastomosis • Upregulating differentiation markers - Gene expression: *VWF*, *VEGFR2*, *VE-cadherin*, *CD31*, *VEGFR1*, *EphrinB2*, Tie2, and *ANGPT* - Protein expression: vWF, VEGFR2, VE-cadherin, CD31, Tie-2, F8	N/A	(124,130,151,156,169-182)
NGF	Inducing migration of glial cells	N/A	• Improving pulpal architecture and cell organization • Upregulating gene expressions of differentiation markers: *DSPP*, *DMP1*, and *TGFB1*	N/A	• Inducing neuronal and glial differentation • Promoting axonal sprouting and growth • Upregulating differentiation markers - Gene expression: *Nestin* - Protein	(150,182-185)

					expression: S100, NF, and p75NTR	
BDNF	Increasing migration of DPSCs	N/A	N/A	N/A	• Inducing neuronal and glial differentation • Upregulating protein expressions of differentiation markers: DCX, NeuN, S100B and p75NTR.	(186-187)

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Manuscript Review Form

Reviewer	:	Reviewer 2
Manuscript #	:	MR2023042
Manuscript Title	:	Combining Dental Stem Cells, Scaffold, and Signaling Molecules for Pulp-Dentin Complex Regeneration

No.	Manuscript Components	Yes	No
1.	Does this manuscript present new ideas or results that have not been previously published?	**v**	
	Notes:		
2.	Are the title and abstract of the manuscript appropriate?		v
	Notes: The title needs to be added with the word 'Proper' in front of the title: Proper Combining Dental Stem Cells, Scaffold, and Signaling Molecules for Pulp-Dentin Complex Regeneration		
3	Do the title and abstract reflect the study result/content?	v	
	Notes:		
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	Notes:		
5.	Are the research study methods technically correct, accurate, and complete enough to be reproduced/cited by other scientists?		v
	Notes: It's better to add with discussion part (outside of the topic of Future Perspectives on the Use of Dental Stem Cells, Scaffold, and Signaling Molecules Combination in		

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	Regenerative Endodontics) to discuss more systematic, intensive and specific about the steps especially for clinicians to do the laboratory and clinical procedure started for the simple case.		
6.	Are the results, ideas, and data presented in this manuscript important enough for publication?	v	
	Notes:		
7.	Are all figures and tables necessarily presented?		v
	Notes: the abbreviation term of N/A need to be written at the bottom of Table		
8.	Is there a logical flow of argument in the Discussion which elucidate all the presented/obtained data?	v	
	Notes:		
9.	Are the conclusions and interpretations valid and supported by the data?	v	
	Notes:		
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	Notes:		

Specific Reviewer's Comments and Suggestions:

(These comments may be in addition to or in lieu of reviewer comments inserted into the text of the manuscript. Use as many lines as needed.)

- The title becomes: Proper Combining Dental Stem Cells, Scaffold, and Signaling Molecules for Pulp-Dentin Complex Regeneration
- Manuscript added with discussion part contain about the steps has to be done especially by clinicians for doing pulp dentin complex regeneration according to the diagnosis.

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Reviewer's Recommendation (Please tick only one option)	√
Accept Submission (No significant alterations suggested)	v
Revisions Required (Suggest changes to the manuscript as specified in this review)	
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Further Reviewer's Comments Regarding Disposition of the Manuscript:

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February 8, 2023

Reviewer 2

UNIVERSITAS TRISAKTI

Ferry Sandra <ferry@trisakti.ac.id>

[InaBJ] MR2023042 Editor Decision Round 1 - Revisions Required

Ferry Sandra <ferry@trisakti.ac.id> Mon, Feb 20, 2023 at 10:57 AM
To: Secretariat of InaBJ <secretariatinabj@gmail.com>

Dear Secretariat of The Indonesian Biomedical Journal,

Thank you for providing the review results. Enclosed is the updated version of the manuscript MR2023042 titled "Crucial Triad in Pulp-Dentin Complex Regeneration: Dental Stem Cells, Scaffolds, and Signaling Molecules". The manuscript has been revised in accordance with the feedback received.

Thank you.

Regards,
Ferry Sandra
[Quoted text hidden]
--
Ferry Sandra, D.D.S., Ph.D.
Head of Medical Research Center
Universitas Trisakti

2 attachments

Round 1 Revision from Author.docx
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Round 1 Revision from Author - Tables.xlsx
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Crucial Triad in Pulp-Dentin Complex Regeneration: Dental Stem Cells, Scaffolds, and Signaling Molecules

Abstract

Background: Pulp damage can lead to dentinogenesis impairment, irreversible pulpitis, or pulp necrosis. Despite being the most used endodontic procedure to treat damaged pulp, root canal therapy only results in nonvital teeth which are prone to fractures and secondary infection. Pulp-dentin regeneration has a potential to regenerate structure similar to normal pulp-dentin complex, and can be achieved by combining dental stem cells, scaffold, and signaling molecules. This article reviews the role of various types of dental stem cells, scaffolds, signaling molecules, and their combinations in regenerating pulp-dentin complex.

Content: Dental pulp stem cell (DPSC), stem cell from human exfoliated deciduous teeth (SHED), and dental follicle stem cell (DFSC) were reported to regenerate pulp-dentin complex *in situ*. SHED might be more promising than DPSCs and DFSCs for regenerating pulp-dentin complex, since SHED have a higher proliferation potential and higher expression levels of signaling molecules. Scaffolds have characteristics resembling extracellular matrix, hence providing a suitable microenvironment for transplanted dental stem cells. To accelerate the regeneration process, exogenous signaling molecules are often delivered together with dental stem cells. Scaffolds and signaling molecules have different regenerative potential, including induction of cell proliferation and migration, formation of pulp- and/or dentin-like tissue, as well as angiogenesis and neurogenesis promotion.

Summary: Combinations of dental stem cells, scaffold, and signaling molecules are important to achieve the functional pulp-dentin complex formation. Current trends and future directions on

regenerative endodontics should be explored. The right combination of dental stem cells, scaffold, and signaling molecules could be determined based on the patients' characteristics. Incomplete pulp-dentin regeneration could be overcome by applying dental stem cells, scaffold, and/or signaling molecules in multiple visits.

Keywords: pulp-dentin regeneration, regenerative endodontics, dental stem cells, scaffold, signaling molecules

Introduction

Dental pulp, the only soft tissue in the tooth, plays a critical role in sustaining tooth homeostasis. However, this tissue is vulnerable to various stimuli, including infections, iatrogenic causes, and trauma.(1) If not treated properly, pulp damage can lead to dentinogenesis impairment and irreversible pulpitis or even pulp necrosis, since this tissue has a limited self-repair capacity.(2)

Damaged pulp can be treated by several procedures. Root canal therapy, the most used endodontic procedure, replaces inflamed or injured pulp with bioinert material fillings. However, this procedure results in nonvital teeth, which are prone to fractures and secondary infection.(3) Regenerative endodontic treatment or pulp-dentin regeneration is an alternative procedure based on the tissue engineering principle. Pulp-dentin regeneration is more holistic than other endodontic procedures since this procedure has a potential to regenerate structure similar to normal pulp-dentin complex. The main goals of pulp regeneration are pulp-dentin complex formation as well as angiogenesis and neurogenesis in the newly regenerated pulp.(4)

Tissue engineering combines dental stem cells, scaffold, and signaling molecules to mimic a suitable microenvironment for regenerating pulp-dentin complex. Numerous studies have been established to examine the effects of dental stem cells, scaffold, signaling molecules, and their combinations in pulp regeneration, providing a new insight in the field of regenerative dentistry and opening a great opportunity for further clinical applications. This article reviews the role of various types of dental stem cells, scaffolds, signaling molecules, and their combinations in regenerating pulp-dentin complex. The right combination of these components could increase pulp-dentin regeneration therapy efficiency.

Role of Dental Stem Cells in Regenerative Endodontics

Based on the locations, dental stem cells are classified as dental pulp stem cell (DPSC), stem cells from human exfoliated deciduous teeth (SHED), stem cells from the apical papilla (SCAP), dental follicle stem cell (DFSC), periodontal ligament stem cell (PDLSC).(5,6) DPSCs, SHED, and DFSCs were reported to have potential in regenerating pulp-dentin complex *in situ*, both in animal models (Table 1) and human subjects (Table 2).

