Regenerative Endodontic Procedures: A Perspective from Stem Cell Niche Biology

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Abstract

Introduction: Endodontics uses cell therapy strategies to treat pulpal and periapical diseases. During these therapies, surgeons aim to reconstruct the natural microenvironments that regulate the activity of dental stem cells. **Methods:** We searched more than 400 articles in PubMed using the following key words: regenerative endodontics and dental stem cell biology. In 268 articles, we reviewed what factors may influence histologic results after preclinical dental treatments that use regenerative endodontic procedures after pulpectomy. **Results:** Several factors, such as the origin of stem cells, the biomimicry of scaffolds used, and the size of lesions, are considered to influence the histologic appearance of the regenerated pulp-dentin complex after treatments. Information is accumulating on transcription factors that generate the pulp-dentin complex and survival/trophic factors that would benefit niche recovery and histologic results. **Conclusions:** In this article, we discuss the noninterchangeability of stem cells, the influence of dentin-entrapped molecule release on pulp regeneration and survival of stem cells, and the need of positional markers to assess treatments histologically. The *ex vivo* amplification of appropriate dental stem cells, the search for scaffolds storing the molecular diversity entrapped in the dentin, and the use of positional transcription factors as histologic markers are necessary to improve future preclinical experiments. *(J Endod 2016;■:1–11)*

Key Words

Biomaterial, endodontics, equivalence, regenerative endodontic procedures, stem cell niche, translational research

Significance

Clinical results of REPs will be improved with new approaches to reconstruct DPSC niches and microenvironments, which take into account the noninterchangeability of stem cells and include the use of scaffolds storing survival/trophic factors entrapped in the dentin.

Regenerative dentistry aims to restore tooth anatomy and function after dental damage (1). In this discipline, regenerative endodontics is a set of biology-based procedures designed to heal periapical lesions and replace cells and dentin of the pulp-dentin complex in a damaged tooth (2). Regenerative endodontic procedures (REPs) are bioengineering therapies that seek to restore the physiological functions of the dental pulp (3–9). These techniques involve a triad of elements: stem cells, growth factors, and biomaterials, also named scaffolds or templates (10–14).

A dental stem cell is a self-renewable cell type in the tooth involved in the maintenance of adult or developing dental tissues (12). These dental stem cells and their daughter cells grow and differentiate dependent on growth factors released by their surrounding tooth microenvironments (15, 16), the dental stem cell niches (1, 17). During REP therapies, the triad aims to reconstruct these microenvironments (18, 19).

Although these techniques clearly induce revascularization after pulpectomy to resolve pulp necrosis and apical periodontitis successfully, scarce results have been published on actual pulp-dentin regeneration (20–25). Applied to the root canal space in animals, REP may generate cementumlike, bonelike (20, 26–30), or periodontal-like (31) tissues instead of a normal dental pulp. Furthermore, REPs applied to human teeth with open apices may also induce the formation of tissues of the periodontium (connective tissue, bone, and cementum) into the root canals (32). Scaffolds impregnated with growth factors and stem cells from other tissues normally induce tissue growth, but its matching to healthy functional tissue is not fully accomplished (33, 34). These nonsatisfactory results suggest that some important factors are not being considered in current REPs.

Although research has paid special attention to the technical expertise of dental stem cell isolation and scaffold developments (12, 29, 35–39), the dental microenvironments that generate the pulp-dentin complex have been much less investigated. The nonsatisfactory results obtained may be caused by the partial reconstruction of these microenvironments, and this could be solved by stimulating either exogenous or endogenous stem cells or growth factors (1).

In this review, we discuss preclinical histologic results of REP treatments and *in vivo* REP-related experiments under this hypothesis of microenvironmental

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regulation. From this, some experimental improvements and translational developments are discussed to speed the application of REPs to humans.

Dental Stem Cells under the Developmental Biology Paradigm

To better understand technical failures in REPs, the triad of elements used will be first evaluated under the scope of modern developmental biology. Potential metabolic causes of poor dental pulp regeneration will not be discussed here. "Nonequivalence," "hierarchical combinatory of transcription factors," and "positional memory" (40) are cell biology concepts that highlight how signal molecules from scaffolds are influencing stem cells. As a result, the noninterchangeability of dental stem cells or the extreme heterogeneity of released signal molecules are revisited in this article for future progress. Although anti-inflammatory or immunomodulatory treatments are very important topics in REP treatments (13, 41, 42), they are not discussed in this review.

