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Stem Cell and Biomaterials Research in Dental Tissue Engineering and Regeneration

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INTRODUCTION

The ultimate goal for tissue engineering and regenerative medicine is to develop therapies to restore lost, damaged, or aging tissues using engineered or regenerated products derived from either donor or autologous cells. Various approaches have been considered in tissue engineering and regenerative medicine, but currently the most common is to use a biodegradable scaffold in the shape of the new tissue that is seeded with either stem cells or autologous cells from biopsies of damaged tissues.^{1,2} The scaffold provides an environment that allows the implanted cells to proliferate, differentiate, and form the desired tissue or organ. Several biomimetic scaffold materials have been used for this purpose, including naturally occurring macromolecules such as collagen, alginate, agarose, hyaluronic acid derivatives, chitosan, and fibrin,³ and man-made polymers such as polyglycolic acid (PGA), polylactic acid (PLA), poly(caprolactone) (PCL), poly(dioxanone), poly(methyl methacrylate) (PMMA), and poly(glycerolsebacate).^{4–8}

The approach of combining adult stem cells with biomimetic scaffolds and bioactive molecules is in varying stages of development for the treatment of disorders such as diabetes, arthritis, Parkinson disease, Alzheimer disease, atherosclerosis, cancer, and heart disease. This article focuses on dental diseases such as caries and periodontitis, which are

pandemic, cause a permanent loss of tissues and functions, and affect the health of populations in all age groups worldwide.

PAST AND PRESENT APPROACHES IN TISSUE REGENERATION

Over the past few decades, new technologies in tissue engineering such as microfabrication, self-assembled biomimetic peptides, and 3-dimensional (3D) printing have rapidly developed. These technologies have enabled the building of simple tissues such as skin epithelium and production of composite tissues such as bone, kidney, and bladder.^{9–14}

Regeneration of Nondental Tissues

The first tissue-based therapies for skin grafting were developed in India around 3000 BCE, but the synthesis of substitute materials for skin and various grafting techniques (eg, autologous and allografts) were not developed until the eighteenth century.¹⁵ The first engineered skin tissues were generated by Howard Green and colleagues in 1975.^{16,17} This product, which contained only a few layers of cells and did not contain dermis, led to the development of the first commercial skin product, named Epicel (Genzyme, Cambridge, MA, USA), which contains sheets of autologous keratinocytes. Another engineered product for skin was generated using bovine type I collagen and shark chondroitin 6-sulfate.^{18,19} These compounds were crosslinked and packed into a porous matrix with a silicone sheet attached onto one side as a temporary epidermis-like barrier. A composite product of reconstituted dermis and epidermis has led to the development of a commercial skin graft product called Apligraf (Organogenesis, Canton, MA, USA).^{20,21} The strategy of combining cells and extracellular matrix in skin-graft products was also used to successfully produce cartilage-graft materials. Cell-based cartilage repair techniques were first described in 1994.²² This technology led to the development of the first commercial product for cartilage grafts, called Carticel (Genzyme). Since 2008, significant advances in tissue engineering have been made for other tissues such as bone, kidney, bladder, blood vessels, and liver.^{9–14,23–26}

Unlike other tissues, the skin and cartilage do not require an extensive vascular supply.²³ An important challenge in organ regeneration is the acquisition of a functional vascular supply for the engineered organ. Endothelial cells and the paracrine factors that regulate them, such as vascular endothelial growth factor, were shown to induce angiogenesis and facilitate the integration of transplanted tissues/organs into the host. This finding led to a new treatment strategy in regenerative medicine by using peripheral blood-derived or bone marrow-derived endothelial progenitor cells to induce de novo vessel formation in regenerated organs.^{27–29} Vascular endothelial cells can also be generated from human embryonic stem (ES) cells. These cells can integrate into the host and form chimeric vasculature.³⁰ Vascular endothelial cells can facilitate the differentiation of ES cells into various cell types such as pancreatic insulin-producing cells,³¹ cardiomyocytes,³² neurons, and glial cells.³³ 3D cardiac tissues with endothelial cell networks have been created and implanted onto infarcted rat hearts, which regain function after the surgery. The improvement of cardiac function was dependent on the endothelial cell densities within the engineered cardiac tissues. The number of capillaries in the transplanted tissues with the endothelial cell network is also greater than those without the endothelial cells.³⁴

Regeneration of Dental Tissues and Supporting Structures

The regeneration of periodontium was the first tissue-engineering technology in dentistry, and was invented by Nyman and colleagues³⁵ in 1982. This procedure, termed guided tissue regeneration (GTR), involves inserting a barrier membrane under the periodontal tissue flap to prevent the ingrowth of gingival epithelium and connective tissue, while creating a space

on the root surface for progenitor cells from the periodontal ligament including cementoblasts, fibroblasts, and osteoblasts to migrate in and form new periodontal structures including cementum, periodontal ligament, and alveolar bone. Various types of bonegraft materials such as autogenous grafts, allografts, alloplasts, or xenografts have been placed in the space above root surfaces to facilitate bone formation.^{36–38}

There are 2 main types of barrier membranes, resorbable and nonresorbable. The nonresorbable membranes require a second surgical procedure to remove the membranes at 4 to 6 weeks after the initial surgery. Two types of commonly used non-resorbable GTR barrier membranes include expanded polytetrafluoroethylene (ePTFE), also known as Gore-Tex, and nonexpanded polytetrafluoroethylene (nPTFE). The resorbable barrier materials were more recently developed and are available in 2 formats, synthetic polymers and natural barrier materials. The synthetic polymer GTR materials consist of a lactide/glycolide copolymer or PLA blended with a citric acid ester. The natural barrier membranes include those made from collagen, calcium sulfate, or enamel matrix proteins.^{38–40}

The regeneration of periodontium with these products requires the presence of at least one bony wall at the treatment site, most likely to provide progenitor cells and vascular supply, allowing the repair and regeneration of the periodontal tissues. To improve on the limited level of success, strategies using exogenous growth factors and stem cells have been studied and await translational application to clinical practice. Potential growth factors for periodontal regeneration include bone morphogenetic proteins, platelet-derived growth factor, amelogenin proteins, and fibroblast growth factors.^{38,41–46}

Current therapeutic approaches involve replacing the missing tooth structure with artificial materials as the capacity of adult human dental tissues to regenerate is virtually nonexistent, particularly for enamel, due to the absence of ameloblasts in formed teeth. The regeneration and repair of inner-tooth dentin can be obtained only if the healthy dental pulp tissue is still present and if bacterial contamination is completely removed.^{47,48} Typically, mechanical removal of decayed enamel and dentin is completed and artificial materials are used to fill in the prepared cavity, to prevent bacterial contamination and induce the formation of reparative dentin onto the dentinal floor of the cavity.