Cell Number, Proliferation Rate, and Immunomodulatory Properties of DPSCs, SHED and DFSCs

DPSCs, SHED and DFSCs are different in several aspects, including the number of cells isolated from the tissues, proliferation rate, and immunomodulatory mechanisms. DPSCs and SHED have relatively high cell numbers in original cultures compared with DFSCs, because dental pulp, both in permanent and deciduous teeth, have relatively high amounts of stem cells compared with dental follicles of developing tooth germ. Since dental follicle tissues are smaller

in size, contain small amounts of cells, and located in sites that are relatively not easy to be accessed, DFSCs are difficult to be obtained and distinguished from other types of dental stem cells.(4)

DPSCs have been reported to have a higher proliferation rate compared with bone marrow mesenchymal stem cell (BMMSC), while SHED have a higher proliferation rate than DPSCs.(33) It has been demonstrated that the proliferation rate of DFSCs is notably higher than DPSCs.(34) Moreover, in a recent study, DFSCs were shown to have a higher proliferation rate than SHED.(35) Thus, DFSCs might have the highest proliferation rate, followed by SHED and DPSCs. High proliferation of DFSCs implies that they are more immature, since this type of stem cells are isolated from developing tissues (36), and consequently they might be more plastic compared with other dental stem cells. In summary, DPSCs, SHED, and DFSCs vary in their proliferation rates, which could be determined by the developmental stages of the stem cell sources.

Mesenchymal stem cell (MSC), including DPSCs, SHED and DFSCs have been reported to modulate the immune system through several mechanisms (37). DPSCs have been demonstrated to modulate the adaptive and innate immune responses through interaction with B cells, T cells, macrophages, dendritic cells (DCs), and natural killer (NK) cells. For instance, the production of B cell immunoglobulin and proliferation of T cell proliferation are inhibited in co-culture of peripheral blood mononuclear cells (PBMCs) and DPSCs. Transforming growth factor (TGF)-β secreted by DPSCs plays a crucial role in this inhibition and the addition of interferon (IFN)-γ to DPSCs culture enhances the inhibitory effects.(38) DPSCs markedly decrease $CD4^+$ and $CD8^+$ T cell proliferation, irrespective of hypoxia-inducible factor (HIF)-1α expression level in DPSCs. However, overexpression of HIF-1α increases the DPSCs inhibitory effect on DCs

proliferation. Expression of HIF-1α by DPSCs also enhances the recruitment and differentiation of macrophages with M2 characteristics. Furthermore, NK cell-mediated cytotoxicity is suppressed in HIF-1α-overexpressed DPSCs.(39)

SHED have been shown to modulate T cells, macrophages and DCs. This type of stem cell restrains the differentiation of T helper (Th) 17 cells, and has greater immunomodulatory potential compared with BMMSCs.(40) SHED have been reported to promote phenotypic polarization of macrophage toward M2-like phenotype in transwell co-culture systems and increase the number of macrophages with M2-like phenotype in rat model of periodontitis.(41) A study demonstrates that SHED affect differentiation, maturation, and T cell activation ability of DCs. The same study also shows that SHED augment T regulatory (Treg) cell induction ability of DCs. SHED-treated DCs have a lower level of IFN-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-2, as well as higher level of IL-10.(42)

Meanwhile, DFSCs have immunomodulatory properties toward T cells and macrophages. A study demonstrates that DFSCs increase the number of Treg cells as well as suppress $CD4^+$ T cell proliferation via TGF-β and indoleamine 2,3-dioxygenase (IDO) pathways.(43) In lipopolysaccharide (LPS)-induced macrophage, this type of stem cell is involved in phenotypic polarization to M2 by secreting thrombospondin-1 and TGF-β3.(44) Therefore, the immunomodulatory activities of DPSCs are exerted on B cells, T cells, macrophages, DCs, and NK cells. SHED regulates T cells, macrophages and DCs, while DFSCs show immunomodulatory activities toward T cells and macrophages.

DPSCs, SHED and DFSCs Play a Crucial Role in Regenerating Pulp-dentin Complex

Dental stem cells are involved in pulp-dentin complex formation *in situ*. When transplanted into an emptied root canal or a tooth construct, DPSCs, SHED, and DFSCs generate tissue that has characteristics resembling dental pulp. Several biomarkers have been used to detect the presence of the regenerated pulp, such as thyrotropin-releasing hormone-degrading enzyme (*TRH-DE*), *syndecan 3*, and *tenascin*. Furthermore, magnetic resonance imaging (MRI) can also be utilized to assess pulp regeneration by dental stem cells in the root canal (Table 1, Table 2). After pulpectomy, the signal intensity of MRI is relatively low compared with those in the normal teeth. The signal intensity in the pulpectomized tooth then increases several days after transplantation and keeps decreasing until it is similar to normal pulp, indicating complete pulp regeneration.(21)

Formation of dentin-like structure by DPSCs, SHED, and DFSCs has also been documented by the generation of dentin matrix deposition that causes dentin thickening and the presence of odontoblast-like cells on the canal dentinal walls which express both specific and non-specific odontoblast markers. Specific odontoblasts markers include dentin sialoprotein (DSP), dentin sialophosphoprotein (DSPP) and dentin matrix acidic phosphoprotein (DMP) 1 (13-15), while non-specific odontoblasts markers include bone sialoprotein (BSP) and osteopontin (OPN).(11,14) There are several viewpoints regarding the use of non-specific odontoblasts markers for detecting newly regenerated dentin. Some investigators consider that enhanced expression of these markers suggest greater dentin regeneration potential (8,10,14,15,28) since they involve in dentin formation.(45) Other investigators consider these markers as osteogenic markers instead of odontogenic markers.(29,46) The increase in the expression of these markers implies that the regenerated structure has similar characteristics with bone instead of dentin.(46). Therefore, these markers should not be more strongly expressed in

dentin than the expression of odontoblast-specific markers.(11,14,29) Besides detection of odontoblast markers, cone beam computed tomography (CBCT) can be used to assess dentin formation (Table 2), which is demonstrated by a reduction in low-density areas, indicating a decrease in pulp volume and an increase in dentin thickness.(32) Studies that use ectopic and semiorthotopic pulp-dentin regeneration models are not included in Table 1, since these models do not provide similar conditions as the human oral cavity.(4)

Additionally, the research that assesses the ability of DFSCs to form pulp-dentin complex is more limited than those conducted using DPSCs and SHED. DFSCs are usually used to simultaneously regenerate pulp-dentin and cementum-periodontal complexes.(29) This may be caused by the tendency of DFSCs to regenerate periodontal tissue and tooth root rather than pulp-dentin complex. Transplantation of treated dentin matrix that contains DFSCs regenerates periodontal-like tissue in subcutaneous space and cementum-like tissue in the outer surface of dentin.(47) Moreover, combination of DFSCs and treated dentin matrix which is transplanted to the alveolar fossa of rats have a potential to induce root formation.(48) Thus, DFSCs are better to use in periodontal tissue and root regeneration, although they might also have a potential to regenerate pulp-dentin complex. Despite the large number of studies that explore the regenerative potential of DPSCs, SHED might be more promising than DPSCs, since SHED have a higher proliferation potential (33) and higher expression levels of signaling molecules which may contribute to the pulp-dentin regeneration.(49)

DPSCs, SHED and DFSCs are Involved in Angiogenesis

Angiogenesis has been reported to occur in pulp-like tissue regenerated by DPSCs and SHED *in situ*. There are limited studies that demonstrate the involvement of DFSCs in the

angiogenesis process in regenerated pulp tissue (Table 1). The angiogenic potential of DFSCs has been reported to be lower compared with DPSCs and SCAP.(50) The new vessels provide oxygen and nutrition to the newly regenerated pulp, hence support the survival of the transplanted stem cells, and facilitate further regeneration process. Blood vessels in the regenerated pulp can be detected using immunostaining of *Griffonia* (*Bandeiraea*) *simplicifolia* lectin 1 (BS-1 lectin) and CD31 (Table 1).

