The Crowning Achievement:
Getting to the Root of the Problem

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Martha J. Somerman, D.D.S., Ph.D.

Abstract: An ideal goal of oral-craniofacial dental reconstructive therapy is to establish treatment modalities that predictably restore functional tissues. One major area of focus has been in the area of dental materials with marked improvements in the design of materials used to restore teeth/periodontium/bone lost as a consequence of disease or disorders. With advances in understanding the cell and molecular controls for development and regeneration of tooth structures, it is now possible to consider therapies that promote regeneration of lost tissues, along with replacement of these tissues. This review presents a background on our current knowledge as to the composition of the tooth/periodontium followed by a discussion on successes to date, both in vitro and in vivo, toward regenerating a whole tooth and next steps required to regenerate a functional tooth.

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Oral-craniofacial researchers and clinicians have long applied principles of bioengineering to oral tissue restoration. A major concentration has been in the area of dental materials with improvements seen in materials used to restore tooth structure (crown) in terms of adherence, aesthetics, and endurance and in impression materials used for fabrication of crowns, dentures, partials, and other restorative approaches. In the last decade, significant advances have been made in approaches used to replace missing teeth, including enhancements in bridge design and materials as well as marked expansion and success in the use of implants for tooth replacement, from single abutments to multiple abutments. An alternative approach for replacement of single teeth is auto-transplantation, but this is limited to individuals having teeth for transplantation (e.g., third molars), and further there are mixed reports on the success of this procedure.

Beyond the focus on designing materials to replace lost tissues, substantial progress has been seen in the use of materials/factors to promote regeneration of tissues lost as a consequence of diseases or disorders; however, to date many of these procedures have been of limited success and/or are not predictable. For example, materials have been applied to areas of deep decay, often with pulp exposure, in attempts to repair the damaged tissues (pulp and dentin). In an effort to restore periodontal tissues and/or to increase bone at a specific site, including sites for implantation of teeth, various barrier membranes often coupled with grafts/polymers/factors, etc. have been used with varying success.

Thus, substantial achievements have been made toward improving the safety and efficacy of materials/methods used to restore oral tissues. With the more recent advances in understanding the cell and molecular basis controlling the development and regeneration of tooth structures, the potential for designing predictable therapies is great. The goals of this article are to outline current information as to the proteins and cells associated with tooth structures; describe achievements to date in attempts to use cell/gene/protein therapy to bioengineer a tooth; and finally, discuss possible next steps required to regenerate a functional tooth (e.g. enamel, dentin, pulp, cementum, surrounding bone, and a functional periodontal ligament).

Background

Enamel

Enamel is the most mineralized biological hard tissue in the human body. It is also a tissue that lacks self-healing capabilities. Developmental defects
(pathological/genetic) or damage to this tissue by either mechanical overloading or decay due to poor oral hygiene/disease requires replacement with various synthetic materials: ceramics, metal alloys, or composites. While these synthetic materials post various degrees of success, they bear little resemblance to the enamel tissue in their chemical and structural makeup. Enamel is indeed a complex tissue and deserves a comprehensive understanding of its biominalization process, structure, and physical properties to appreciate the requirements for regeneration of this complex tissue.

**Biomineralization of Enamel.** Enamel is formed via an orchestration of cell signaling coupled with protein-protein and protein-mineral interactions. The result is a unique mineralized tissue with characteristic structural features at several size scales. From the early stage of formation to the final mature tissue, the enamel tissue transforms from a completely organic substance to a mineralized tissue with almost no organic substance. Throughout this transformation process, cellular and molecular events guide the timing of the mineral-to-organic substitution process as well as the structural development of the mineralized phase.