The regeneration of dentin is usually not possible in necrotic teeth. However, in children with incompletely formed teeth with wide-open root apices, pulp tissue can be regenerated through the opened root apices. Findings from prior revascularization studies of traumatized teeth showed that the success of pulp-tissue regeneration in replanted avulsed teeth depends on the diameter of the opening of root apices.^{49–51} A diameter of 1 mm (1000 μm) of the opening of root apices has been suggested as a minimum requirement to allow new tissues with neural and vascular structures to regrow into the tooth.⁴⁹ Because diameters of the neural, vascular, and cellular structures are less than 100 μm (ie, 10–30- μm diameters for eukaryotic animal and human cells; 0.2–20- μm diameters for nerve fibers; and <100- μm diameters for most arteries in the dental pulp),^{52–54} theoretically the regeneration of pulp tissues may not need as much as a 1000- μm -diameter opening. However, the positive correlation of clinical success in revascularization of the replanted teeth and a 1-mm minimum apical opening requirement may be due to the existence of stem cells or progenitor cells in the apical area. Further studies are needed to test this notion.

Several case series showing clinical success of pulp-tissue regeneration in immature necrotic teeth led to the growing recognition of the regenerative potential of tissues at the apical end of these immature teeth.^{55–61} The recent identification of adult mesenchymal stem cells in these tissues also suggests that this cell population regrows into the tooth and regenerates the

dentin-pulp complex of such immature necrotic teeth. However, the exact mechanisms by which such precursor cells contribute to clinical outcomes remain unknown.

USES OF STEM CELLS IN TISSUE REGENERATION

Cell-based therapies are the most common approaches in regenerative medicine. Challenges in applying this approach clinically are to acquire the appropriate source of cells, to identify methodologies to induce cell proliferation and differentiation, to maintain cell survival, and to remove unwanted cells.

As stem cells possess a remarkable potential to proliferate and develop into many different cell types to form the desired organ, these cells hold great promise for regenerative therapy. The progeny of stem cells may remain as unspecialized progenitors to serve as an internal source of repair and replenishment, or may differentiate into specialized cells to form the desired tissue. The most commonly used and studied stem cells are (1) ES cells, (2) somatic or adult stem cells, and (3) induced pluripotent stem cells.

Embryonic Stem Cells

ES cells are derived from the inner cell mass of early embryos, called blastocysts. ES cells were first isolated from mouse embryos in 1981.^{62,63} The success of this work led to the derivation of human ES cells from in vitro fertilized human blastocysts in 1998.⁶⁴ ES cells are capable of dividing and renewing themselves for long periods without differentiating, whereas most somatic or adult stem cells cannot. In the appropriate environment, these cells can acquire epigenetic marks in their DNA to modulate their gene expression, allowing them to differentiate into any specialized cells. Various types of specialized cells derived from ES cells⁶⁵ include retina cells,^{66,67} cardiomyocytes,^{32,68} neurons,^{69,70} hematopoietic cells,^{71,72} hepatic cells,^{73–75} trophoblasts,^{76,77} pancreatic insulin-producing cells,³¹ vascular endothelial cells,^{30,78,79} pituitary hormone-producing cells,³⁰ and osteoblasts.^{80,81}

Somatic or Adult Stem Cells

In general, the regenerative capacity of adult tissues depends on tissue-specific stem-cell populations that maintain stable numbers by self-renewal and possess the ability to differentiate into distinct cell lineages. Regeneration and renewal in adult mammals has been studied in several organs, including blood, mammary glands, gut, brain, skin, muscle, and hair. These tissues contain adult stem cells such as hematopoietic, endothelial, mammary, intestinal, neural, skin, muscle, and hair-follicle stem cells. Similarly, teeth and supporting structures contain multiple lineages of somatic stem cells, including:

1. mesenchymal stem cells isolated from the dental pulp of permanent teeth, termed Dental Pulp Stem Cells (DPSC)⁸² and from the dental pulp of exfoliated deciduous teeth termed Stem cells from Human Exfoliated Deciduous teeth (SHED)⁸³
2. mesenchymal stem cells isolated from the periodontal ligament⁸⁴
3. mesenchymal stem cells isolated from the apical end of developing tooth roots, termed Stem Cells from the Apical Papilla (SCAP),^{85–87} and
4. epithelial stem cells isolated from the labial cervical loop of rodent incisors.^{88–91}

In primates, incisors cease growth once their roots are completely formed, whereas in rodents the incisors continue to grow throughout postnatal life because of the presence of epithelial and mesenchymal stem cells that have the capacity to self-renew and differentiate into all of the cell types of adult teeth, including ameloblasts, odontoblasts, and the stratum intermedium (SI). Thus, rodent incisors provide a model for determination of signaling mechanisms that coordinate cell-fate decisions, stem cell self-renewal, and maintenance.

The labial cervical loop (CL), but not the lingual CL, of rodent incisors contains stem cells that give rise to ameloblasts and the SI (Fig. 1).⁹² Labeling experiments demonstrated that cells in the dental epithelium move in a proximal to distal direction.⁹³ In the labial CL, the stem-cell progeny contribute to a population of transit-amplifying (T-A) cells (see Fig. 1A, B). T-A cells undergo several rounds of cell division before they move distally and differentiate into ameloblasts. The incisor epithelia seem to function as a conveyor belt, moving cells from a proximal, undifferentiated source to regularly repopulate the tooth with specialized cell types.