In addition, laser Doppler flowmetry can be used to assess angiogenesis and analyze the blood flow in the regenerated pulp tissue, as demonstrated by several studies. Blood flow in the pulp tissue regenerated by DPSCs is not remarkably different compared with that in normal pulp tissue, implying complete functional angiogenesis.(18) Human tooth with symptomatic irreversible pulpitis which is treated with DPSCs and normal tooth have low mean perfusion units. Blood perfusion in both teeth is indicated by pulse characteristics.(30) In addition, SHED-transplanted teeth experience an increase in the average of vascular formation.(27)

DPSCs, SHED, and DFSCs are involved in angiogenesis through differentiation toward endothelial cells (28) or angiogenic factors secretion. Several angiogenic factors that are expressed by these stem cells includes vascular endothelial growth factor (*VEGF*) (16,28,29), *HIF1A* (28), granulocyte-monocyte colony-stimulating factor (*GM-CSF*), matrix metalloproteinase 3 (*MMP3*) (16), selectin E (*SELE*) (18), angiopoietin (*ANGPT*), and von Willebrand factor (*VWF*).(15) These factors stimulate vessel formation by modulating local endothelial cells in a paracrine manner.(16) Several subsets of DPSCs have been reported to secrete angiogenic factors but they do not incorporate to the newly formed blood vessels, such as dental pulp $CD31^-$ side population cells (16) and granulocyte colony-stimulating factor (G-CSF) mobilized DPSCs.(18,51)

Angiogenesis in pulp-like tissue can be induced further by culturing dental stem cells under hypoxic conditions. Hypoxia mimics conditions in the dental pulp cavity (52), which increases the expression of *HIF1A*. Upregulation of this transcriptional factor activates the expression of angiogenesis-related genes.(25) Hypoxia culture on nanofibrous spongy microspheres increases angiogenesis potential of human DPSCs (hDPSCs) as indicated by more CD31-stained blood vessels in the regenerated pulp-like tissues.(13) Another research demonstrates that the expression levels of *HIF1A* in hypoxia preconditioned DPSCs (hpDPSCs) are two times higher compared with those in mobilized DPSCs, while *VEGF* expression levels in both DPSCs are similar. hpDPSCs have been demonstrated to have a similar neovascularization potential compared to mobilized DPSCs.(25) DPSCs from permanent and deciduous teeth that are cultured under hypoxic conditions have similar expression levels of *VEGF* and *GM-CSF*, as well as *in situ* neovascularization potential.(26) Furthermore, co-culture of dental stem cells with endothelial cells has also been demonstrated to enhance angiogenesis. Crosstalk between transplanted stem cells with endothelial cells has been shown to increase the expression of angiogenic factors in both cells by activating specific pathways, such as nuclear factor κB (NF-κB).(53)

DPSCs, SHED and DFSCs are Involved in Neurogenesis

DPSCs, SHED, and DFSCs have a potential to induce neurogenesis, as shown by the studies that reported the presence of nerve fibers in pulp-like tissue after stem cell transplantation. Newly formed nerve fibers in orthotopic pulp regeneration models are detected using immunostaining of protein gene product 9.5 (PGP9.5), neuronal nuclei (NeuN), neurofilament, calcitonin gene-related peptide (CGRP), and transient receptor potential cation

channel subfamily V member 1 (TRPV1) (Table 1). The expression of other neurological markers, such as sodium voltage-gated channel alpha subunit 1 (*SCN1A*) and *neuromodulin* genes (16,18), as well as tubulin-βIII (TUBB3) (29), nestin, and transient receptor potential cation channel subfamily M member 8 (TRPM8) protein (27), has also been detected in cultured or subcutaneously implanted stem cells. Electric pulp test is another common technique utilized for detecting nerve fibers in regenerated pulp tissue (Table 1, Table 2).

Mechanisms of neurogenesis induction are similar to the angiogenesis induction by DPSCs, SHED, and DFSCs. These types of stem cells have been reported to differentiate toward neural cells.(54,55). In addition, various neurogenic factors are expressed by DPSCs and SHED, including nerve growth factor (*NGF*), glial cell-derived neurotrophic factor (*GDNF*), brain-derived neurotrophic factor (*BDNF*), neuropeptide Y (*NPY*), and neurotrophin 3 (*NTF3*).(16,56) Investigations on neurogenic factors secreted by DFSCs are still limited. Hypoxic conditions could enhance the expression of neurogenic factors in dental pulp stem cells. *NGF* and *BDNF* expression levels are notably higher in hpDPSCs compared with those in mobilized DPSCs, but *GDNF* expression level is lower. It has been reported that hpDPSCs have a similar reinnervation potential compared to mobilized DPSCs.(25) A recent study revealed that DPSCs from deciduous teeth had a markedly higher mRNA expression of *BDNF* compared with those obtained from permanent teeth, but not *NGF* or *GDNF*. However, both of these stem cells had a similar BDNF protein expression level and reinnervation potential.(26)

Factors Affecting the Regenerative Potential of DPSCs, SHED and DFSCs in Pulp-Dentin Complex Regeneration

Several factors may affect the regenerative potential of DPSCs, SHED, and DFSCs. Aging has been reported to cause the reduction of DPSCs regenerative potential. An animal study demonstrated that about 60% of root canal area is covered by pulp-dentin complex after 120 days in teeth of aged dogs (5–6 years of age) transplanted with autologous mobilized DPSCs.(19) This percentage is much lower than that in young dogs (8-10 months of age), which shows regeneration volume of more than 90% after 60 days.(18) SHED, which are obtained from dental pulp of younger individuals, have a higher expression of neuronal markers when compared with adult DPSCs, suggesting lower neurogenic potential in DPSCs.(57) In dental follicle cells, cell senescence is correlated with a decrease in osteogenic potential and lower WNT5A expression, although the role of WNT5A may be less significant in regulating the expression of osteogenic markers.(58)

Dental diseases, such as caries, are reported to have no effect or even increase regenerative potential of dental stem cells. SHED obtained from carious deciduous teeth has a similar osteogenic potential compared to those that were obtained from sound deciduous teeth.(59) Meanwhile, DPSCs isolated from teeth with deep caries have greater proliferation and angiogenesis abilities, as well as higher expression of odontoblast differentiation markers.(60,61)

Dental stem cells can differentiate not only to odontoblasts and dental pulp cells, but also to other types of cells, since it has been reported that transplantation of DPSCs regenerates periodontal ligament-, bone-, and cementum-like tissues instead of pulp-like tissue. Signals sent from tissues surrounding the root canal, such as alveolar bone and periodontal ligament, might affect the fate of transplanted dental stem cells.(46) Taken together, the success of stem cells-mediated pulp-dentin complex regeneration may be affected by aging, dental diseases, and signals sent from the surrounding tissues.

Recent Advances on the Use of Dental Stem Cells in Regenerative Endodontics

Dental stem cells have been demonstrated to regenerate functional pulp-dentin complex in human subjects in several studies, most of them using autologous dental stem cells (Table 2). Combination of autologous mobilized DPSCs and good manufacturing practice (GMP)-grade G-CSF are transplanted into the teeth of five adult irreversible pulpitis patients.(31) Mobilized DPSCs are subsets of DPSCs isolated through G-CSF-induced cell mobilization.(51) Four weeks after transplantation, four patients show a positive electric pulp test result. Lateral dentin formation is observed in three patients as shown by CBCT imaging. Interestingly, all patients do not experience any adverse events or toxicity caused by mobilized DPSCs transplantation.(31)

Successful pulp regeneration using autologous DPSCs obtained from inflamed pulp has also been reported. DPSCs are obtained from the permanent tooth with symptomatic irreversible pulpitis. These stem cells are implanted with leukocyte platelet-rich fibrin (L-PRF) obtained from the patient's blood into the root canal of the same tooth. After 36 months, no tenderness to palpation or percussion, and no adverse events are observed. Laser Doppler flowmetry results demonstrate that both untreated and DPSCs-implanted teeth have pulse characteristics, implying blood perfusion in the teeth, although the mean perfusion units in those teeth are low.(30)

Transplantation of autologous hpDPSCs seeded on atelocollagen scaffold containing G-CSF in multirooted molars of two patients affected by symptomatic or asymptomatic irreversible pulpitis has been successfully demonstrated. No periapical radiolucency is observed by CBCT and radiographic examination after 48 weeks. Moreover, no adverse events or systemic toxicity are experienced by these patients as shown by the results of clinical and laboratory evaluation.(32)