Events that lead to the formation of enamel start with a series of signals at the epithelial and mesenchymal interface involving several signaling protein families: bone morphogenetic protein (BMP), fibroblast growth factor (FGF), hedgehog (Hh), and Wnt (an amalgam of wingless and int) at the budding stage of tooth morphogenesis (Table 1A).8,9 These signaling molecules guide the differentiation of tooth-specific cells: mesenchymal odontoblasts and epithelial ameloblasts, which produce the dentin and enamel matrices, respectively. Initiation of enamel occurs between the cap and bell stages and begins with ameloblasts secreting a matrix at the dentin-enamel junction (DEJ). From this point on, the ameloblasts migrate away from the DEJ and begin secretory function at their basal surface. In the wake of the ameloblasts are the protein matrix and ions necessary for the biomineralization of hydroxyapatite (Hap). At the DEJ, crystals first nucleate within the protein matrix then grow to eventually overtake the enamel space. The matured mineral phase possesses unique structural features identifiable at the nanoscale—closely packed elongated Hap crystallites ~20 nm wide and mesoscale—bundles of Hap crystallites that make up the enamel rods and interrods.10-12 These hallmark structural features of enamel (as opposed to plate-like nanocrystals in collagen matrix as seen in bone, cementum, and dentin) are a consequence of the molecular and cellular control of the mineralization.

**Molecular Control of Mineralization.** Amelogenin, the major extracellular protein matrix of developing enamel, has been shown to be critical for the proper regulation of enamel crystallites.12-14 In *vivo* models of amelogenesis imperfecta that lack the expression of amelogenin exhibit very weak

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<th>Table 1A. Summary of tissue information: the mature enamel complex</th>
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enamel tissue due to low mineral density and lack of proper enamel structure.\textsuperscript{11,13,15,16} In vitro as well as in vivo studies have demonstrated that amelogenin undergoes self-assembly.\textsuperscript{13,17-19} Incorporation of defective amelogenin lacking self-assembly ability, in transgenic mouse models, results in poor organization of amelogenin nanospheres and poorly organized crystal habits at the nucleation stage.\textsuperscript{11}

For the enamel to increase mineral density as it matures, elimination of the protein matrix must take place concurrent with the growth of the mineral phase. Previous studies have shown that ameloblasts secrete proteinases in addition to secreting matrix proteins.\textsuperscript{12,20,21} Several types of proteinases have been identified, with notable ones being matrix metalloproteinase 20 (MMP-20) and kallikrein 4 (KLK4), also known as enamel matrix serine proteinase 1 (EMSP-1).\textsuperscript{20} Degradation of proteins occurs rapidly, leading to the majority of mineral growth occurring after proteins have been digested. The absence of protein enables the enamel crystals to grow without restriction until they are pressed against each other allowing the mineral phase to occupy nearly 100 percent of the enamel space.

**Cellular Control of Mineralization.** The enamel rod structure is dependent on the ameloblasts via Tomes’ process, a process on the basal surface of ameloblasts. The directionality of the enamel rods are regulated by the migratory path of the ameloblasts with the Hap crystallites within the enamel rod oriented approximately 90º from the secretory surface of Tomes’ process. The genetic signals that direct the ameloblast pathways and the relationship between ameloblasts and interrods remain largely unknown,\textsuperscript{10,12} although cellular control of rod and interrod patterns is highly specific. For example, micro-patterning of enamel rods in rodent incisors are significantly different from that of primates.\textsuperscript{18,22}

**Physical and Mechanical Properties.** Matured enamel is more than 95 percent mineral by weight with trace amounts of proteins and water. The mineral by itself is not the one top performing ceramic materials for mechanical loading (for example, silicon carbide, silicon nitride, and aluminum oxide are ceramics with far superior mechanical properties). However, through structural design, the enamel tissue is able to endure repeated loads for long lengths of time. The hardness and wear resistance of enamel, though, inferior to advanced ceramics, are sufficient to withstand long-term masticatory loads. One of the notable structural features is the anisotropic properties of the enamel rods. Mechanical tests have revealed that, given low enough loads, the elongated rods, while brittle and susceptible to cracks, are capable of containing cracks and, therefore, avoiding catastrophic failure.\textsuperscript{23-25} This is an elegant example of nature’s ability to fabricate a mineralized tissue with sophisticated structural design to optimize its intended function of repeated biting and chewing.