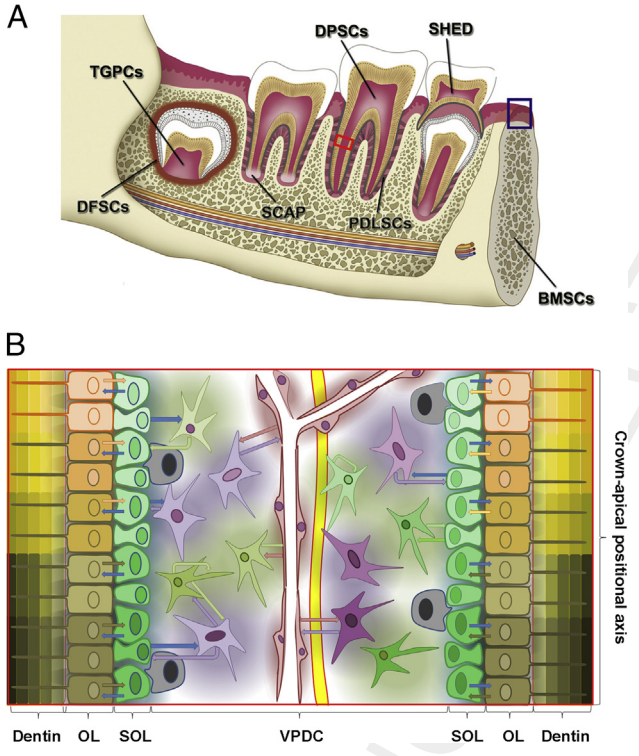

Figure 1. Dental stem cells and signal molecules. (*A*) A drawing of a mandible that shows the most important dental stem cells. Dental stem cell initials are described in the text. Reproduction after *Journal of Prosthodontic* research (Egusa et al, 2012a;56:151–165) (12). (*B*) A schematic of signal molecule distributions in the pulp-dentin complex. Positional identities of cells are in different colors. Graded colors around cells are specific compositions of released signal molecules. Colors in the margins are different compositions of dentin-entrapped signal molecules. Both well-documented dentin-derived signal molecules TGFβ1, BMP2, DMP1 and HGF, and MSC survival, proangiogenic, morphogens, trophic factors, or cytokines would comprise these compositions. Variations in colors along the crown-apical axis are potential changes in positional identities. OL, odontoblast layer; SOL, subodontoblast layer; VPDC, vascularized pulp-dentin complex. The *arrows* are suggested interactions in the literature. The crown-apical positional axis is shown in the margin. The scheme corresponds to the *red rectangular region* indicated in Figure 1*A*.

Several populations of adult stem cells have been identified in the tooth (Fig. 1*A*). Dental pulp stem cells (DPSCs) (11, 12, 38, 43) and [F1] stem cells from human exfoliated deciduous teeth (SHEDs) have been isolated from the pulp-dentin complex. Cells derived from them differentiate *in vitro* into odontoblasts, adipocytes, osteoblasts, or chondroblasts and form dentin/pulplike tissues after *in vivo* transplantation (11, 12). Periodontal ligament stem cells or stem cells of the developing root apical papilla (SCAPs) have also been isolated. Cells derived from these cells can differentiate into odontoblasts, cementoblastlike cells, adipocytes and connective tissue, both *in vitro* and *in vivo* (11, 12). Finally, dental follicle stem cells (DFSCs), potential progenitor cells of periodontal ligament stem cells, tooth germ progenitor cells (12) (Fig. 1*A*), inflammatory periapical progenitor cells, and dental mesenchymal stem cells (DMSCs) (44, 45) (not shown in Fig. 1*A*) are other dental stem cells isolated from the tooth. Stem cells from other mandible positions have also been isolated (eg, bone marrow stem cells [BMSCs]) (Fig. 1*A*). Both DMSCs and BMSCs are considered similar to mesenchymal stem cells (MSCs) (12). Also useful for therapies, induced pluripotent stem cells (iPSs) could be genetically modified to acquire a pluripotent dental-like state (1, 13).

The regenerative potential of these dental cells is clarified by both natural and experimental conditions. Differentiated "dentinoblasts," also named secondary odontoblasts, produce new dentin in response to dental pulp injury. This regenerativelike process is named "reparative tertiary dentinogenesis." Recruitment of endogenous dental stem cells has been proposed to underlie this type of dentinogenesis (46, 47). Furthermore, cells derived from isolated DPSCs (Fig. 1*A*) and DMSCs are sufficient for the generation of a tooth with normal histology but nonsatisfactory morphology (44, 48). Also, epithelial cells and either dentinogenic mesenchymal cells in collagen drops (48, 49) or iPS-derived neural crest cells in reaggregates (50) give functional teeth in mice after coimplantation in the mandible. Both the natural regenerative capacity of the tooth and the behavior of cells derived from isolated dental stem cells or developing dental tissues are of paramount importance in REP preclinical research. New advances at the cellular and molecular levels are providing interesting information to better understand this regenerative potential.

In developmental biology terminology, "equivalence" is a condition by which 2 cells from different embryonic origins (eg, DPSCs and BMSCs) could be interchanged without any developmental perturbation (1, 51, 52). Although iliac, tibia, femur, and orofacial BMSCs may regenerate bone, they are molecularly and functionally different in both human and animal models (33, 53–55). Similar evidence is also accumulating in favor of the "nonequivalence," and thus noninterchangeability, of dental stem cells and MSCs (13, 56–58). This could also be applied to their responses to the niches surrounding them (1, 17, 57, 59, 60). These differences would reside in the diverse developmental origins of these cells.

During animal development or regeneration, cells orchestrate the generation of tissues and organs by the deployment of a "genetic program" (61). This program comprises a hierarchical, combinatorial network (62, 63) of specific transcription factors (TFs). In the cell nuclei, the specific TFs regulate the transcription of genes coding for signal molecules. These molecules would be released to surrounding cells, which in turn would regulate gene transcription in neighbor cells by activating new TFs (Fig. 1*B*) (17). Besides this, other molecular mechanisms may also regulate this basic process (eg, acetylation/deacetylation [64] or microRNAs [65–69]). TFs provide positional prepatterning, size control, and cell differentiation (57). Once an initial pattern of cell specifications is achieved, a "positional memory" mechanism (40) further maintains it to generate the final histologic appearance.