SHED transplantation into injured human teeth markedly increases dentin thickness and root length, as well as reduces apical foramen width compared with the apexification procedure. An increase in vascular formation is observed in SHED transplantation group. In contrast, a decrease in vascular formation is observed in the apexification group. Teeth transplanted with SHED show a significantly higher mean decrease in sensation than those treated with apexification procedure. No adverse events were observed at 24 months after transplantation.(27)

Besides dental stem cells, induced pluripotent stem cells (iPSCs), which are obtained by introducing reprogramming factors including octamer-binding transcription factor 4 (*Oct4*), Kruppel-like factor 4 (*Klf4*), sex determining region Y-box 2 (*Sox2*), *l-myc*, *c-myc*, and *Lin28* to somatic cells, can also be used in pulp-dentin regeneration.(62-65) Stem cells, such as DPSCs (63), and differentiated cells, such as fibroblasts (64) could be used to generate iPSCs. Generation of odontoblasts-like cells could be performed by directly inducing iPSCs.(63) In addition, iPSCs could be induced toward iPSCs-derived neural crest-like cells (iNCLCs), which in turn can be differentiated further into odontoblasts-like cells.(63,64) Differentiation to odontoblasts and generation of pulp-like tissue from iPSCs can be induced by transfection of specific genes (62), as well as addition of exogenous growth factors (63,64) and scaffold.(64)

Whole tooth regeneration is another promising advance in endodontic therapy. This method relies on the interaction between the dental mesenchyme and the dental epithelium to generate a bioengineered tooth bud.(66) Cells of the dental mesenchyme and the dental epithelium can be isolated from embryonic (67-69) or postnatal (67) dental tissues. Autologous (67), allogeneic (69), and xenogeneic (68) cells have been used in tooth bud production. Both types of cells are combined in collagen gel drop and cultured *in vitro* (67-69) or seeded in a

scaffold.(70) The bioengineered tooth bud is then transplanted to the jaw bone to regenerate the new tooth.

Role of Biomaterial Scaffolds in Regenerative Endodontic Therapy

Along with dental stem cells, the use of biomaterial scaffold (bioscaffold) also becomes a notable consideration in regenerative endodontics, especially for the formation of dental tissues. These biomaterials are expanded *in vitro* to environmentally mimic the *in vivo* condition.(71,72) Ideal scaffolds for regenerative endodontic therapy should resemble the extracellular matrix (ECM) of pulp-dentin complex in terms of dimensional stability, sufficient porosity with adequate particle size, similar biodegradability rate, as well as physical and mechanical strength (71,73,74), since biocompatibility is highly important to prevent adverse tissue reactions.(75)

Bioscaffold for regenerative endodontic therapy includes broad ranges of applications and sources. Based on the scaffold geometry, the existing biological constructs are porous scaffolds, fibrous scaffolds, microsphere/microparticle scaffolds, and solid free-form scaffolds.(76) Meanwhile, based on the material sources, bioscaffold can be classified into blood-derived scaffolds, natural-derived biomaterial scaffolds, and synthetic biomaterial scaffolds. Each scaffold has different regenerative properties and potential, including pulp and dentin regeneration, vascularization, as well as stem cell proliferation and differentiation (Table 3).

Blood-derived Scaffolds

Induction of bleeding and formation of intracanal blood-clot (BC) in the root canal is a well-known used method in regenerative endodontic therapy that applies the strategy of

bioscaffold for pulp-dentin regeneration and dental tissue ingrowth.(78,106) BC is a gel-like lump obtained during the blood state alterations from liquid to solid.(74) This technique usually includes canal preparation and disinfection, followed by induction of BC from the periapical region.(107)

The practicality and success of regenerative endodontic therapy using BC, including in treating permanent or immature teeth with apical periodontitis and necrotic pulps, have been reported. In terms of pulp and dentin regeneration, BC bioscaffold therapy showed that it was able to give substantial results in increasing root length and thickness, thickening dental wall, improving bone density, providing apical closure, as well as periapical healing.(78-80,82,84,108) Immature symptomatic apical periodontitis teeth treated with BC scaffold showed a similar root morphology compared to other teeth that underwent normal development.(74,109)

Although has been performed a lot previously, yet the failure in inducing apical bleeding or in achieving adequate blood volume within the canal space remain as the common problems during the therapy with BC bioscaffold. The percentage of discoloration was also significantly greater in teeth with BC scaffold therapy compared with teeth with other platelets concentrates.(86) Hence, lately the use of autologous platelet concentrates, including platelet-rich plasma (PRP) and platelet-rich fibrin (PRF), have been explored as the possible scaffold source for regenerative endodontics therapy.(83,85)

PRP, an autologous first-generation platelet concentrate, is a high concentrate of autologous platelet obtained by centrifugation of autologous blood that may be source for several types of growth factors such as TGF-β, insulin growth factor (IGF), platelet-derived growth factor (PDGF), VEGF, as well as fibroblasts growth factor (FGF).(110,111) PRP preparation process consists of the removal of erythrocytes that would be expected to undergo necrosis

shortly after clot formation. The PRP clot is composed of fibrin, fibronectin, and vitronectin, which are cell adhesion molecules required for cell migration.(78) PRP is an ideal scaffold regenerative endodontic treatment since it is comparably easy to prepare in a dental setting, rich in growth factors, and forms a 3D fibrin matrix that helps attract the growth factors.(77)

As a comparable autologous bioscaffold, PRP has been able to show results of further root development (including root lengthening and thickening), periapical lesion resolution, improvement of periapical bone density, and continued apical closure compared with BC in the regenerative treatment of teeth with necrotic pulps.(77,78,81,86) Most blood-derived bioscaffolds showed the ability to improve pulp vitality response. However, PRP was found to be more effective than BC in revascularization. Even though not significant PRP treatment showed highest vitality test response compared with BC treatment, which suggests the higher occurrence of pulp's blood supply.(83,85) PRP has also been proved to be successfully stimulating the collagen production, sustained release of growth factors, as well as enhanced recruitment, retention, and proliferation of undifferentiated mesenchymal and endothelial cells from periapical area.(77,82) At a certain concentration of range, PRP also may increase the proliferation of fibroblasts and osteoblasts.(111)

PRF, a second-generation platelet concentrate, is a non-thrombonized autologous fibrin mesh that responsible as a reservoir for the slow, continuous release of growth factors PRF is an unadulterated centrifuged blood which consists of autologous platelets and leukocytes present in a complex fibrin matrix, that is able to achieves polymerization naturally. PRF is composed of fibrin membranes enriched with platelets, growth factors, and cytokines (86,112). The PRF clot is an autologous biomaterial and not an improved fibrin glue. Unlike the PRP, the strong fibrin

matrix of PRF does not dissolve quickly after application, instead, it is formed slowly in a similar way to a natural BC.(80)

Although composed of almost similar fibrin membranes, PRF has lower risk than PRP during the application since there is no bovine thrombin and anticoagulants present. PRF also shows better potency in accelerating wound and tissue healing, as well as better efficiency for cell proliferation and migration than PRP (113,114). PRF clots acted as successful scaffolds for the regeneration of dentin and pulpal contents in immature teeth with necrotic pulps because of its ability to increase root length, increase dental wall thickness, and healing the periapical lesion better than BC and PRP.(80,85) Meanwhile, in terms of clinical sign and symptom resolution, PRF achieved comparable outcomes to BC in regenerative endodontic therapy.(84) In the therapy of necrotic immature permanent teeth, revascularization/revitalization utilizing PRF also showed to be highly successful.(87)

When being combined with stem cells, PRP and PRF also show better regeneration potential. Human DPSCs was co-cultured with 10% of PRP showed higher expression levels of fetal liver kinase (Flk)-1, VEGF, PDGF, and stromal cell-derived factor 1 (SDF-1) mRNA compared with the combination of hDPSCs and fetal bovine serum (FBS). This suggests that PRP can promote vasculogenesis better than FBS in hDPSCs culture.(115) Both combinations of hDPSCs + PRP and hDPSCs + liquid-PRP showed significant increase of cell migration, proliferation, and differentiation compared with hDPSCs only. Though in hDPSCs + liquid-PRF, the cell migration was observed faster than hDPSCs + PRP.(116)