**Dentin-Pulp Complex**

Dentin is a living connective tissue with biomechanical properties similar to bone and cementum (Table 1B). Odontoblasts are the cells responsible for dentin secretion and for maintenance of the mature tissue. A series of epithelial-mesenchymal interactions regulate the differentiation of odontoblasts from neural crest cells in the first branchial arch and frontonasal processes\textsuperscript{26} (see also Thesleff\textsuperscript{27} and Thesleff and Mikkola\textsuperscript{28} for a discussion of the signaling molecules and transcription factors advancing tissue differentiation). The first tissue deposited in development, the mantle dentin, includes a thin, superficial layer at the dentin-enamel junction. Mineral deposition begins in the mantle matrix and progresses across the DEJ to contribute to enamel crystal growth.\textsuperscript{29} Underlying the mantle dentin, the intertubular dentin forms the bulk of the coronal and radicular dentin.\textsuperscript{30} Peritubular (intratubular) dentin defines the walls of tubular compartments within the intertubular dentin, and odontoblast cell bodies lining the periphery of the dentin/pulp border extend elongated cell processes into fluid-filled dentin tubules. Although dentin tubules traverse the tissue thickness, the extent of the odontoblast processes within the dentin is subject to controversy.\textsuperscript{31} Dentin structure includes a dense meshwork of collagen fibrils and a wide variety of non-collagenous proteins (Table 1B). The collagen fibers are oriented perpendicular to the tubules and serve as a substrate for mineral crystallites.\textsuperscript{32} This organic matrix constitutes ~20 percent of dentin structure (by wt.) with hydroxyapatite mineralization of the matrix (70 percent) and H\textsubscript{2}O (10 percent) forming the remaining components. The lesser mineralization of dentin relative to enamel confers greater deformability to the dentin and enables it to function in supporting the enamel crown.\textsuperscript{33}

The dental pulp includes the tooth’s living tissues that respond to pain or damage or initiate repair. As in dentin, the pulp cells differentiate from neural crest-derived ectomesenchyme during tooth development. The odontoblast cell layer forms the
Table 1B. Summary of tissue information: the mature dentin-pulp complex