Identification of organ-/tissue-specific adult stem-cell populations can be challenging, because stem cells often reside in heterogeneous niches intermingled with support cells. A useful characteristic of stem cells that has aided in their identification *in vivo* is the relatively slow cell-division kinetics of many stem cells relative to surrounding tissue.⁹⁴ Slow-cycling cell populations have largely been identified through label-retention experiments, traditionally using 5-bromo-2'-deoxyuridine (BrdU) incorporation, because cells that divide slowly do not dilute the BrdU label as quickly as their rapidly dividing neighbors. Using this technique, BrdU label-retaining cells (LRCs) were identified in the labial CL of cultured perinatal incisors and in adult incisors *in situ*. Another approach to label retention is the use of transgenic mice harboring a tetracycline-sensitive, histone H2B conjugated with a green fluorescent protein cassette (H2B-GFP) under the control of a tissue-specific transactivator (see Fig. 1C, D).⁹⁵ Expression of H2B-GFP is initially activated in all cells of the tissue of interest followed by a "chase" period when the transgene is repressed by exposure of the animal to doxycycline, such that dividing cells dilute the label. This technique was used to identify LRCs in the outer enamel epithelium of the adult labial CL.⁹¹ The LRCs of the dental epithelium expressed *Gli1*, a target gene of sonic hedgehog (SHH) signaling, and lineage-tracing experiments demonstrated that the *Gli1*-expressing cells were indeed stem cells.⁹¹

Understanding the regulation of adult stem-cell populations is key to the future use of such cells for clinical therapies. How stem cells are maintained at the appropriate number, what signals regulate their differentiation, and how they are established within the context of the developing organism are important questions in stem-cell research. Many signaling molecules and pathways are implicated in the development and homeostasis of stem cells. These signaling components include SHH, WNT, NOTCH, bone morphogenetic protein (BMP), and fibroblast growth factor (FGF) superfamily proteins that are important regulators of stem cell self-renewal and differentiation.^{88,90,92,96-98} During the development of epithelial-mesenchymal-derived organs such as teeth, these proteins mediate critical interactions between epithelial and mesenchymal cells that lead to the various stages of tooth development (Fig. 2). Gene-expression data on growth factors and bioactive molecules at each stage of tooth development can be found at www.biteit.helsinki.fi. An extensive review of dental stem cells and growth factors is provided in articles elsewhere in this issue.

CELL-REPROGRAMMING TECHNIQUES

ES cells possess the capacity to multiply indefinitely. Under an appropriate microenvironment, these cells can differentiate into any cell types and are very useful in research and clinical applications in tissue engineering and regenerative medicine. However, undifferentiated ES cells have the potential to form tumors.⁹⁹⁻¹⁰¹ The derivation of human ES cells with matched immunogenotypes from fertilized human embryos also raises ethical issues. By contrast, somatic stem cells or adult stem cells have limited applications. These cells are capable of generating cell types of the tissue in which the cells reside but not cells of a very different origin. For example, hematopoietic stem cells are blood-forming adult stem cells that give rise to various blood cells but not cells of different tissues.

The challenges of working with human ES cells and somatic stem cells in part led to the development of new techniques for obtaining stem cells. Two such techniques are transdifferentiation and induced pluripotent stem cells.

Transdifferentiation

This process converts a given cell type directly into another specialized cell type without bringing the cells back to a pluripotent state. The success of this approach was shown for the conversion between two closely related cell types. For example, a transcription factor, MyoD, was used to convert dermal fibroblasts, chondroblasts, gizzard smooth muscle cells, and pigmented retinal epithelial cells into elongated postmitotic mononucleated striated myoblasts.¹⁰² Similarly, adult mesenchymal stem cells from teeth and bone marrow were shown to normally differentiate only into other mesenchymal cell types such as chondrocytes and adipocytes.^{82,103} However, a combination of 3 neural transcription factors, ASCL1, BRN2, and MYT1L, converted mouse embryonic and postnatal fibroblasts into functional neurons in vitro.¹⁰⁴ Furthermore, the transcription factor OCT4 and cytokine treatment converts human dermal fibroblasts into granulocytic, monocytic, megakaryocytic, and erythroid lineage cells.¹⁰⁵ Another set of transcription factors, C/EBP β and C/EBP α , were used to convert fibroblasts into macrophages.¹⁰⁶ Even so, the transdifferentiation approach remains an area of great debate.

Induced Pluripotent Stem Cells

This technique was developed using a quartet of transcription factors, OCT3/4 (Pou5f1), SOX2, KLF4, and c-MYC, to reprogram somatic cells into pluripotent stem cells.^{107–109} The first induced pluripotent stem (iPS) cells were developed from adult mouse cells by Yamanaka and Takahashi in 2006^{107,108} and from adult human cells by the same group in 2007.¹¹⁰ This breakthrough discovery provided a new way to dedifferentiate cells while maintaining donor-specific immunocharacteristics necessary to prevent rejection by the immune system. The iPS cells possess almost identical properties to the ES cells in that they can multiply almost indefinitely without losing their potential to differentiate into any cells of the 3 germ layers: endoderm, mesoderm, and ectoderm.^{110,111}

iPS cells can be produced from both normal and diseased tissues. For example, iPS cells were derived from human amniotic fluid cells collected for diagnosis from patients with β -thalassemia¹¹² and those from cystic fibrosis lung removed from patients.¹¹³ Instead of transdifferentiation, this approach is also useful to reprogram adult stem cells to generate specialized cells of different origins. For example, endothelial CD34⁺ progenitor cells were derived from the iPS cells of bone marrow,¹¹⁴ and functional neurons were produced from the iPS cells of skin dermal fibroblasts.¹¹⁵

STRATEGIES FOR SELECTIVE REMOVAL OF UNDIFFERENTIATED STEM CELLS

Challenges in the clinical application of stem-cell-based therapies are not only to differentiate the cells into the desired specialized cell types but also to establish strategies to remove residual undifferentiated cells to prevent tumor formation. Both positive and negative selection systems have been proposed, including:

- Engineered human ES cells to express herpes simplex virus thymidine kinase so that the ES cells can be killed by ganciclovir at concentrations that are nonlethal to other cell types^{116–118}

- Magnetic activated cell sorting using antibodies for differentiation markers for positive selection and/or a selective killing of residual undifferentiated cells by the cytotoxic monoclonal antibody mAb 84.^{119–121}

SCAFFOLDS IN TISSUE ENGINEERING

The type of scaffolding material that stem cells will require to generate specific tissues is an area of great interest. The 2 basic methods for tissue engineering are a top-down approach and a bottom-up approach. The more traditional method is the top-down approach, whereby cells are seeded in a preformed 3D scaffold made from polymer, natural porous materials, or decellularized native extracellular matrix. In the bottom-up approach, various methods have been used to aggregate cells to form distinct subunits that could eventually be used as building blocks to engineer whole organs. Examples of these methods are cell printing, microwells, cell sheets, and self-assembled hydrogels. This section describes the various types of scaffold materials used in tissue engineering, the types of methods in which these materials are used (top-down vs bottom-up), and a few examples of how these materials are being applied to dental pulp regeneration. An extensive review of scaffolds in dental tissue generation is provided in an article elsewhere in this issue.