During tooth development, cranial neural crest–derived mesenchymal cells (70) reciprocally interact with the odontogenic epithelium to regulate morphogenesis and differentiation. During pulpal healing, cells derived from the subodontoblastic layer migrate and differentiate into odontoblasts after odontoblast layer destruction following cavity preparation (71). These studies stress the existence of specific cell lineages and interlayer interactions during tooth morphogenesis. Mechanisms controlling positional and cell lineage specification would be at the heart of tooth growth during development and regeneration. A conserved set of TFs, such as SOX9, RUNX2, OSTERIX, and DLX5, is known to regulate both osteoblast (72) and tooth (70, 73–77) differentiation. Moreover, BARX1, MSX1, MSX2, LEF1, DLX2, PAX9, PITX2, and TWIST1 of nuclear factor I-C are other TFs involved in the dental "genetic program" (70, 77–80). This network of TFs may occur during both tooth development and regeneration (81). After PubMed searches using combinations of key words, more than 400 articles have been selected. The list of key words includes "dental-pulp," "tooth," "dentinogenesis," all dental stem cell initials, and all signal molecules or TF initials. References according to the name of researchers in the field (eg, "Smith," "Cooper," "Galler," "Mao," "Nör," and "Mitsiadis") as well as articles in specific journals, such as *Journal of Endodontics* and *Biomaterials*, have been searched for. Besides this extensive search, no articles on cell lineage analysis during tooth development or regeneration were found, and just 1 article on dental pulp "positional memory" (56) was discovered. The HOX and cofactor TALE genes provide positional memory to most tissues in animals (40, 82), but they were not expressed during tooth positioning in the jaw (83, 84). Nevertheless, some articles suggested their involvement during tooth germ formation (85, 86). Picchi et al (56) described differences between the pattern of expression of these genes in human stromal stem cells from bone marrow and dental pulp by quantitative real-time polymerase chain reaction (PCR). HOXA10 has been shown to be increasingly expressed by DPSCs until osteogenic differentiation is completed (56).

The knowledge of this information is crucial both to understand the process (81) and to design REPs in humans based on genetic modifications. Overcoming these preliminary results, further information from studies on signal molecules is suggesting interesting alternatives.

TGFβ1, BMP2, dentin matrix protein 1, or hepatocyte growth factor are some dentin-derived signal molecules (9, 37, 87–89). These molecules regulate cell-cell interactions, controlling cell proliferation and differentiation during tooth development. During this process, they are entrapped in the dentin matrix where they remain functional during life (90–92) (Fig. 1*B*). These molecules have been detected to be released from dentin entrapping by cariogenic bacteria (93, 94) or after treatments with EDTA (91, 95), phosphoric acid (92), calcium hydroxide–containing materials (96), mineral trioxide aggregates (97), tricalcium silicate–based cement (98), or some dental adhesives (99). Other signal molecules regulating TFs during dental pulp proliferation or differentiation in animals or humans are WINT; HEDGEHOG; BMPs; FGFs (70, 100); EGFs; PDGF; VEGF; IGFs (101, 102); and cytokines, such as CXCL14 or MCP1 (103). Some of these biomolecules are MSC survival (104–109), proangiogenic (103, 110), or trophic (103) factors. All these other molecules could also remain entrapped in the dentin matrix at very low concentrations. Because dentin-entrapped factors are potentially controlled by TFs regulating "positional memory" during development, their exact concentration in the tooth dentin could also be highly heterogeneous (Fig. 1*B*). General REP treatments that release them from dentin (9, 111) would also be benefited by this heterogeneity, naturally stored during tooth development. The treated dentin would act as an extracellular niche, potentially modulating growth and differentiation of neighboring odontoblasts (9) during pulp regeneration. This would be beneficial during REPs, even in the absence of any molecular information.

To improve this benefit, appropriate scaffolds should be designed to let dentin-released molecules be accessible to dental stem cells. A variety of biomaterials (112) have been tested during preclinical REPs, which include tissue extracts, such as blood clot (28, 113), platelet-rich plasma (PRP) (114, 115), and platelet-rich fibrin (PRF) (28, 116, 117); ceramics such as calcium hydroxyapatite (23, 118, 119), tricalcium phosphate (120, 121), and mineral trioxide aggregate (58, 113); synthetic polymers such as polylactic (119) and polylactic coglycolic (122, 123) acids; biopolymers such as collagen (23, 113), hyaluronan (124), and chitosan (125); and self-assembling peptide hydrogels (37). In recent years, new synthesis routes using nanotechnology have developed a variety of biomaterials in the form of nanoparticles (125, 126) or nanofibers and nanocomplexes (127, 128), offering scaffolds that show unique mechanical and physical properties. This industrial diversification has been reached driven by the enormous heterogeneity of dental stem cells and signal molecules, whereas it adapts to each specific dental application (13, 18). Nevertheless, the homing of the dentin-derived molecular heterogeneity in biomaterials has not been screened yet.

Results on pulp-dentin regeneration after REP treatments of necrotic teeth in animals or after REP-related experimental approaches in animal models with human cells are discussed in the following sections. REPs are quick and efficient in the dentist chair, and they preclude the long-lasting growth of a tooth (1), but they hide some drawbacks when they are histologically and molecularly studied in these models.