<table>
<thead>
<tr>
<th>The Mature Dentin-Pulp Complex</th>
<th>Dentin</th>
<th>Pulp</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Odontoblasts</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cells</strong>&lt;sup&gt;5,31&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroblasts (heterogenous population)</td>
<td>Undifferentiated mesenchymal cells</td>
<td>Dendritic cells, macrophages, lymphocytes, mast cells</td>
</tr>
<tr>
<td><strong>Origin</strong>&lt;sup&gt;26&lt;/sup&gt;</td>
<td>Neural crest-derived ectomesenchyme</td>
<td></td>
</tr>
<tr>
<td><strong>Structural Organization</strong>&lt;sup&gt;31&lt;/sup&gt;</td>
<td>Mantle, intertubular, and peritubular dentin consisting of collagen fibril matrix. Odontoblast cell processes extend into dentin tubules</td>
<td>Odontoblast, cell-free, cell-rich zones and pulp core</td>
</tr>
<tr>
<td><strong>Mesoscale</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nanoscale</strong>&lt;sup&gt;32&lt;/sup&gt;</td>
<td>Plate-like mineral crystals attached to collagen fibrils</td>
<td></td>
</tr>
<tr>
<td><strong>Proteins in Tissue</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Collagens</strong></td>
<td>COL1 (98%), COL3 (1-2%), COL5 (1%)</td>
<td>COL1 (56%), COL3 (41%), COL5 (2%), COL6 (0.5%)</td>
</tr>
<tr>
<td><strong>Non-Collagenous Proteins</strong>&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Phosphorylated (SIBLINGS), non-phosphorylated matrix proteins, proteoglycans, growth factors, metalloproteinases, alkaline phosphatase serum derived proteins, phospholipids (SIBLINGS: small integrin binding ligand N-linked glycoprotein)</td>
<td></td>
</tr>
<tr>
<td><strong>Regulators of Tissue Formation</strong>&lt;sup&gt;27,28&lt;/sup&gt;</td>
<td>Epithelial-mesenchymal interactions with signaling molecules and transcription factors regulating differentiation</td>
<td></td>
</tr>
<tr>
<td><strong>Organic/Inorganic Ratio</strong></td>
<td>~70% hydroxyapatite, 20% organic, 10% H₂O (by wt)</td>
<td>100% ionic &amp; organic laden fluid</td>
</tr>
<tr>
<td><strong>Mechanical Properties</strong>&lt;sup&gt;32,33,40,101&lt;/sup&gt;</td>
<td>Less stiff than enamel, strong in tension, resists propagation of cracks</td>
<td>Soft connective tissue conferring viscoelasticity to dentin-pulp complex</td>
</tr>
<tr>
<td>Nanoindentation hardness:</td>
<td>0.77 ± 0.14 GPa</td>
<td></td>
</tr>
<tr>
<td><strong>Function(s)</strong>&lt;sup&gt;31&lt;/sup&gt;</td>
<td>Support of the enamel crown</td>
<td>Response to pain, damage, repair</td>
</tr>
<tr>
<td><strong>Other Tissue Interactions</strong>&lt;sup&gt;29,31&lt;/sup&gt;</td>
<td>Dentin crystallites extend across DEJ</td>
<td>Nerve fibers extend into odontoblast cell layer and tubule, highly vascularized</td>
</tr>
<tr>
<td>Not vascularized, or directly innervated although nerve fibers extend into tubular compartment</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The outer boundary of the pulp and is closely associated with an underlying network of dendritic cells. A cell-free zone occurs underlying the odontoblast cell layer and includes nerve fibers and blood vessels passing from the deeper pulp layers to the odontoblasts. Capillary loops and nerve fibers can form close associations with odontoblast cell bodies, and some nerve fibers extend into the dentin tubule. The cell-rich zone occurs directly beneath the cell-free zone and includes more dendritic cells, as well as the main matrix-producing cells, a heterogenous population of fibroblasts. These cells, as well as undifferentiated mesenchymal stem cells, occur in the deeper layer of the pulp core.<sup>5,31</sup> Existing data provide evidence that the dental pulp contains a stem cell population (DPSCs).<sup>34,35</sup> In combination with BMP-2, DPSCs have been found to differentiate into dentin-secreting odontoblasts, offering great potential for clinical dentin repair/regeneration.<sup>36</sup> Additional cells of the pulp core function in pulpal defense, such as macrophages, lymphocytes, and mast cells also reside in this deeper layer. The pulp core is also the origin of larger blood vessels and nerves. As in dentin, the pulp matrix includes a variety of collagenous and non-collagenous proteins (Table 1B)<sup>2</sup>, but lacks the mineralization of the overlying tissue. Dentin sensitiv-
ity and pain are attributed to movement of fluid within
dentin tubules and triggering of closely associated
nerve fibers. The pulp can respond to dentin tissue
damage either by secreting new dentin with mem-
bers of the original odontoblast population or, if the
original cells have been impaired, with the genera-
tion and secretory activity of a secondary popula-
tion of odontoblast cells.

Periodontium

The periodontium consists of tissues support-
ive of the tooth crown—namely, cementum, peri-
odontal ligament (PDL), and alveolar bone. It is a
unique environment in the body in that there are three
mineralized tissues in close proximity: bone, cemen-
tum, and root dentin, as well as a non-mineralized
interface, the PDL. Table 1C summarizes the three
tissues of the periodontium.

Cementum. Cementum is a thin mineralized
layer that covers the root dentin. Developmentally,
as root dentin is formed, cementum is deposited in
step on the surface, effectively sealing the dentinal
tubules. Root dentin and cementum may be stimu-
lated by signals from the epithelial root sheath (ERS),
an apical extension of the enamel organ that disinte-
grates around the time of root formation, leaving a
few select cells to become cell rests of Malassez that
lie within the PDL space. The first cementum laid
down in the cervical portion of the root is acellular.
More apically, toward the root tip, cementum be-
comes both thicker and more cellular. Cementoblasts,
the cells responsible for synthesizing the extracellu-
lar matrix (ECM) and promoting mineralization of
the cementum, become entrapped within the matrix
of this thicker layer and are then termed cemen-
tocytes.