Natural-derived Biomaterial Scaffolds

Natural-derived polymers are usually used as biomimetic materials for scaffold in regenerative endodontic therapy. Most of the natural polymers are bioactive, containing cellular binding motifs, thus promoting cell adhesion, and/or present soluble signaling factors that are capable in regulating cell behaviour, hence it is also known to provide better biocompatibility compared with synthetic polymers.(96,117) Natural polymers are also known to be rapidly degradable compared with other types of scaffolds, hence allowing easier replacement with natural tissues after the degradation.(110,118) Natural polymers consist of natural polypeptides of the ECM, such as collagen, fibrin, gelatin and keratin, as well as polypeptides that are chemically similar to natural glycosaminoglycans, such as alginate, chitosan and hyaluronic acid (HA).(96)

For the regeneration of pulp and dentin-like tissue, polymers like collagen, gelatin, fibrin, chitosan, and HA have shown the ability to improve root development, including increase root length, root thickness, and enhance the mineralization of root canal (73,89,91,93,94,96-98). While being used as a single scaffold, those natural polymers also showed better ability in increasing intracanal connective tissue formations and narrowing apical width compared with BC, healing the periapical lesion, increasing dental wall thickness, as well as resuming the maturation process for the immature teeth.(73,88-90,93,98)

Natural polymers are often combined and crosslinked with other bioscaffold or chemical agents to improve its potential in regenerative therapy.(119) Dental pulp regeneration through cell homing approaches can be improved by using the combination of HA hydrogel and BC, as well as combination of chitosan hydrogel and BC scaffolds.(73,120) Meanwhile, to fill root canal space with new vital tissue and to enhance the root canal mineralization, the combination of gelatin sponge and BC scaffold as well as collagen and BC scaffold can be used, and have shown

better results compared with BC scaffold only.(92,93) To enhance scaffolds physical properties, the crosslinking between collagen hydrogel and cinnamaldehyde (CA) had shown to be successful. It resulted in the enhanced physical properties of collagen by CA, which upregulated the cellular adhesion compared with the collagen only. This means that this property was promoted in the presence of CA.(121)

In terms of its vascularization function, while being used as a single bioscaffold, both fibrin and HA have shown the potential of increasing vascularization better than the control.(73,97) On the other hand, chitosan, when being used alone, does not show vascularization potential, however when being combined with sodium hyaluronate or pectin, both combinations were able to increase vascularization of connective tissues.(95)

Besides its advantages in dental-pulp regeneration and vascularization, natural-derived bioscaffolds that are classified into moldable porous scaffold, such as chitosan and collagen as single scaffold, or even combination of gelatin/collagen hydrogens bioscaffold, also have the ability to promote cell adhesion, migration and proliferation (96,110,119,122). And to induce hDPSCs cell migration, adhesion, and proliferation, which later followed by a culminated amount of mineralized matrix, scaffold from chitosan and collagen matrix can also be combined with calcium-aluminate.(123) In the combination with SCAP, cell viability promotion, mineralization, and odontoblastic-like differentiation can also be achieved by using HA-based injectable gel scaffold.(124)

Synthetic Biomaterial Scaffolds

While natural-derived polymers scaffolds offer good biocompatibility and bioactivity, synthetic polymers scaffolds offer more flexible and controllable physical and mechanical

properties to fit for specific applications.(76,125) Polylactic acid (PLA) and polyglycolic acid (PGA), as well as their copolymers such as poly-L-lactic acid (PLLA), polylactic-polyglycolic acid (PLGA), and polycaprolactone (PCL) have been successfully reported to be used as bioscaffold for regenerative endodontics therapy.(74)

Synthetic polymers scaffolds and its combination with other scaffold materials are able to induce pulp-dentin regeneration. The increase of mineralization, as well as tissue and bone formation, can be reached by using the combination of PLGA and magnesium scaffold, PLLA combined with DPSC or minced-pulp mesenchymal stem cell (MSC), as well as combination of PCL and PDLSC.(100,103,104) Other than that, culture of hDPSCs on either side of PLGA scaffold was also able to enhance surface closing in the opened side of scaffold. Meanwhile, in terms of pulp vascularization and neurogenesis, the enhancement of neurovascular regeneration through angiogenic and neurogenic paracrine secretion has been reported after the therapy with PLGA scaffold on hDPSCs culture.(102)

PLLA and PLGA scaffolds while being cultured in DPSC are able to improve DPSC differentiation and proliferation, it also induces longer cell replicative lifespan (99,100,102). PLLA scaffold was also used for human minced-pulp MSC, and the results found that the combination showed even better ability to increase cell differentiation and replication better than in DPSC.(100) Although not being used as scaffold as much as PLLA and PLGA, the use of PCL scaffolds in SCAP and hDPSCs seeding were also reported to be able to increase the cell proliferation and differentiation.(105,126)

A PLGA microsphere combined with hDPSCs, was able to increase hDPSCs proliferation and adhesion to the scaffold, as well as increase expression levels of *DMP1*, *DSPP*, *COL1*, and *OPN* genes.(101) While increased expression of *DMP1*, *DSPP*, runt-related

transcription factor 2 (*RUNX2*), osteocalcin (*OCN*), secreted phosphoprotein 1 (*SPP1*), collagen type I alpha 1 (*COL1A1*), and growth differentiation factor 5 (*GDF5*) genes was obtained with the combination of PCL and fluorapatite.(105) In the construction of dental and periodontal pulp for the preservation of periodontal ligament fibroblasts (PDLF), the use of PLGA scaffold combined with PRF has shown the ability to sustain fibroblast viability.(74,127)

Role of Signaling Molecules in Regenerative Endodontics

Various signaling molecules, including growth factors and cytokines have been recognized to enhance the proliferation, migration and differentiation of dental stem cells. These molecules are naturally contained in the pulpal cells and dentin matrix, and involved in modulating dentin-pulp complex homeostasis.(128) In the pulp-dentin regeneration process, the remaining periapical and pulpal cells, adjacent dentin, or implanted platelet concentrates, blood clot scaffold, or stem cells are responsible for the release of signaling molecules. To accelerate the process, exogenous signaling molecules are often delivered together with dental stem cells in a scaffold. Addition of signaling molecules to transplanted dental stem cells is expected to mimic the signaling cascades that occur during the formation of pulp-dentin complex.(129)

Signaling Molecules Related to Cell Migration

Bone morphogenetic protein (BMP)-2, TGF-β1, basic FGF (bFGF), PDGF, VEGF, NGF, and BDNF have been reported to stimulate cell migration (Table 4). Induction of cell migration by these molecules is important, since cells must reach the damaged sites to regenerate the tissues. Several signaling pathways have been identified to be induced by these molecules in stimulating cell migration. For example, via PDGFR-β/Akt pathway, PDGF contributes in

recruiting smooth muscle cells to blood vessels (168); BDNF accelerates DPSCs migration via extracellular signal-regulated kinase (Erk) phosphorylation (193); VEGF increases the migration of DPSCs through VEGF receptor (VEGFR) 2 activation and its downstream focal adhesion kinase (FAK) / phosphoinositide 3-kinase (PI3K) / Akt and p38 signaling.(181,182)

Signaling Molecules Related to Cell Proliferation

After reaching the damaged sites, cells must proliferate to increase the number of cells. BMP-2, TGF-β1, bFGF, PDGF and VEGF have been reported to increase proliferation (Table 4). However, the proliferation process is inhibited when cells start to enter the differentiation stage. Thus, signaling molecules which have proliferation-related functions may both inhibit proliferation and induce differentiation in a specific time point, as discussed in the subsequent sections. Several signaling pathways have been identified to be induced by these molecules in stimulating cell proliferation. BMP-2-induced cell proliferation involves BMP-2 receptor (BMP2R) activation as well as Erk1/2 and small mothers against decapentaplegic (Smad) 1/5 phosphorylation (131), while bFGF modulates the expression of cyclin B1 (CCNB1) and cell division control 2 (CDC2), which are related to cell-cycle regulation via mitogen-activated protein kinase kinase (MEK)/Erk pathway.(154) VEGF activates the Akt signaling pathway and increases cyclin D1 expression levels, which in turn promotes proliferation of DPSCs.(182)