Experimental Studies of Regenerative Endodontics

Conventional apexification with calcium hydroxide in several visits (129) or apexification sealing the open apex with mineral trioxide aggregate in 1 visit (130) have been, until very recently, the treatment options for immature permanent teeth with pulp necrosis. However, these treatments leave short roots and thin dentinal walls, increasing the risk of future root fracture (131, 132). As an alternative to this, REPs have become part of the therapeutic endodontic spectrum in the treatment of necrotic immature teeth (95, 133–136). In clinical practice, these REPs (46) are almost exclusively focused on the treatment of immature teeth with pulp necrosis (137).

REPs are diverse applications of a common procedure that initiates with disinfection with hypochlorite irrigation and medication by intracanal administration of calcium hydroxide or antibiotics with variable durations. These techniques also include *in situ* biopolymer administration (eg, blood clot, PRP/PRF or collagen sponge, and sealing with intracanal coronal bioceramic barriers) (9, 113). Biopolymer administration may be as simple as a bleeding at the root canal by which a blood clot is formed, serving as a natural scaffold for invading MSCs. Both MSCs and signals at the clot induce revascularization and formation of new hard tissue (46). A more complex technique isolates PRP from a blood sample, which is applied to the empty root canal (114, 138). Recently, these techniques have also been applied to the treatment of human mature necrotic teeth with apical periodontitis (25, 139, 140) or root-filled teeth with persistent apical periodontitis (24). Beside these advances, the histologic analysis of some of these applications is revealing inconsistent results (4, 30, 32) (see later). Potential improvements to these techniques are arising from experiments that aim to regenerate dental pulp after pulpectomy using various human dental tissues and *in vivo* animal models.

TABLE 1. Histologic Results of Preclinical Studies *In Vivo* with Human or Animal Dental Pulp Cells and Animal Models

Groups	Stem cells	Scaffolds	Signal molecules	*In vivo* models	References
1	Endogenous	PRP/BC	ND/NA	REP in human	(21)
		BC/MTA	ND/NA	REP in human	(155)
		BC/collagen/MTA	ND/NA	REP in dog	(20)
		BC/PRP	ND/NA	REP in dog	(30)
		BC/PRP	ND/NA	REP in ferret	(28)
		Collagen	ND/NA	Orthotopic REP in rats	(27)
	CNCC	HA/TCP	BMP4/FGF2/FGF4/noggin/ activin ba	Subcutaneous implantation in mouse of mouse tooth germ	(33)
	DPSC/CC	PGA	FGF9	Allogenic subcutaneous implantation in mouse of human cells	(156)
	SCAP		Wnt3a/BMP9	Subcutaneous implantation in mouse	(80)
2	Endogenous	Collagen	bFGF/VEGF/PDGF/NGF	Subcutaneous implantation in mouse of complete human tooth and REP	(26)
		BC/MTA	EDTA	REP in humans	(32)
		Collagen	bFGF	Subcutaneous implantation in mouse of complete human tooth and REP	(157)
		PLGA	BMP4/BMP7/Wnt3a	Renal implantation in mouse of gel drops with rat *in vitro* germs	(49)
	DFSC	TDM	ND/NA	Subcutaneous implantation in mouse of human tooth root	(158)
	DPSC	PuraMatrix/MTA	ND/NA	Subcutaneous implantation in mouse of treated human tooth root	(19)
		Collagen	GCSF	Autologous transplantation in dog	(145)
		Collagen	SDF-1	Autologous transplantation in dog	(144)
		Alginate	GF	Subcutaneous implantation in mouse of human tooth root and REP	(29)
		Collagen/gelatin	FGF2/VEGF/PDGF	Femur and autologous implantation of rat tooth chambers	(34)
		PLGA/HA/TCP/CDHA/collagen	ND/NA	Renal implantation in mouse of human and rat cells	(120)
		TCP	ND/NA	Subcutaneous implantation in mouse of human cells	(16)
	DPSC/SCAP/BMSC	PLG	ND/NA	Subcutaneous implantation in mouse of human tooth root	(154)
	SHED	PLLA	ND/NA	Subcutaneous implantation in mouse of treated human tooth slice	(88)
		PuraMatrix	VEGF	Subcutaneous implantation in mouse of human tooth root	(4)
	MSC	Collagen	CXCL14/MCP1	Subcutaneous implantation in mouse of treated human or pig tooth roots	(103)
3	Dental pulp		VEGF/FGF2	Subcutaneous implantation in mouse of human tooth slice	(159)
	DFSC	PLGA/gelatin/TDM/DPEM	ND/NA	Subcutaneous implantation in mouse of human or pig cells	(123)
	DPSC	PRF	ND/NA	Subcutaneous implantation in mouse of human or dog tooth root and autologous orthotopic transplantation	(117)
		Hydrogel	VEGF/FGF2/TGFb1	Subcutaneous implantation in mouse of human tooth slice	(111)
		Collagen	SDF-1	Autologous treatments of dog tooth root	(143)
		Collagen/PCL/HA	SDF-1/BMP7	Review of experiments with many species	(5)
	DPSC/iPS	Collagen	GCSF	Subcutaneous implantation in mouse of human tooth root	(142)
	DPSC/MSC	Collagen	ND/NA	Subcutaneous implantation in pig of pig tooth root	(160)

(*continued*)

TABLE 1. (*continued*)