Cementoblasts are thought to be derived from
neural crest cells, making cementum an ecto-
mesenchymal tissue, as are the other two tissues of
the periodontium: the PDL and alveolar bone. The
more immediate precursors for all of these periodon-
tal tissues are proposed to be the dental follicle cells.
While the dental follicle region disappears as tooth
formation proceeds, cells retaining pluripotential
properties reside in the periodontal ligament, where
they may be available to differentiate and repair dam-
age to the periodontium in adult tissues. A compet-
ing hypothesis maintains that cementoblasts are the
result of an epithelial-mesenchymal transformation
event that ERS cells undergo.

Like dentin and bone, cementum is a collag-
enous mineralized tissue that hardens upon forma-
tion of carbonated hydroxyapatite. Compared to the
other tooth mineralized tissues, cementum is similar
in hardness to bone and dentin, but less hard than
tooth enamel.

While cementoblasts are phenotypically simi-
lar to osteoblasts, and cementum resembles bone in
mineral composition and hardness, cementum is not
known to undergo any significant turnover during
the lifetime. In external tooth root resorption, repara-
tive material may be formed; however, it is unclear
whether this is true cementum or a more bone-
or dentin-like material. Cementum, in fact, seems to be
highly resistant to resorption by osteoclastic cells
compared to the adjacent bone, and while cases of
idiopathic root resorption are observed in humans,
they are relatively rare.

Periodontal Ligament (PDL). PDL is a space
interlying the cementum and alveolar bone, a replace-
ment of the follicle region surrounding the develop-
ing tooth in cap and bud stages of development. At
about the same time as the disintegration of ERS and
the initiation of cementum formation, fibroblasts and
collagen bundles reorganize to orient perpendicular
to the root surface. Fibers inserted into the cemen-
tum layer may be of follicle origin (termed Sharpey’s
fibers) or cementoblast origin (in cellular intrinsic
fiber cementum). The PDL matures during tooth
eruption, preparing to support the functional tooth
for the occlusal forces in store. In the mature PDL,
major collagen bundles (principal fibers) span the
entire PDL, embedding in both cementum and al-
veolar bone. Fibers are arranged in specific orienta-
tions to maximize absorption of the forces to be
placed on the tooth during mastication.

Mature PDL contains mostly periodontal fibro-
blasts, as well as stem cells capable of becoming fi-
broblasts, osteoblasts, or cementoblasts. The origin
of these stem cells includes a small population of
cells within the PDL region, as well as cells associ-
ated with the blood vessels and bone marrow in the
local region (stromal stem cells). While containing
cemento- and osteo-progenitor cells, the PDL seems
to be quite resistant to mineralization, a necessary
defense to protect against narrowing of the PDL space
from infringement by the cementum or bone on ei-
ther side. Occasionally, as a result of pathologic con-
ditions, PDL space is lost to overzealous mineral-
ized repair tissues, which may even extend over the
entire space, to cause ankylosis between cementum.
### Table 1C. Summary of tissue information: the periodontium complex

#### The Periodontium Complex

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cementum&lt;sup&gt;145&lt;/sup&gt;</th>
<th>PDL</th>
<th>Bone&lt;sup&gt;43,45&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cells</strong></td>
<td>Cementoblasts</td>
<td>Fibroblasts (heterogeneous population)</td>
<td>Osteoblasts</td>
</tr>
<tr>
<td></td>
<td>Cementocytes</td>
<td>Undifferentiated progenitor (stem) cells?</td>
<td>Osteocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell rests of Malassez (remnants from ERS cells)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cementoblasts</td>
<td>Monocytes, macrophages</td>
<td>Osteoclasts</td>
</tr>
</tbody>
</table>

#### Cell Origin

Neural crest-derived ectomesenchyme; potential stem cells in PDL for repair

#### Structural Organization

| Acellular extrinsic: Fibers (collagen) interface with cementum and bone, typically in bundles |
| Cellular intrinsic: Interstitial spaces contain blood vessels and nerves |
| Mixed intrinsic and extrinsic: apical and furcations Ground substance contains amorphous material |
| Acellular afibrillar: patches over CEJ Size 150-380 µm |

#### Proteins

| Collagenous proteins: COL-1 (90% of organics), COL-3, COL-12 | Collagenous proteins: COL-12, COL-1, COL-3 | Collagenous proteins: COL-1 (90% of organics), COL-3, COL-12 |
| Noncollagenous proteins: Extracellular matrix proteins (ECM) | Noncollagenous proteins: Many in ground substance | Noncollagenous proteins |