Scaffold Materials

Whether the approach is top-down or bottom-up, the role of a scaffold is to provide support for delivering cells and/or growth factors to the proposed site of tissue regeneration. Toward these goals, there are important features to consider in scaffold selection, including the physical and mechanical aspects of the material, its biocompatibility, and its degradation timeline. These physical aspects of a 3D scaffold include the porosity (pore volume fraction of the scaffold), pore size (pore diameter), pore structure (shape), and all aspects that can influence how well the cells adhere to the material.^{122–124} Hydrogels are polymeric structures that are crosslinked and swell in water. For a hydrogel, important aspects include swelling behavior and diffusivity of the hydrogel.

Important mechanical properties of a scaffold material include the viscoelasticity and the tensile strength. For dental regeneration purposes, the tensile strength may be not as important as the viscoelastic properties of the scaffold materials. In general, scaffold materials should reflect the microenvironment of target tissues/organs to facilitate cell growth and ultimately integration to the host. A beneficial clinical feature for dental pulp regeneration would be if the scaffold is injectable, as are some of the natural scaffold materials and hydrogels. In these cases, the gelation time would need to be taken in to consideration when seeding cells in a scaffold for implantation into a host.

An essential clinical feature for scaffold selection is biocompatibility. Naturally derived scaffold materials have the advantage that they are generally well tolerated, do not lead to immunogenic response, and do not involve the use of harsh chemicals during processing. However, a major drawback is the lack of control over the pore size and heterogeneity of the scaffold.

The degradation process of the scaffold is important, and should closely follow the rate of tissue regeneration. When using synthetic polymers, the release of acidic degradation products must be taken into consideration, as well as the resulting drop in pH in the surrounding microenvironment and how that affects the immune response, surrounding tissue, and other factors. Ceramics and bioactive glasses have only recently been studied in terms of how their dissolution products affect cell behavior, and further research is needed to completely understand the mechanism by which the cells and these by-products react.¹²⁵

Synthetic Scaffolds

Biodegradable scaffold materials—Polyglycolic acid (PGA) is a simple, linear, aliphatic polyester that was first used as a biodegradable suture. The PGA suture was brought to market under the trade name Dexon. PGA in scaffolds was first introduced in the 1980s, alone as a mesh to investigate renal injury,¹²⁶ and blended with Dacron (polyethylene terephthalate), to study tendon and ligament repair.^{127–129} Large-scale production of fibrous PGA scaffolds with consistent porosity was achieved in the early 1990s, which was used to regenerate cartilaginous tissue.¹³⁰ The degradation rate was studied in vitro, whereby only 30% of the polymer remained after 8 weeks.

The first copolymer mixture to gain approval from the Food and Drug Administration was the mixture of PGA with a more hydrophobic polymer, polylactic acid (PLA). This copolymer, poly(lactic-co-glycolic acid) (PLGA) was first available as a suture material under the trade name Vicryl in 1974. PLGA scaffolds were used in the early 1990s toward engineering bone¹³¹ and liver,¹³² and were famously used in the tissue engineering of cartilage in the shape of a human ear.¹³³ PLGA in a 50:50 mixture has a degradation time of about 8 weeks.¹³⁴ PLGA can also be blended with other polymers as well as natural materials, such as gelatin,¹³¹ which was used to study trabecular bone regeneration.

PLA is another biodegradable aliphatic polyester, more hydrophobic than PGA. There are 2 racemic isoforms, poly-L-lactic acid (PLLA) and poly-D-lactic acid (PDLA). The racemic mixture can be termed poly-D,L-lactic acid (PDLLA) or simply PLA, without indication of which chiral form is present. PLA in scaffolds is usually found in a copolymer mixture (see above), although a few early studies looked at the use of PLA scaffolds for cartilage repair¹³⁵ and nerve regeneration.¹³⁶ PLLA fibrous scaffolds maintained integrity for a 42-day period, during which PDLLA fibrous scaffolds shrunk significantly after only 3 days.¹³⁷

Poly(ϵ -caprolactone) (PCL) is a slowly degrading polymer that was first tested as a bulk material for dermal fibroblast growth.¹³⁸ PCL scaffolds have been used toward tissue engineering efforts in bone, either alone^{139,140} or combined with hydroxyapatite (HA).¹⁴¹ PCL scaffolds are attractive for the longer term, as it degrades over 2 years.^{142,143}

Non-biodegradable scaffold materials—In addition to biodegradable scaffolds, nonbiodegradable scaffolds have also been investigated for tissue-engineering purposes. These materials in some cases can be osseointegrated and are well tolerated by the body.