Groups	Stem cells	Scaffolds	Signal molecules	*In vivo* models	References
	iPS	Alginate-chitin	ND/NA	Subcutaneous implantation in mouse of human cells	(161)
	BMSC		ND/NA	Renal implantation in mouse of human tooth slice	(141)

BC, ■■■; bFGF, ■■■; BMP, ■■■; BMSC, ■■■; CC, ■■■; CDH, ■■■; CNCC, ■■■; DFSC, dental follicle stem cell; DPSC, dental pulp stem cell; FGF, ■■■; GCSF, ■■■; GF, ■■■; HA, ■■■; iPS, induced pluripotent stem cell; MSC, mesenchymal stem cell; MTA, mineral trioxide aggregate; NA, not exogenously added; ND, not determined; NGF, ■■■; PCL, ■■■; PGA, ■■■; PLGA, ■■■; PLLA, ■■■; PRF, platelet-rich fibrin; PRP, platelet-rich plasma; REP, regenerative endodontic procedure; SCAP, stem cell of the apical papilla; SHED, stem cell from human exfoliated deciduous teeth; TCP, ■■■; TDM, ■■■.

Groups 1, 2, and 3, respectively, are poor, partially good, and good histologic appearance of regenerated pulp.

Preclinical Studies *In Vivo* with Human Dental Pulps and Animal Models

Scientific research with animals has facilitated the histologic evaluation of pulp regeneration. *In vitro* culture of an engineered human tooth (98); transgenic mice (78); subcutaneous implantations in mice of engineered human dental tissues, such as tooth slices (141), tooth roots (19, 103, 111, 142), or dental stem cells (16); orthotopic transplantation of treated teeth (117, 123); and experimental REPs (ie, pulpectomized teeth filled with the previously mentioned triad) (117, 143–145) are some of the most important techniques used with animals. These studies are combined with histologic descriptions of the regenerated pulps. These pulps are stained with normal procedures (eg, hematoxylin-eosin or Masson trichrome) (19, 30, 78, 103, 123, 146), immunostaining (19, 103, 123, 146), *in situ* hybridization (78, 103), or cell fluorescence labeling (19). Several molecular techniques are also used to evaluate messenger RNA (mRNA) or protein presence in pulp samples from humans and animals. Some of these techniques are real-time reverse transcription PCR (RT-PCR), semiquantitative RT-PCR (147, 148) or quantitative RT-PCR (149), RNA sequencing (16), microarrays (148, 149), atomic absorption spectroscopy (97), ID (96, 97) or SDS (91)-polyacrylamide gel electrophoresis, Western blot (91), and enzyme-linked immunosorbent assay (95–97). Information on cell distribution or gene expression domains obtained after these techniques should be used to select molecular markers appropriate for histologic evaluations of treatments (17, 111).

Although improvements have involved the control of hypochlorite irrigation, the screening of appropriate scaffolds (150, 151), the design of anti-inflammatory or anti-infection reagent-releasing scaffolds (27, 42, 152), and the development of new techniques for pulp implantation (153) as a search for pain relief and inflammation or infection elimination, focus will be placed here exclusively on improvements suggested after the histologic evaluation of pulp regeneration. In principle, these experiments in animals have searched for different combinations of host tissues and guest stem cells to improve REPs under a purely empirical approach (137). These experimental combinations have provided important results, and modifications to the general procedure have also been suggested.

Histologic Results Supporting Noninterchangeability of Stem Cells and Dispensability of Growth Factors. Histologic results after REPs (26–28, 32) or subcutaneous transplantation of treated dental tissues (19, 30, 142, 154) from animals or humans range from nonsatisfactory to rather satisfactory ones. Nonsatisfactory results are characterized by incomplete regeneration of the pulp; ectopic formation of dentin, bone, or cementum in the pulp; poor formation of the odontoblast layer; irregular dentin deposition (21, 155); or excessive formation of the vascular supply (Table 1, group 1). This has been observed when cells from other origins, such as neural crest (33), bone marrow, or fat tissue (144), are seeded during experimental REPs in animals (Table 1). Also, human dental pulps have been regenerated in organ culture *in vitro* after pulpotomy with nonsatisfactory histologic results (98).

However, partial histologic results have been published after REP applications (26, 29) in which specific dental stem cells, such as animal DPSCs (144, 145) or human SHEDs (3, 88), are seeded in scaffolds with growth factors after *in vitro* amplification. In these articles, a general good histologic appearance of the regenerated pulp is accompanied with some of the previously mentioned features of poor regeneration (Table 1, group 2). These results have also been observed after subcutaneous implantation of dental tissues treated with human DPSCs (19, 103, 120, 154), SHEDs (3, 6, 88), or SCAPs (154, 162). Nevertheless, apparently, optimal results (Table 1, group 3) have been described when REP-derived applications use DPSCs to seed scaffolds (117, 143) or when human DPSCs (111, 117, 142) and SHEDs (4) are used to treat root canals or tooth slices previous to subcutaneous implantation in immunodeficient mice. These good results are also observed when human BMSCs (141) or DFSCc (123, 158) are used in ectopic transplantations (Table 1). In summary, although nonsatisfactory histologic results can be obtained after *in vivo* (33, 60, 141) or *in vitro* experiments (98), amplified human DPSCs and SHEDs (4, 111, 117, 142) have been mostly successful in animal models for regenerative endodontics.