Signaling Molecules Related to Dentinogenesis and Pulp Regeneration

BMP-2, TGF-β1, bFGF, PDGF, VEGF, and NGF have been reported to enhance dentinogenesis (Table 4). These molecules have been demonstrated to increase differentiation and mineralization of both dental pulp cells and dental stem cells as indicated by an increase in

alkaline phosphatase (ALP) activity and mineralization, as well as upregulation of osteo-/odontogenic marker expression *in vitro*.(132,151,157,170,188) *In vivo,* these molecules are observed to stimulate dentin formation.(132,145,159,171,187)

TGF-β1 has been demonstrated to enhance ALP activity via activation of Smad2/3, TGF-β activated kinase 1 (TAK1), as well as Erk1/2 and p38.(148) BMP-2 has been known to induce phosphorylation of Erk1/2 and Smad1/5.(131) bFGF could induce mitogen-activated protein kinases (MAPKs) (p38, JNK, and Erk), PI3K/Akt, protein kinase C (PKC), and NF-κB (194), BMP or Wnt signaling.(195) Meanwhile, VEGF has been known to activate Akt, MAPKs (p38, JNK, and Erk), and NF-κB.(157)

Intriguingly, induction of differentiation and mineralization by TGF-β1 and BMP-2 is often associated with a decrease in cell proliferation (136,151). In addition, TGF-β1 increases the expression of early marker genes of odonto-/osteo-genic differentiation and decreases the expression of late-stage mineralization genes.(151) VEGF might not be able to trigger full osteo-odontogenic differentiation, and facilitate only the early stage of cell differentiation.(187) VEGF potential in inducing mineralization is lower compared with bFGF (157) and NGF.(188) The potential of PDGF in enhancing hard tissue formation has been shown to be lower than other materials, such as enamel matrix derivative (EMD) and mineral trioxide aggregate (MTA).(196) Furthermore, PDGF-BB has been reported to inhibit the formation of mineral nodules.(14) Therefore, PDGF should be used in combination with other materials to increase the mineralization potential.(171,172) However, studies regarding signaling pathways that are involved in PDGF and NGF-induced dentin formation are limited.

bFGF, TGF-β1, and NGF are known to contribute to pulp regeneration (Table 4). bFGF regulates growth of dental pulp cells, upregulates the expression of CDC2, CCNB1, and tissue

inhibitor of metalloproteinase 1 (TIMP1), as well as inhibits ALP activity and collagen I production through activation of FGF receptors (FGFRs) and MEK/Erk signaling.(154) Meanwhile, TGF-β1 has been demonstrated to increase TIMP1 production, collagen content, and procollagen I, but slightly attenuate MMP3 production, which are related to the activation of activin receptor-like kinase-5(ALK5)/Smad2/3, TAK1, MEK/Erk, and p38 signaling.(143,148) NGF has been reported to upregulate the expression of healing and repair-related genes (188), as well as improve pulp cell organization and pulpal architecture.(189) Thus, bFGF, TGF-β1 and NGF are involved in pulp regeneration by altering matrix turnover and dental pulp cell proliferation, as well as modulating pulp repair-related gene expression.

Signaling Molecules Related to Angiogenesis

VEGF, PDGF, bFGF, and TGF-β1 have been reported to induce angiogenesis (Table 4) by promoting differentiation of dental stem cells toward endothelial (162,175) or smooth muscle cells (149,150), as shown by upregulation of several differentiation genes.(144,150,162) These signaling molecules also induce the formation of capillary-like structures, both *in vitro* (162,170,175) and *in vivo*.(170,176)

VEGF has been demonstrated to accelerate angiogenesis, since angiogenesis could occur even in the absence of this molecule.(176) This molecule induces angiogenesis by inducing VEGFR phosphorylation and activating downstream Akt, MAPKs (p38, JNK, and Erk), NF-κB.(157) Besides formation of new blood vessels, VEGF has been reported to induce anastomosis of DPSCs-derived blood vessels by increasing vascular endothelial (VE)-cadherin expression through the activation of MEK1/Erk, which in turn causes E-26 transformation-specific-related gene (ERG) transcription factor binds to *VE-cadherin* promoter.(184) VEGF-

induced angiogenesis could be enhanced by inhibiting specific pathways or combining it with other molecules. Combination of VEGF with SB-431542, an inhibitor of TGF-β1 signaling, has been shown to markedly promote SHED differentiation toward endothelial cells, since Smad1/2 inhibition is correlated with VEGFR2 activation.(175) IGF-1 (182) and SDF-1α (179) were also reported to have a synergistic effect in enhancing angiogenesis when combined with VEGF.

PDGF-BB alone induces capillary sprouting, and this phenomenon could be enhanced by bFGF.(168) bFGF alone could induce angiogenesis, but its angiogenic potential is lower than VEGF.(157) PDGF-BB has been reported to promote blood vessels maturation by regulating the investment of smooth muscle cells to DPSCs-derived capillaries through PDGFRβ and Akt phosphorylation in both types of cells.(168) In addition, DPSCs-derived smooth muscle cells that are produced after TGF-β1 treatment have been reported to stabilize blood vessels through ANGPT1/Tie2 and VEGF/VEGFR2 signaling.(149) Combination of PDGF-BB and TGF-β1 induces the expression of smooth muscle-specific early, mid, and late markers, as well as enhances contraction ability in DPSCs, although the cells do not undergo morphological alterations toward smooth muscle-specific cell shapes.(150)

Signaling Molecules Related to Neurogenesis

NGF, BDNF and bFGF have been reported to induce neurogenesis (Table 4). In several neurogenesis induction studies, NGF and BDNF are combined with other neurotrophin and non-neurotrophin signaling molecules.(156,188,193) Meanwhile, bFGF is usually combined with epidermal growth factor (EGF) for neural induction.(167) Addition of these molecules increases the expression levels of neural markers and promotes morphological alterations of the treated

cells toward neuronal and glial cells.(156,192,197) These molecules have also been reported to induce axonal sprouting and promote axonal growth.(167,191)

NGF and BDNF induce neurogenesis via non-specific activation of p75 neurotrophin receptor (p75NTR). In addition, NGF specifically activates tropomyosin-related kinase A (TrkA), while BDNF specifically activates TrkB.(198) Meanwhile, bFGF induces neurogenesis via activation of FGFR (199). Activation of these receptors have been reported to induce the phospholipase C (PLC)-γ pathway, which in turn promotes neuronal differentiation.(198,199) Besides, combination of bFGF and NGF also stimulates neuronal differentiation via PI3K/Akt and Erk pathways.(156)

Future Perspectives on the Use of Dental Stem Cells, Scaffold, and Signaling Molecules Combination in Regenerative Endodontics

Numerous studies have reported successful pulp-dentin complex regeneration using specific combinations of dental stem cells, scaffold, and signaling molecules. Despite most of the ongoing regenerative endodontics studies using these combinations are conducted in animal models (23,200), these combinations were also reported to induce pulp-dentin regeneration in human subjects. Several examples of dental stem cells, scaffold, and signaling molecules combination that have been known to regenerate human pulp-dentin complex are combination of hpDPSCs, G-CSF, and atelocollagen scaffold (31,32), as well as combination of DPSCs and L-PRF (30), which acts as scaffold and contains PDGF and TGF-β.(201) Indeed, the regenerative endodontics field is constantly growing. There will be new findings and innovation regarding dental stem cell biology, the development of new types of scaffolds, and the best way to deliver stem cells and signaling molecules to the root canal, which open a new perspective on a new era

of endodontic therapy. Thus, current trends and future directions on regenerative endodontics should be further explored.

In most pulp-dentin regeneration studies using human subjects, a scaffold that already contains dental stem cells and immobilized signaling molecules is directly transplanted to the root canal in a single appointment.(30-32) Despite the success of this current protocol in regenerating functional pulp-dentin complex, the current procedure might not be similar to the natural process of pulp-dentin regeneration, which involves specific cellular processes. Additionally, regeneration of the pulp-dentin complex may be incomplete in some patients due to differences in pulp-dentin damage severity. To achieve complete pulp-dentin regeneration, additional dental stem cells and/or signaling molecules could be applied in the several next appointments. Since scaffolds have different physical characteristics and biocompatibility, different types of scaffolds could be used to facilitate pulp-dentin regeneration in different parts of teeth. Different types of dental stem cells, signaling molecules, and scaffolds could also be combined with other endodontic procedures, such as apexification and pulp revascularization (202) to enhance the regeneration process in different parts of teeth. Therefore, dental stem cell, scaffold, and/or signaling molecules application could be performed in multiple appointments to mimic the cellular processes that are involved in the regeneration process, hence gradual pulp-dentin regeneration could be achieved.