Polymethyl methacrylate (PMMA) is biocompatible and has been studied for its potential in drug delivery¹⁴⁴ and dermal fillers,¹⁴⁵ but a few studies have been done on its potential as a 3D scaffold,¹⁴⁶ toward bone and cartilage repair,^{145,147} as well as a template for nerve regeneration.¹⁴⁸

Polytetrafluoroethylene (PTFE), more commonly known by its commercial name Teflon, is a polymer made up of repeating carbon and fluorine subunits. It has been extensively studied for its use in vascular grafts.¹⁴⁹ Tissue-engineering efforts using this material outside of vascular work have been sparse, although successful culture of adipocytes¹⁵⁰ and cartilage from the temporomandibular joint have been reported.¹⁵¹

Polydimethylsiloxane (PDMS) is a silicon-based polymer, most commonly used in soft lithography processing of microfluidic devices. PDMS scaffolds have been used in tissue engineering of the heart,^{152,153} bone,^{154,155} liver,¹⁵⁶ and muscle.¹⁵⁷

Other synthetic scaffold materials—Polyethylene glycol (PEG)-based scaffolds are the most widely used hydrogels. These scaffolds have been synthesized as copolymer

solutions and come in variable weights. Poly(2-hydroxyethyl methacrylate) has been used since the late 1960s as a contact-lens material.¹⁵⁸ Studies using the material as a hydrogel for cell encapsulation/tissue engineering began in the 1980s and have been used toward the regeneration of several tissues, including spinal cord/nerve,¹⁵⁹ cardiac tissue,¹⁶⁰ bone,¹⁶¹ and skin.¹⁶² There have been a few published studies of this material and composites in dental applications, discussed in the section on dental pulp regeneration. Polyvinyl alcohol has long been used to investigate islet encapsulation,^{163–166} and has been used as a drug-delivery material¹⁶⁷ and in tissue engineering of the cornea¹⁶⁸ and cartilage.¹⁶⁹

Naturally Derived Materials

Naturally derived scaffolds—There are several naturally derived materials used as either a coating, alone as a hydrogel, or in combination with synthetic materials.

Alginate is a hydrogel comprising 1,4-linked β -D-mannuronic acid and α -L-guluronic acid, typically derived from brown seaweed and also bacteria.¹⁷⁰ The advantages of alginate are its biocompatibility, low toxicity, and slow gelling time (20–60 minutes), depending on the concentration and temperature.¹⁷¹ Disadvantages of the material are the inability to control its degradation rate in vivo and its low viscoelasticity, although this can be improved by increased crosslinking or addition of other substances, such as HA.¹⁷² Several studies using alginate and alginate/HA mixtures have been performed in bone and cartilage tissue engineering.^{173,174}

Agarose is well known for its use in nucleic acid electrophoresis, but it is also a useful hydrogel for cell encapsulation. It has been used in neuronal^{148,175} and cartilage^{176,177} tissue engineering, as well as in composites for engineering of bone¹⁷³ and cornea¹⁷⁸ with HA and fibrin, respectively.

Chitosan is a polymer derived from the deacetylation of chitin, the major component of crustacean exoskeletons. It can be formulated into an injectable hydrogel, and has been used in the study of epithelial wound healing,¹⁷⁹ repair after myocardial infarction,¹⁸⁰ and for intestinal¹⁸¹ and central nervous system¹⁸² tissue engineering. Chitosan is also used as a copolymer with other natural materials^{183–185} and synthetic materials.^{186,187}

Collagen, fibrin, gelatin, hyaluronic acid, and pectin have been used as natural materials in conjunction with one of the other materials described previously, and are discussed here only regarding their contributions toward dental tissue engineering.

Bioceramics and Metals

Bioceramics and metals have long been used as implant materials for joint and tooth replacement. HA is a natural bioceramic constituting various hard tissues such as bone, dentin, and enamel.¹⁸⁸ The HA-based materials have been widely used for dental tissue and bone engineering^{141,145,173,174} and are often used in conjunction with tricalcium phosphate (TCP).^{189–191}

Titanium is the most widely used metal for implants because of its biocompatibility and a capacity to osseointegrate, a beneficial feature for dental implants. Titanium can be coated with various polymers.

3D Organ Printing

3D organ printing involves 3 sequential steps: (1) preprocessing or development of blueprints for organs, (2) processing or actual organ printing, and (3) postprocessing or organ conditioning and accelerated organ maturation. The 3D cell printers can print single

cells or cell aggregates onto the previously printed successive layers of thermo-sensitive gels in a layer-by-layer fashion. These sequential layers are assembled to create the 3D organ.^{192–194} Recently, a new technique termed micro-masonry was introduced for the formation of engineered tissues or organs in 3 dimensions. The shape-controlled PEG microgels are mixed in a prepolymer solution and spread onto the surface of a template made from PDMS. The microgels assemble and closely pack to form a brick-wall-like structure on the surface of the template. The microgels are then illuminated to crosslink the polymer and create a 3D replica of the PDMS template. Cells can be incorporated into the prepolymer solution with a high survival rate ($83.1\% \pm 2.3\%$).¹⁹⁵

SCAFFOLDS IN DENTAL PULP TISSUE ENGINEERING

Humans have long used both natural and synthetic materials as replacements for lost teeth. The earliest known dental implant was made of iron and found in a Roman male, believed to be dated around 200 CE.¹⁹⁶ The first tooth made from a natural material was found in a Mayan woman, estimated around 600 CE, and was made of nacre, or mother of pearl, from sea shells.¹⁹⁷ Although dental implants continue to be used today, more recently tissue engineering has been used to recreate dental tissues. Not surprisingly, both natural and synthetic materials have been explored for this use, each with encouraging results. In dental tissue engineering, a wide variety of biomaterials have been used such as human bone derivatives, natural porous materials, bioceramics, and synthetic polymers.

Synthetic Scaffolds

The most extensively studied scaffold system for dental tooth regeneration is the use of biodegradable PGA scaffolds. The first reported studies maintained human adult dental pulp on a PGA scaffold for more than 60 days in culture.¹⁹⁸ Follow-up studies used PGA scaffolds with human dental pulp and found upregulation of type I collagen, fibronectin, and several BMPs and their receptors, suggesting the capacity of this scaffold to maintain cell vitality and support the differentiation of human dental pulp cells.¹⁹⁹

More recently, mixtures of PGA with both synthetic copolymers and other macromolecules were used for dental tissue engineering. PLGA scaffolds have 2 different pore sizes: 150 to 180 μm and 180 to 300 μm . These scaffolds were evaluated in rabbits using autologous DPSCs and were shown to induce osteodentin formation after subcutaneous implantation for 2 and 6 weeks.²⁰⁰

PGA scaffolds were compared with β -tricalcium phosphate (B-TCP), fibrin, and collagen scaffolds for their capacity to grow dental structures when seeded with tooth germs from 6-month-old minipigs.²⁰¹ On fibrin and collagen gels, the porcine third molar tooth bud maintains its epithelial structure, resembling tooth buds, whereas on PGA and B-TCP, the implanted tooth buds produce more dentin-like material.