#### Regulators of Tissue Formation

Growth factors, adhesion molecules, epithelial factors, collagens, Gla proteins

#### Organic/Inorganic Ratio

50-55% organic, 45-50% hydroxyapatite Nonmineralized, elastic 30-35% organic, 60-65% hydroxyapatite

#### Mechanical Properties

Similar to dentin but more compliant. Nanoindentation hardness: 0.64 +/- 1.0 GPa<sup>104</sup>

Viscoelastic, highly compliant

Similar to dentin but more compliant. Nanoindentation hardness: 0.53 to 0.66 GPa<sup>41</sup>

#### Function(s)

Seals surface of root dentin Anchors tooth to alveolar bone via PDL Supportive Sensory Nutritive Supportive of teeth

#### Interacting Tissues

Embedded collagen (Sharpey’s) fibers continuous with PDL Interacts with both cementum and bone via embedded Sharpey’s fibers Embedded collagen (Sharpey’s) fibers continuous with PDL No blood or nerve supply Rich nerve and blood supply Blood and nerve supply
and bone. PDL cells seem to be regulated by mechanical forces, with cell shape and protein production affected by forces.

**Alveolar Bone.** Alveolar bone is the primary support tissue for the mature tooth. While similar in many ways to other bony tissues of the body, alveolar bone is anatomically complex and very responsive to masticatory forces, with the ability for rapid turnover in response to demands.

During development, tooth buds form within bony crypts that lie in the jaw, and at about the time of root formation, teeth move axially and erupt through the overlying bone and gingiva to assume their final position in the oral cavity. For eruption to occur, osteoclasts must be prompted to resorb bone in the eruption pathway to make way for the tooth to emerge. Alveolar bone crests remain between the erupted teeth, and Sharpey’s fibers spanning the PDL insert into the bundle type bone lining the tooth socket.

Like other bones of the body, cells of alveolar bone mainly consist of osteoblasts, which are actively secreting matrix for bone formation, and osteocytes, mature cells that reside in the lacunae of the bone and extend long cell processes through canaliculi. Upon maturation, osteoblasts may also become bone-lining cells that cover quiescent bone surfaces. The other major cell type is the osteoclast, responsible for bone resorption by the release of hydrolytic enzymes and acids in a localized compartment under the osteoclastic cell. Osteoclasts are derived from the monocyte/macrophage lineage and mature and differentiate in response to molecular signals given by other cells. Osteoblast and osteoclast activities are typically coupled, but in pathologic conditions such as periodontal disease, activities of one cell type may overwhelm the other.

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**Success to Date**

The potential for generating tooth structure outside of the oral cavity was observed in early experiments transplanting dental soft tissues to new growth environments. As early as the late nineteenth century, isolated and transplanted dental pulp demonstrated dentin formation. The importance of epithelial-mesenchymal interactions in tooth development was uncovered when enamel formation was found to depend upon close apposition between ameloblast and odontoblast layers. Furthermore, numerous studies in the early twentieth century developed techniques for culturing both isolated dental tissues and whole teeth, establishing the basis for future experimental manipulation of dental tissues (see Glasstone for a review).

More recent experiments used the technique of isolating and recombining dental tissues to characterize the regulatory role of epithelial-mesenchymal interactions in tooth formation (Figure 1). Epi-

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**Figure 1. Epithelial/mesenchymal interactions**