Although studies regarding tissue engineering-based pulp-dentin regeneration show promising results, there are several challenges for its future clinical translation that need to be addressed. Regenerated pulp-dentin complex should have a precise and highly ordered histological structure as compared to that in normal teeth.(4) Besides, different oral diseases, such as irreversible pulpitis and necrotic pulp, as well as the presence of residual bacteria and

lipopolysaccharide may affect the root canal microenvironment, which in turn alter the fate of transplanted dental stem cells.(203,204) Other factors, including age and the presence of systemic diseases might also affect the regeneration potential of stem cells.(4,205) Since each type of dental stem cell, scaffold, and signaling molecule has unique characteristics and functions, they can be utilized to address these challenges by combining these components together to achieve successful regeneration. Thus, the right combination of dental stem cells, scaffold, and signaling molecules is needed to enhance the pulp-dentin regeneration process.

Conclusion

Combinations of dental stem cells, scaffold, and signaling molecules mimic the cellular microenvironment that is suitable for regeneration, hence they are important to achieve the functional pulp-dentin complex formation. Since regenerative endodontics is a constantly growing field, current trends and future directions in this field are still needed to be further explored. The right combination of dental stem cells, scaffold, and signaling molecules could be determined based on the patients' characteristics. Incomplete pulp-dentin regeneration, which may occur in some cases, could be overcome by applying dental stem cells, scaffold, and/or signaling molecules in multiple appointments to achieve gradual pulp-dentin regeneration.

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Table 1. Regenerative potential of DPSCs, SHED, and DFSCs in animal model of pulp-dentin regeneration.

Type of Dental Stem Cells	Species	Regenerative Potential			Reference
		Pulp- and/or Dentin-like Tissue	Angiogenesis	Neurogenesis	
DPSC	Dog	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DSPP	Histology: Blood vessels in regenerated pulp	N/A	(7-10)
	Mini-pig	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DSP, DMP1, and BSP	Histology: Blood vessels in regenerated pulp	N/A	(11)
	Ferret	Histology: Formation of osteodentin mixed with loose connective tissue.	N/A	N/A	(12)
	Rat	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DMP1, DSPP, DSP, and OPN	Histology: Blood vessels in regenerated pulp Positive immunostaining: CD31	N/A	(13-15)
DPSC $CD31^-$	Dog	Histology: - Pulp tissue regeneration - Dentin formation Gene expression: *MMP20, syndecan 3, TRH-DE*	Positive immunostaining: BS-1 lectin	Positive immunostaining: PGP9.5	(16)

DPSC $CD105^+$	Dog	Histology: Pulp tissue regeneration	Histology: Blood vessels in regenerated pulp	N/A	(17)
Mobilized DPSC	Dog	Histology: - Pulp tissue regeneration - Dentin formation Gene expression: *tenascin C*, *syndecan 3*, *TRH-DE*, *MMP20*, *DSPP* Positive immunostaining: TRH-DE MRI: Signal intensity of transplanted teeth was similar compared with that in normal teeth.	Positive immunostaining: BS-1 lectin Laser Doppler flowmetry: Blood flow in regenerated pulp tissue is similar compared to that in normal pulp tissue.	Positive immunostaining: PGP9.5 Electric pulp test: Positive pulp sensibility response	(18-25)
hpDPSC	Dog	Histology: - Pulp tissue regeneration - Dentin formation	Positive immunostaining: BS-1 lectin	Positive immunostaining: PGP9.5	(25,26)
hpDPSC from deciduous teeth	Dog	Histology: - Pulp tissue regeneration - Dentin formation	Positive immunostaining: BS-1 lectin	Positive immunostaining: PGP9.5	(26)
SHED	Mini-pig	Histology: - Pulp tissue regeneration - Dentin formation	Histology: Blood vessels in regenerated pulp Positive immunostaining: CD31	Positive immunostaining: NeuN, neurofilament, CGRP, and TRPV1	(27,28)

DFSC	Mini-pig	Histology: - Pulp tissue regeneration - Dentin formation Positive immunostaining: DMP-1, DSPP, COL1, COL3	N/A	N/A	(29)

N/A: Not applicable; DSPP: Dentin sialophosphoprotein; DSP: Dentin sialoprotein; DMP1: Dentin matrix acidic phosphoprotein 1; BSP: Bone sialoprotein; OPN: Osteopontin; MMP20: Matrix metalloproteinase 20; Thyrotropin-releasing hormone-degrading enzyme: TRH-DE; BS-1 lectin: *Bandeiraea simplicifolia* lectin 1; PGP9.5: Protein gene product 9.5; NeuN: Neuronal nuclei; CGRP: Calcitonin gene-related peptide; TRPV1: Transient receptor potential cation channel subfamily V member 1; COL1: Collagen type I; COL3: Collagen type III.

Table 2. Regenerative potential of DPSCs, SHED, and DFSCs in case reports and clinical trials of pulp-dentin regeneration.

Type of Dental Stem Cells	Regenerative Potential			Reference
	Pulp- and/or Dentin-like Tissue	Angiogenesis	Neurogenesis	
DPSC	CBCT: - Formation of dentin bridge - Apical canal calcification	Laser Doppler flowmetry: Blood perfusion in the transplanted tooth with low mean perfusion unit.	N/A	(30)
Mobilized DPSC	MRI: Complete pulp regeneration CBCT: - Formation of lateral dentin - Decrease in dental pulp volume	N/A	Electric pulp test: Positive pulp sensibility response	(31)
hpDPSC	MRI: Complete pulp regeneration CBCT: - Formation of lateral dentin - Decrease in dental pulp volume	N/A	Electric pulp test: Positive pulp sensibility response	(32)
SHED	Histology: Regenerated pulp with odontoblast layer, connective tissue, and blood vessels. CBCT: Increase in dentin thickness	Laser Doppler flowmetry: An increase in vascular formation as indicated by high perfusion units.	Positive immunostaining: NeuN Electric pulp test: Positive pulp sensibility response	(27)

N/A: Not applicable; CBCT: Cone beam computed tomography; MRI: Magnetic resonance imaging; NeuN: Neuronal nuclei.

Table 3. Regenerative potential of blood-derived, natural-derived polymer, and sythetic polymer bioscaffolds.

Types of Scaffolds	Regenerative Potential		References
	Pulp-dentin Regeneration	Vascularization	
Blood-derived			
BC	- Increasing root length and thickness - Increasing dental wall thickness - Improving bone density - Narrowing apical width - Healing the periapical lesion	- Improving vitality response (blood pump)	(77-86)
PRP	- Increasing root length and thickness - Increasing dental wall thickness - Improving bone density - Narrowing apical width - Healing the periapical lesion	- Improving vitality response (blood pump)	(77,78,80-83,85-87)
PRF	- Increasing root length and thickness - Increasing dental wall thickness - Improving bone density - Narrowing apical width - Healing the periapical lesion	- Improving vitality response (blood pump)	(80,83,84,85,87)
Natural-derived polymers			

Collagen - BC	- Increasing root length - Enhancing mineralization of root canal - Increasing dental wall thickness - Narrowing apical width - Healing the periapical lesion - Increasing intracanal connective tissue formation	N/A	(88-92)
Gelatin - BC	- Increasing root lenght and thickness - Increasing root length - Increasing dental wall thickness - Narrowing apical width - Increasing intracanal connective tissue formation	N/A	(93,94)
Chitosan - BC - Sodium hyaluronate - Pectin	- Increasing root length and thickness - Increasing dental wall thickness - Enhancing mineralization of root canal - Narrowing apical width - Healing the periapical lesion - Increasing intracanal connective tissue formation	- Increasing vascularization	(95,96)
Fibrin	- Increasing root length and thickness - Enhancing mineralization of root canal - Narrowing apical width - Healing the periapical lesion	- Increasing vascularization	(94,97)

HA	- Increasing root length - Enhancing mineralization of root canal - Increasing dental wall thickness - Narrowing apical width - Healing the periapical lesion - Increasing intracanal connective tissue formation	- Increasing vascularization	(73,98)
Synthetic biomaterial			
PLLA - DPSC - Minced-pulp MSC	- Enhance tissue mineralization - Increase expression levels of *DMP1, DSPP, COL1* , and *OPN* genes	N/A	(99-101)
PLGA - DPSC - Magnesium	- Increase bone height and volume - Enhance bone mineralization - Enhance surface closing	- Initiate neurovascular regeneration	(102,103)
PCL - PDLSC - Fluorapatite	- Enhance bone formation in defect tissue - Improve periodontium neogenesis - Increase expression of *DMP1, DSPP, RUNX2, OCN, SPP1, COL1A1* , and *GDF5* genes	N/A	(104,105)

N/A: Not applicable; DMP1: Dentin matrix acidic phosphoprotein 1; DSPP: Dentin sialophosphoprotein; COL1: Collagen type I; OPN: Osteopontin; RUNX2: Runt-related transcription factor 2; OCN: Osteocalcin; SPP1: Secreted phosphoprotein 1; COL1A1: Collagen type I alpha 1; GDF5: Growth differentiation factor 5.