The mixtures of PGA fiber mesh scaffolds with porous or nonporous HA/B-TCP were used to seed porcine dental pulp-derived cells and were implanted subcutaneously for 6 weeks. Newly-formed hard tissues were observed in all implants but the dentin-like structure with expression of dentin sialoprotein (DSP), collagen type I, osteonectin, and bone sialoprotein (BSP) was only seen in the PGA-cell implants with porous HA/beta-tricalcium phosphate.²⁰²

PGA/PLLA and PLGA scaffolds were used in pioneering work in which scaffolds were formed in tooth molds, seeded with porcine third molar dissociated tooth buds, and allowed to grow in the omenta of athymic rats. After 20, 25, and 30 weeks, tooth-like structures containing pulp, dentin, and enamel were observed, with surrounding cells expressing BSP

and amelogenin.²⁰³ Similar results were obtained by seeding rat tooth bud cells on both PGA and PLGA scaffolds for 12 weeks in the omentum²⁰⁴ or rat jaw.²⁰⁵

Studies comparing PLGA with HA, B-TCP, or calcium carbonate hydroxyapatite found that human DPSCs proliferated best on PLGA with B-TCP and were able to form mineralized structures. After 4 to 5 weeks, the rat tooth bud cells differentiated and expressed DSP.¹⁸⁹

PCL scaffolds were used for the regeneration of various mineralized tissues such as bone, cartilage, and dentin.^{206–209} The PCL scaffolds support adhesion, proliferation, and odontoblastic differentiation. The incorporation of HA into PCL scaffolds enhances odontoblastic differentiation of human DPSCs.²⁰⁹

PEG is also known as polyethylene oxide or polyoxyethylene. These scaffold materials can support cell growth and differentiation as well as decelerate the degradation of fibrin, thus creating a new hybrid material, PEGylated fibrin gel, for cell delivery.²¹⁰

Naturally Derived Materials

Naturally derived scaffolds—Alginate has been used in dental engineering to deliver cells and/or growth factors. The alginate hydrogel with either transforming growth factor (TGF)- β 1 or acid treatment was applied to slices of human teeth with vital dentin-pulp complex tissues and maintained in culture. Hydrogel with TGF- β 1 or acid treatment, but not the untreated control hydrogel, induced dentin matrix secretion and formation of new odontoblast-like cells in the human tooth slices.²¹¹

Collagens, particularly type I collagen, are major constituents of dentin and have been used to provide a 3D culture environment for various types of cells, including stem cells from the dental pulp.²¹² Compared with other natural scaffold products including gelatin and chitosan, the dental pulp cells cultured in the type I and III collagen gel exhibited a higher degree of odontoblastic differentiation as shown by alkaline phosphatase activity and expression of osteocalcin, dentin sialophosphoprotein (DSPP), and dentin matrix protein 1 (DMP1).^{212–216} Collagen gel can be used alone or in combination with growth factors (eg, TGF- β 1, BMP4, FGF2)²¹⁷ and other scaffold materials such as chitosan.²¹⁸

Chitosan/HA blend (polyelectrolyte complex) was used for compatibility studies with mesenchymal stem cells. In a 2:1 blend (HA/chitosan), cells were viable for 72 hours and no cytotoxicity was apparent.¹⁸⁵ The same group used chitosan/pectin scaffolds for bone regeneration with similarly positive results.¹⁸⁴ Chitosan/collagen scaffolds adsorbed with BMP7 were seeded with human adult dental pulp cells and stained positive for dentin matrix proteins DSPP and DMP1, whereas scaffolds without BMP7 were negative.²¹⁸

Hyaluronic acid sponges were used as 3D scaffolds for the regeneration of dental pulp. In comparison with the collagen sponge, the hyaluronic acid sponge can support cell growth in culture and in vivo from the amputated dental pulp of rat molars, with fewer immunologic reactions as shown by expression of inflammatory cytokines tumor necrosis factor (TNF)- α and interleukin-6, as well as leukocyte infiltration.²¹⁹ However, when used as an injectable hyaluronic acid gel for soft-tissue augmentation, adverse hypersensitivity reactions were reported, due to impurities and bacterial contamination.²²⁰

Fibrin consists of the blood proteins fibrinogen and thrombin, which are produced naturally in the body after injury to establish hemostasis and enhance wound healing. Because of these properties, fibrin glue, fibrin sealant, and fibrin in other forms were produced to aid bleeding control, speed wound healing, cover holes instead of sutures, and provide slow-release delivery of antibiotics or other drugs. Because of their biocompatibility,

biodegradability, simple preparation, and manipulation, fibrin scaffolds have been used for multiple purposes (eg, filling in bone cavities, vascular graft, and repairing injuries to urinary tract, liver, and lung) and are also available as mixtures with other polymers such as fibrin-PEG blend.^{210,221–225} Fibrin hydrogel allows the incorporation of growth factors and bioactive molecules via a heparin-binding delivery system, cell seeding through inkjet printing, and self-assembly through a magnetically influenced technique.²²⁵ Blood clots have been used as natural scaffolds for bone healing in the tooth-extraction socket as well as for dental pulp regeneration/revascularization in immature necrotic teeth. Fibrin glue and platelet-rich fibrin can be prepared from whole blood before surgery. The mixture of these 2 components was used as a scaffold for reassembly of porcine tooth bud cells implanted in the extraction socket. After 36 weeks, these implants developed into a complete tooth or an unerupted tooth crown.²²⁶ The mixtures of fibrin and other polymers such as PEGylated fibrin scaffold aid in handling the material. The PEGylated fibrin scaffold is injectable, tunable, degradable, and compatible with dental stem cells. It induces osteoblastic and odontoblastic differentiation as well as the formation of dentin-like collagenous matrix and vascularized pulp-like structure after transplantation in vivo.²²⁶

Nanostructured Films and Self-assembled Peptides

Recently, investigators have been examining scaffold microtopography and nanotopography as a determinant for successful dentin regeneration. Scaffold nanotopography and molecular self-assembly offer new directions for the fabrication of tissues with similar cell and matrix organization to the native tissues at the nanoscale.²²⁷ This nanotechnology can be used not only for tissue engineering but also for the delivery of antimicrobial and/or anti-inflammatory drugs, which will be beneficial for endodontic regeneration. For example, the PGA scaffold was incorporated with an anti-inflammatory peptide, α -melanocortin (α -MSH). The PGA/ α -MSH scaffold promotes the adhesion and proliferation of human pulp fibroblasts while inhibiting inflammatory responses.²²⁸ In another study, the nanostructured, self-assembling peptides were used as a carrier for the anti-inflammatory drug K5, which inhibits the production of inflammatory cytokines TNF- α and prostaglandin E₂ from macrophages.²²⁹ More detailed studies are needed to evaluate the effects of these peptides on various cell types in the dental pulp and the formation of dentin in a more clinical relevant setting.