According to these histologic evaluations, the origin of stem cells used in treatments could be a factor to determine good REPs results, considering DPSCs and SHEDs are more appropriate than cells from neural crest or other dental origins or the simple recruitment of endogenous stem cells. This is in good agreement with the previously mentioned hypothesis of noninterchangeability of stem cells obtained from bone regeneration studies (33, 53–56, 160). Of special interest at this point is the revision performed by Huang et al (60), who also compared results obtained after experiments using different dental stem cells. These authors also consider it important to refocus actual research on dental stem cell niches and their influence on dental stem cells (60).

In summary, exogenous stem cells applied to empty teeth with scaffolds and general growth factors may render nonsatisfactory results in which pulp regeneration is only partial or ectopic bone or dentin is formed (Fig. 2, *arrow 1*). This evidence broadly supports the hypothesis of noninterchangeability of stem cells during REPs (1). Besides the recruitment of endogenous stem cells from neighboring dental tissues (9), future treatments after pulpectomy should use autologous and orthotopic amplified DPSCs or SHEDs when possible.

A significant number of reviews have also been focused on the appropriateness of scaffolds (11, 163–166) or growth factors (3, 7) during these treatments. The mixture of good and poor histologic results described earlier is usually observed after blood clot or PRP treatments or after the use of scaffolds in ectopic or *in vitro* studies.

Some of these scaffolds are collagen (26, 34, 103, 120, 144, 145, 157), hydroxyapatite/ tricalcium phosphate ceramic powder (45), poly(lactide/glycol) (3, 49, 88, 120, 154), PuraMatrix (19), and Biodentin (98). Optimal results have been described after the use of collagen (5, 142, 143, 160), poly(lactide/glycol) (123), or PuraMatrix (4) scaffolds. These results are also observed when root canals (142–145, 154, 160), tooth slices (141, 159), or treated dentin (123, 158) are combined with DPSCs, SHEDs, or SCAPs in allogenic transplantations or after pulpectomy (Table 1).

Although actual REPs use endogenous growth factors provided by the blood clot, the PRP/PRF treatment, or the washed dentin during releasing treatments, preclinical experiments have used an important combination of different growth factors exogenously supplied with a variety of scaffolds and *ex vivo*–amplified stem cells to reconstitute original stem cell microenvironments both *in vitro* and *in vivo*. Following our review, optimal histologic results have been observed after *in vivo* treatments with bFGF or VEGF (111, 159) applied to pulp tissue in *ex vivo*–amplified tooth slices (159) or over DPSCs seeded to hydrogels (165); SDF-1 (5, 143); or even in the absence of any growth factor application over DPSCs in PRF (117), DFSCs in polylactic coglycolic (123) or treated dentin (158), or BMSCs in tooth slices after renal implantation (141) (Table 1). These results open an interesting research field on how scaffolds interact with the natural dentin during pulp regeneration and how to reduce the "cocktail" of growth factors in the potential therapeutic triad.

Molecular Studies Further Supporting a Need for Dental Pulp Positional Markers to Screen Biomimicry of Scaffolds. Molecular analyses of the pulp in carious or fractured teeth, experimental REP-treated teeth, and subcutaneous transplanted dental tissues are suggesting potential translational advances to reduce inflammation or to increase the availability of endogenous signals. Several issues have analyzed the molecular complexity involved in a caries lesion, and comparisons have been made between this molecular profile and the one shown by the healthy pulp (17, 147–149, 167–169). In principle, the expression of genes associated with inflammation is enhanced during caries (9, 46, 168, 170, 171) but the expression of other genes associated to cell growth and differentiation are also upactivated. Other molecular analyses are also providing information from developmental (16, 78, 146) and regenerative (4, 16) studies, but a complete molecular comparison between carious, developing, and regenerating teeth has not been reported.

Several molecules have been extracted from dentin and shown to remain active over pulp-derived or nonderived cells during regeneration (9, 37, 87–92). During dentin caries progression, the release of these molecules would activate reparative tertiary dentinogenesis potentially related with the previously mentioned gene up-regulation (46, 168). As stated previously, several treatments (91, 92, 95–98) increase the release of these molecules, and this is at the heart of a new REP improvement (9). Using these treatments, chemotactic signals released by dentin would recruit stem cells from surrounding tissues, and they would accompany DPSCs or SHEDs to control cell growth and differentiation during pulp regeneration (9, 17) (Figs. 1*B* and 2 [*arrow 2*]). Furthermore, although good histologic results have been obtained after root canal and tooth slice transplantations (Table 1), no good results have been reported in the crown pulp area, a region with a much larger size. If dentin or apex is providing important signal molecules or nutrients for the regenerative process, they may diffuse away because the receiving cells are over a threshold distance from sources. Also, the exogenous administration of growth factors (Table 1) could be solved with the use of survival/trophic factors to maintain stem cells and the cells derived from them alive or with appropriate scaffolds, which could preserve the high heterogeneity of released dentin-entrapped molecules at long distances to the dentin surface.

Some trophic factors that activate cell migration and dental stem cell growth have been tested (9). MSC survival depends on IGF1 (107), and this factor has been found to be entrapped in the dentin (87). Moreover, CXCL14 and MCP1 are also trophic factors active over pulp MSCs (103). Once appropriate combinations of these DPCSs/SHED survival/trophic factors are found, the effective harnessing of the innate tooth capacity for self-repair could be stimulated (14, 152) (Figs. 2 [*arrow 3*] and 3).