Table 4. Regenerative potential of signaling molecules in pulp-dentin regeneration.

Signaling Molecule	Regenerative Potential					Reference
	Cell Migration	Cell Proliferation	Pulp- and/or Dentin-like Tissue	Angiogenesis	Neurogenesis	
BMP-2	Inducing migration of dental pulp cells	Increasing proliferation of dental pulp cells	• Increasing ALP activity and mineralization • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *ALP*, *RUNX2*, *COL1*, *DSPP*, *DMP1*, *DSP*, *MMP20*, *BSP*, *OCN*, and *OSX* - Protein expression: RUNX2, DSPP, DMP1, BSP, and OCN	N/A	N/A	(130-141)
TGF-β1	Inducing migration of dental pulp cells	Increasing proliferation of DPSCs and dental pulp cells	• Increasing ALP activity, mineralization, and collagen content • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *DSPP*, *DSP*, *MMP20*, *RUNX2*, *DMP1*, *COL1A1*, and *BSP* - Protein expression: N-cadherin, TIMP1, COL1A1, DMP1, and BSP • Downregulating protein expression: MMP3	• Inducing smooth muscle cell differentation • Maintaining blood vessels stability • Upregulating differentiation markers - Gene expression: *αSMA, SM22α, CALP, SMTN*, and *MYH11* - Protein expression: αSMA, SM22α, CALP, SMTN, ANGPT1, Tie2, and MYH11	N/A	(137,142-151)

bFGF	Inducing migration of SCAP, mobilized DPSCs, BMMSCs, periodontal ligament fibroblasts, and endothelial cells	Increasing proliferation of SHED, DPSCs, mobilized DPSCs, BMMSCs, dental pulp cells, periodontal ligament fibroblasts, and endothelial cells	• Increasing ALP activity and mineralization • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *DSPP*, *MMP20*, *TRH-DE*, *ALP*, *TIMP1*, *DMP1*, *COL1A2*, *OPN*, and *OCN* - Protein expression: DSPP, DMP1, TIMP1, and COL1	• Enhancing blood vessel formation • Upregulating differentiation markers - Gene expression: *VEGFR2*, *Tie2*, *ANGPT1*, *VWF*, *VE-cadherin*, and *CD31* - Protein expression: VEGFR2, Tie2, ANGPT1, VWF, VE-cadherin, and CD31	• Inducing neuronal and glial differentation • Promoting axonal sprouting and growth • Upregulating differentiation markers - Gene expression: *Nestin*, *TUBB3*, *Sox2*, *VIM*, *NEFM*, *MAP2*, *NEFH*, *GFAP*, and *S100B* - Protein expression: Nestin, NEFM, TUBB3, NeuN, GFAP, S100B, and MAP2	(152-168)
PDGF	Inducing migration of DPSCs, SHED, dental pulp cells, and smooth muscle cells	Increasing proliferation of DPSCs	• Increasing ALP activity and mineralization • Promoting formation of new dentin • Upregulating differentiation markers - Gene expression: *DMP1*, *DSPP*, and *OCN* - Protein expression: DMP1 and DSPP	• Inducing smooth muscle and endothelial cell differentation • Enhancing blood vessel formation • Promoting blood vessel stabilization • Upregulating differentiation markers - Gene expression: *αSMA*, *SM22α*, *CALP*, *SMTN*, and *MYH11* - Protein expression: αSMA, SM22α, CALP, SMTN, VEGFR2, Tie2, CD31, and VE-cadherin	N/A	(142,150,168-174)

VEGF	Inducing migration of DPSCs and endothelial cells	Increasing proliferation of DPSCs and dental pulp cells	• Increasing ALP activity and mineralization • Upregulating odontoblast markers - Gene expression: *ALP*, *OCN*, *OSX*, *DSPP*, *RUNX2*, *DMP1*, *COL1A2*, *BSP*, *TGFB1*, and *OPN* - Protein expression: DMP1, DSPP, and OSX	• Inducing endothelial cell differentation • Enhancing blood vessel formation • Promoting blood vessel anastomosis • Upregulating differentiation markers - Gene expression: *VWF*, *VEGFR2*, *VE-cadherin*, *CD31*, *VEGFR1*, *EphrinB2*, Tie2, and *ANGPT* - Protein expression: VWF, VEGFR2, VE-cadherin, CD31, Tie2, F8	N/A	(130,136,157,162,175-188)
NGF	Inducing migration of glial cells	N/A	• Improving pulpal architecture and cell organization • Upregulating gene expressions of differentiation markers: *DSPP*, *DMP1*, and *TGFB1*	N/A	• Inducing neuronal and glial differentation • Promoting axonal sprouting and growth • Upregulating differentiation markers - Gene expression: *Nestin* - Protein expression: S100, neurofilament, and p75NTR	(156,188-191)
BDNF	Increasing migration of DPSCs	N/A	N/A	N/A	• Inducing neuronal and glial differentation • Upregulating protein expressions of differentiation markers: DCX, NeuN, S100B and p75NTR.	(192,193)

N/A: Not applicable; ALP: Alkaline phosphatase; RUNX2: Runt-related transcription factor 2; COL1: Collagen type I; DSPP: Dentin sialophosphoprotein; DMP1: Dentin matrix acidic phosphoprotein 1; DSP: Dentin sialoprotein; MMP: Matrix metalloproteinase; BSP: Bone sialoprotein; OCN: Osteocalcin; OSX: Osterix; COL1A1: Collagen type I alpha 1; TIMP1: Tissue inhibitor of metalloproteinase 1; αSMA: Alpha smooth muscle actin, SM22α: Smooth muscle protein 22 alpha, CALP: Calponin, SMTN: Smoothelin, ANGPT: Angiopoietin, MYH11: Myosin heavy chain 11; TRH-DE: thyrotropin-releasing hormone-degrading enzyme; OPN: Osteopontin; VEGFR: vascular endothelial growth factor receptor; VWF: von Willebrand factor; TUBB3: tubulin beta III ; Sox2: sex determining region Y-box 2; VIM: Vimentin; NEFM: Neurofilament medium chain; MAP2: Microtubule associated protein 2; NEFH: Neurofilament heavy chain; GFAP: Glial fibrillary acidic protein; S100: S100 calcium binding protein; NeuN: Neuronal nuclei; TGFB1: Transforming growth factor beta 1; F8: Coagulation factor VIII; p75NTR: p75 neurotrophin receptor; DCX: Doublecortin.

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[InaBJ] MR2023042 Editor Decision - Manuscript Accepted

Secretariat of InaBJ <secretariatinabj@gmail.com> Tue, Feb 21, 2023 at 7:44 AM
To: ferry@trisakti.ac.id

Dear Dr. Ferry Sandra,

Good day. We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "**Crucial Triad in Pulp-Dentin Complex Regeneration: Dental Stem Cells, Scaffolds, and Signaling Molecules**."

Our decision is to: **Accept Manuscript.**

Your manuscript will be sent to our publisher for typesetting and you should receive the proofreading in due course.

Congratulations on your interesting research, and thank you for allowing us to publish this valuable material. Please let us know once you have read this email. We wish you a nice day.

Best Regards,

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