SUMMARY

The emergence of tissue engineering and regenerative medicine shed new light on the treatment of patients with degenerative disorders. These approaches combine tools from a variety of fields such as stem-cell biology, biomaterials, and developmental biology. Whereas regenerative medicine places more emphasis on cell-based therapy, particularly stem cells, to repair or replace damaged tissues/organs, tissue engineering focuses on using biomaterials with or without cells to make bioartificial tissues or organs. Various sources of stem cells have been identified and used to generate the desired specialized cell or tissue types. These stem cells are classified into 3 main types: ES cells, somatic/adult stem cells, and induced pluripotent stem cells. In addition, endothelial cells and their paracrine factors such as vascular endothelial growth factor were shown to play important roles in mediating angiogenesis to nurture engineered tissues or organs and facilitate host integration. Other growth factors and bioactive molecules such as those included in the FGF, BMP, Shh, Wnt, and Notch signaling pathways dictate various aspects of tooth morphogenesis and maturation, and thus can be used to guide the formation of engineered tooth tissues/organs in the manner recapitulating development. These key components are summarized in Fig. 3.

Finally, in order to apply stem-cell–based therapies to the treatment of diseases, the appropriate microenvironment must be identified to guide the development of stem cells through the following 6 steps:

1. To increase survival of stem cells in the recipient/transplant.
2. To integrate the transplanted cells into the surrounding tissue without harming the recipient; research strategies must be created to avoid the problem of immune rejection without long-term use of immunosuppressive drugs.
3. To increase the proliferation of stem cells to generate sufficient amounts of tissue.
4. To induce the differentiation of stem cells into the desired cell type(s).
5. To maintain the differentiated cells and retain their functions throughout the recipient's life.
6. To remove unwanted cells.

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KEY POINTS

- Dental caries and periodontal disease are the most common diseases resulting in tissue loss. To replace or regenerate new tissues, various types of stem cells have been identified, including embryonic, somatic/adult, and induced pluripotent stem cells. Somatic and induced pluripotent stem cells can be obtained from teeth and periodontium.
- Endothelial cells and their paracrine factors mediate the formation of vasculature into engineered tissues or organs.
- Growth factors and bioactive molecules dictate various aspects of tooth morphogenesis and maturation and thus can be used to guide the formation of engineered tooth tissues in the manner recapitulating development.
- Various biomaterials can be chosen when designing a scaffold, including synthetic, natural, degradable and non-degradable materials.
- Advances in biomaterial sciences including microfabrication, self-assembled biomimetic peptides, and three-dimensional printing hold great promise for whole organ or partial tissue regeneration to replace teeth and periodontium.

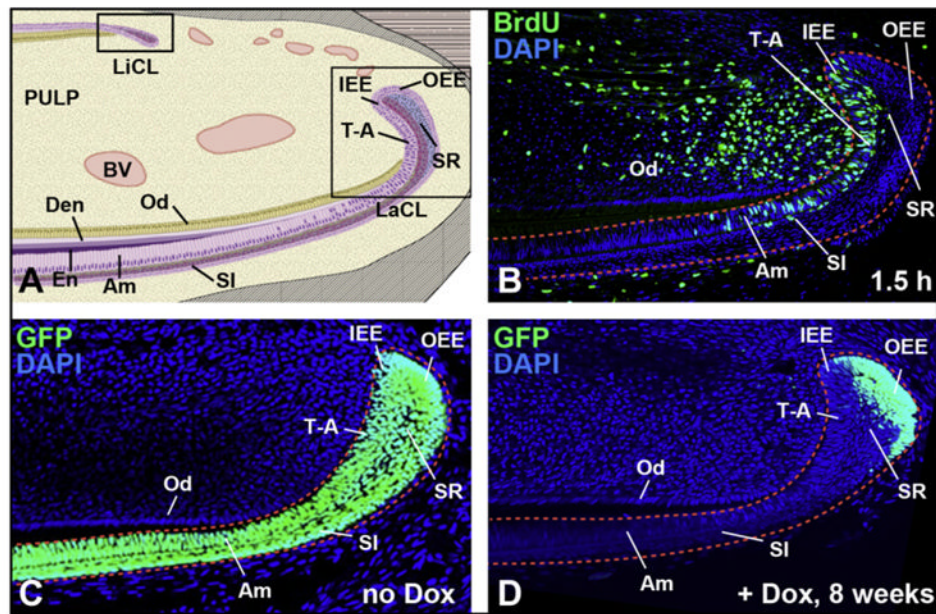


Fig. 1.

Mouse epithelial cervical loop stem cells. (A) Sagittal view of mandibular mouse incisors shows 2 stem-cell compartments in the lingual (liCL) and labial (laCL) cervical loops. The transit-amplifying (T-A) cells and ameloblasts (Am) arise from inner enamel epithelium (IEE), whereas the outer enamel epithelium (OEE) houses the label-retaining cells (LRCs) in the laCL. The LRCs are putative dental epithelial stem cells. D, dentin; E, enamel; Od, odontoblasts; SI, stratum intermedium; SR, stellate reticulum. (B) BrdU labeling (1.5 hours) of rapidly proliferating cells in the T-A region of laCL epithelium and adjacent mesenchyme. (C, D) Images from incisors of *Krt5-tTA*; H2B-GFP mice. In the absence of doxycycline (no Dox; C), GFP is present in all CL epithelial cells expressing *Krt5* including OEE, IEE, SR, SI, and Am. In the presence of doxycycline (+Dox; D) for 8 weeks, H2B-GFP expression was turned off, leading to the retention of GFP in the slowly proliferating LRCs of the OEE.