Appropriate scaffolds should also be screened according to their capability to restore positional identities to pulp cells during regeneration. As stated previously, these new scaffolds would primarily sequester the heterogenous distribution of molecules released from dentin. During the screening of these scaffolds or even of the appropriate combination of factors and stem cells, molecular positional or cell lineage–specific markers would be of paramount importance. The best molecular markers would be those actively involved in pulp regeneration, such as TFs or signaling pathways controlling the positional identity of pulp cells (see earlier). Nestin expression in the odontoblast layer or Notch2 expression in subodontoblastic cells during reparative dentinogenesis (17), *Cbfa1/Runx2*-regulator *Msx1*gene expression in dental mesenchyme (70), and odontoblast differentiation regulator ATF6 expression in odontoblast and ameloblasts would be good candidates (146). Hypoxia-dependent synthesis of proangiogenic factors by SCAPs (172), DPSCs, or fibroblasts from dental pulp (173) would also be good molecular markers. Molecules, such as VEGF, are up-regulated in these conditions, and they would be overexpressed during the excessive vascularization of poor dental pulp regeneration. Also, candidates for good molecular markers are HOX and TALE genes (eg, HOXA10) (56), previously shown to characterize a molecular signature of human stromal stem cells from dental pulp, such as DPSCs (56), but probably the best example of a good molecular marker is *Twist-1* mRNAs (78). This gene is continuously expressed during tooth development as detected by RT-PCR, and its knockout in transgenic mice (78) shows ectopic formation of dentinlike tissue in the dental pulp, similar to that observed as a poor histologic result during REPs (Table 1). A complete description of the pattern of expression of these genes in the dental pulp of the late developing tooth would be crucial for this proposal (70). Poor or partially good histologic results from blood clot, PRP, experimental REPs, or ectopic subcutaneous transplantations of exogenous and heterotopic stem cells (Table 1) could be molecularly assessed by a combination of expressions of these genes or proteins (16) to interpret incorrect allocation of cells or the incorrect activation of positional memory genes in regenerating pulp cells (Fig. 2).

Both international (ISO 10993-1:2009) and European Regulation (CE) no. 1394/2007 suggest procedures to evaluate health risk by direct contact of human tissues with materials or leachables arising from them (174, 175). Nevertheless, the beneficial influences of REPs have not been standardized (176). In the search of these "beneficial assays," the use of positional or cell lineage–specific markers during the histologic evaluation would be useful. These mRNA and proteins could be easily studied by immunostaining or *in situ* hybridization of histologic sections, and modifications in their pattern of expression could be correlated with the histologic appearance of the pulp.

Toward a New Bioengineering Dental Triad

DPSCs (117, 143–145) and SHEDs (3, 88) have been tested in several REP studies displaying satisfactory or partially satisfactory histologic results. Results with DPSCs (117, 143), collagen, or polylactic/glycol scaffolds and with scarce or no growth factors are