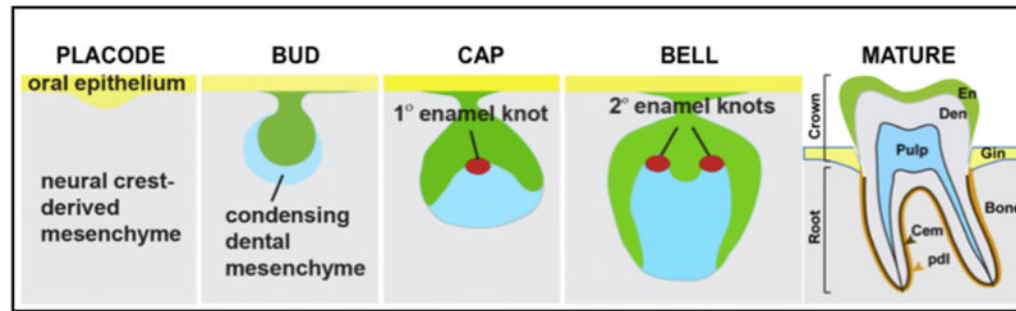


Fig. 2.

Schematic of molar development. (1) Placode stage: thickening of oral epithelium and invagination into the neural crest–derived mesenchyme. (2) Bud stage: neural crest–derived mesenchymal cells condense around the epithelial bud. (3) Cap stage: the primary enamel knot, a signaling center, is formed in the epithelial cap. (4) Bell stage. The secondary enamel knots, the future sites of cusps, are present. At the tip of the future cusps, the dental papilla mesenchymal cells, adjacent to the inner enamel epithelium, differentiate into dentin-producing cells, odontoblasts. Once the odontoblasts lay down the dentin matrix, the inner enamel epithelial cells, adjacent to odontoblasts, differentiate into enamel-producing cells (ameloblasts) and secrete enamel matrix. This process continues until the tooth crown is completely formed. (5) Mature tooth: once the crown is completely formed with mineralized enamel and dentin, the tooth erupts while the roots and periodontal supporting structures are continuously formed until the closure of the apical end of the tooth roots.

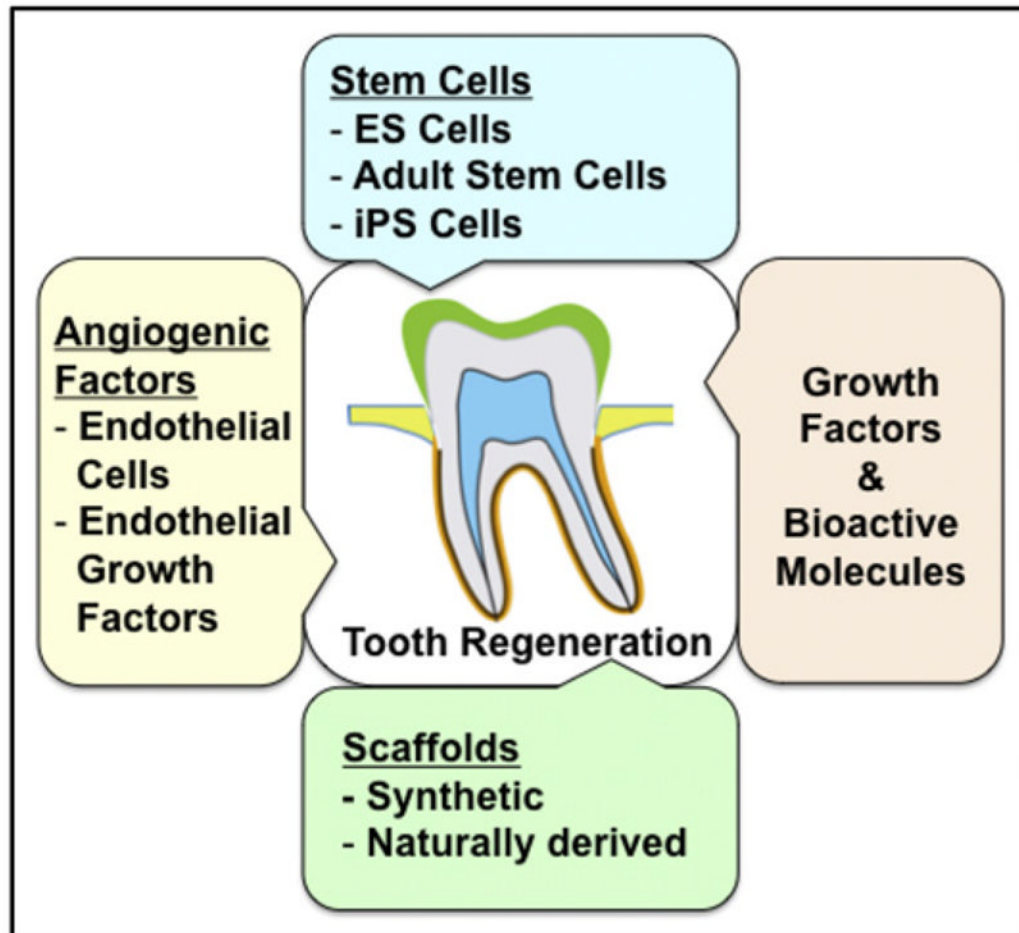


Fig. 3.

Key components of organ/tissue engineering. Key components include (1) stem cells (ie, embryonic stem cells, ES cells; somatic/adult stem cells; induced pluripotent stem cells, iPS cells) or any cells with the capacity to form the desired tissue/organ; (2) angiogenic factors to enhance vascularization of the engineered tissue/organ; (3) growth factors and/or bioactive molecules such as those in the fibroblast growth factor, bone morphogenetic protein, sonic hedgehog, Wnt, and Notch signaling pathways; and (4) scaffold to deliver cells and drugs. Together with embedded growth factors and/or bioactive molecules, the scaffold provides a microenvironment that supports the development and differentiation of stem cells into specialized cells to form the target tissue/organ.