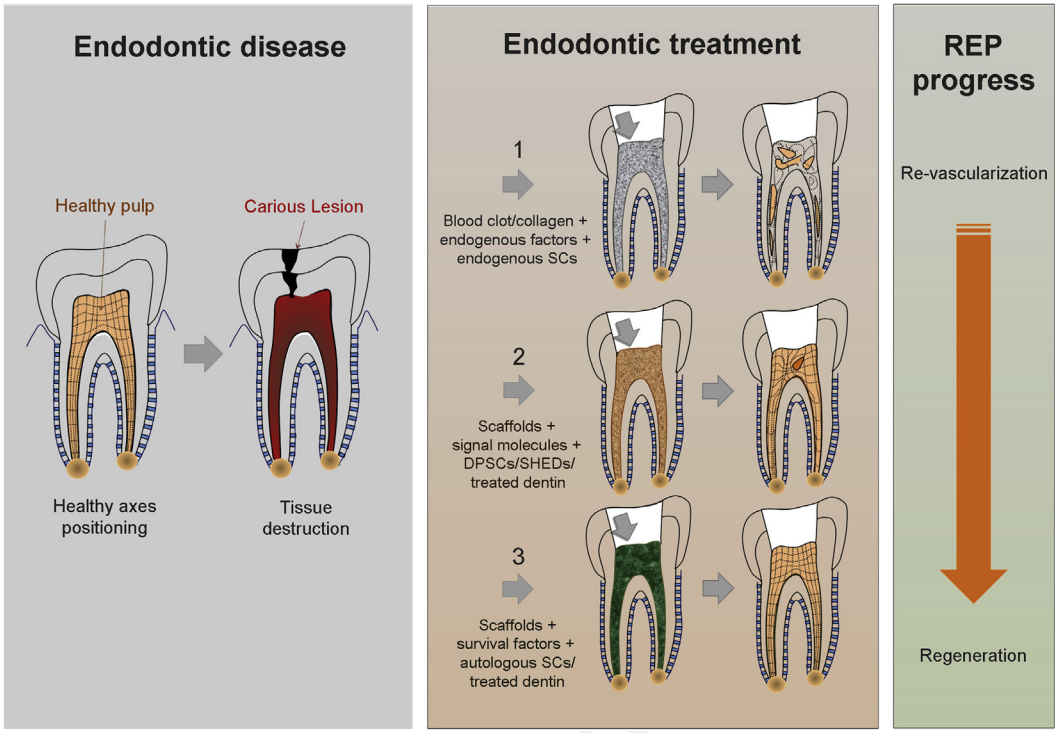

Figure 2. A schematic showing current and advanced endodontic stem cell–based therapies. The *left blue-gray box* shows endodontic disease acting over a healthy tooth (ie, carious lesion, *reddish area*). The axes positioning of the dental pulp is destroyed after irreversible pulpitis. The *middle light ochre box* shows 3 dental stem cell–based treatments (*arrows 1–3*) after pulpectomy. (*1*) Current preclinical and clinical treatments with scaffolds, growth factors, and heterotopic stem cells (ie, PRP) (*gray area* in the left tooth). The right tooth schematic shows the results of abnormal histologic integration described in the text. (*2*) The current preclinical and clinical treatments with scaffolds, chemotactic signals, and migrating homotopic stem cells, together with treated dentin (ie, blood clot) (*light brown area* in the left tooth). The right tooth shows abnormal histologic morphology because of modified positioning of migrating stem cells. (*3*) An alternative approach with scaffolds, survival/trophic factors (survival signals), with treated dentin and stem cells/stem cell niches (*green area* in the left tooth). The right tooth displays potential results of normal histologic regeneration because of correct cell positioning. The right *green box* shows potential progress of REP therapies. From a simple revascularization treatment, potentially true dental pulp regeneration is expected. The *gray arrows* over colored areas indicate REP treatments. The *lines* in the dental pulp schematic show potential positioning cues acting during tooth development, tissue maintenance, and regeneration. The *upper orange area* and the *lower concentric light ochre areas* in each root apex show DPSCs and SCAPs microenvironments, respectively.

optimal when compared with REPs using growth factors but no amplified stem cells (26–29, 35). Several dentin treatments show further good results (Table 1), and these advances should accompany PRP/PRF or collagen gels in REPs and enhance the biomimicry of scaffolds to preserve the diverse concentrations of factors released in the search of regeneration of dental stem cell niches (Fig. 2 [*arrow 3*]). Growth factor research is now at a stage to facilitate cell survival to stem cells much more than to establish appropriate interactions with dentin-released factors. Thus, the screening of more appropriate stem cells, dentin molecule–releasing treatments, scaffolds with good biomimicry, and good positional markers for histologic evaluation of treatments is an exciting activity for future venues of REP improvements.

If dentin provides local complexity of released signal molecules, emerging REP advances may also be applied to human mature necrotic teeth with apical periodontitis after pulpectomy (1, 25, 139) where the root canal or the dental pulp cavity is becoming smaller with age. Furthermore, REPs have been proposed for the treatment of necrotic teeth with small apical dimensions, such as mature apical foramina (137). Nevertheless, only a few studies have suggested reversible pulpitis as a target of REP (140, 143, 145, 177). Vital pulp therapy is the elective treatment option for teeth with reversible pulpitis, aiming to preserve and maintain reversible compromised pulp tissue. Vital pulp therapy requests an accurate diagnosis of the pulp status and careful management of the remaining pulp tissue. Both direct pulp capping and pulpotomy, the main vital pulp therapy procedures, are based on the ability of DPSCs of the remaining vital dental pulp to accomplish repair (137). The presence of dental stem cell niches in remaining vital pulp would represent an advantage and would be crucial to obtain good

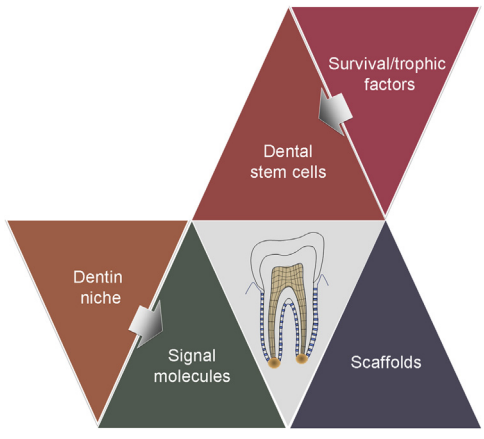

Figure 3. A schematic showing an alternative dental bioengineering approach that includes the 3 classic elements (ie, dental stem cells, signal molecules, and scaffolds) and 2 new ones (ie, stem cell niches and survival/trophic factors). *Arrows* indicate influences acting over dental stem cells from their reconstructed microenvironment.

results in REPs, even in cases with small fragments of residual vital pulp (154). The release of dentin-entrapped molecules from dentin walls could also be stimulated during pulpotomy to better coordinate cell differentiation in the regenerated pulp-dentin complex. If clean dentin surfaces and vitality of microenvironments are sustained, good results could also be expected (Fig. 3).

Conclusions

According to the results reviewed here, good histologic results are accumulating after REPs derived *in vivo* experiments with human cells in animal models. These results suggest modifications of the classic triad of tooth bioengineering (Fig. 3) and restrictions to the use of iPSs as dependent on appropriate TFs. The clarification of the "dentition genetic program," the discovery of good positional or cell lineage–specific markers of the dental pulp, the screening of biomimicry of scaffolds, and the search for trophic factors maintaining pulp-dentin vitality (46, 101–103, 106, 108, 178) are basic research objectives of dental bioengineering (Fig. 3). If these objectives are accomplished, further translational advances to current regenerative endodontics could be achieved.

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