Epithelial and mesenchymal tissues are dissociated from cap stage developing incisors and molars; incisor epithelium is recombined with molar mesenchyme in order to test the inductive capacities and competencies of the respective tissues. The resulting transplant develops a molar morphology with enamel and dentin deposition, demonstrating the capacity of molar mesenchyme to induce crown shape and advance tissue differentiation.
Epithelial and mesenchymal tissue layers were dissociated in teeth of different developmental stages or tooth types, recombined in different combinations, and grown as in vitro cultures or as transplants. The developmental advancement of the recombinant tooth germ was used to test predictions regarding the time-dependent inductive capacities and competencies of the epithelium and mesenchyme. The ability of oral epithelia to initiate tooth development was shown when recombination of mandibular arch epithelia, prior to the bud stage, with “non-dental” mesenchyme from the second branchial arch resulted in viable tooth germs. Recombination experiments using cap and bell stage tissues showed that the inductive potential switches to the mesenchyme in later tooth development. When molar epithelium was recombined with incisor dental papilla, an incisiform tooth germ developed and vice versa. The mesenchymally derived dental papilla was thus found to “instruct” tooth morphogenesis and determine crown shape in addition to promoting epithelial differentiation. The dental papilla’s inductive potential was further shown in tooth germ formation from recombined dental mesenchyme, also from later tooth development, with non-dental epithelia, such as mouse foot epithelium and chick oral epithelia. Further experiments dissociated epithelial and mesenchymal cells prior to recombination, and the resulting tooth structures established that the tooth forming potential of epithelial and mesenchymal tissues did not depend upon the organization of cells in a tissue layer.

Significant advances have been made in defining the molecular basis for the regulation of tooth development through epithelial-mesenchymal interactions. Both epithelial and mesenchymal tissue layers have been found to express signaling molecules that trigger new gene expression at progressive developmental stages. The reciprocal and sequential nature of these inductions has been found to be the basis for advancing differentiation of dental tissues. (See http://bite-it.helsinki.fi/ for a comprehensive list of signaling molecules and target genes expressed during odontogenesis.) A condensation of cells within the enamel organ, known as the enamel knot, has also been shown to secrete signaling molecules that mediate formation of tooth crown shape. Additionally, the organization of the dentition into incisors, canines, premolars, and molars is established through fields of molecular expression in the oral epithelium early in development.

Our understanding of mechanisms for tooth development is now being applied in dental tissue-engineering toward regeneration of teeth. One line of investigation includes isolation of epithelial and mesenchymal cells from developing pig and rat molars and seeding of cells on biodegradable scaffolds. After a period of growth in an in vivo host environment, the implants from both animal models demonstrated the presence of dentin, enamel, and pulp tissues and showed epithelial root sheath structures (Figure 2). Putative cementum formation was also reported; however, it was not clear whether this tissue was correctly positioned or adherent to the adjacent dentin. Such work makes use of the age-specific nature of cell-signaling that directs epithelial and mesenchymal tissue differentiation in selected cells for scaffold seeding. For example, pulp

Figure 2. Engineering tooth formation
Epithelial and mesenchymal cells are dissociated from developing teeth and seeded on a biodegradable scaffold. After a period of in vivo growth, tooth implants develop enamel, dentin, pulp tissue, and epithelial root sheath structures.
and enamel organ cells are used in the regeneration of implants from pig tissue, and epithelial and mesenchymal cells comparable to the cap stage are used from rat tissue. In both cases the seeded cells are sufficiently mature to continue the reciprocal inductions initiated in early tooth formation prior to tissue collection and cellular dissociation, but have not fully differentiated or begun hard tissue apposition. These studies resemble earlier tissue recombination studies in the pairing of epithelial and mesenchymal tissues in an in vivo growth environment, but are novel in the use of a biodegradable scaffold for cell seeding. The growth and differentiation of dental tissue cells in the scaffold environment circumvent the enamel knot’s mediation of developing tooth shape and establish the differentiating cells and developing hard tissues on the scaffold’s predetermined form. In this way, the potential exists for tissue engineers to design live implants with morphological characteristics specific to individual needs.

A second line of investigation has introduced stem cell lineages as building blocks for tissue-engineered tooth implants. Rather than using cells from developing dental tissues in implant formation, mouse stem cells from embryonic, neural, and bone marrow origin have been investigated for tooth-forming potential. Pellets of each stem cell type were overlaid with oral epithelium, cultured in vivo, and subsequently analyzed for expression of molecular markers of tooth development. Thus, these experiments rely on the early oral epithelium’s capacity for initiation of tooth formation to test stem cell types for competency in responding to odontogenic signals. Each stem cell type responded by expressing odontogenic genes, and epithelial recombinations with bone marrow-derived cells formed teeth, including enamel, dentin, pulp, and surrounding alveolar bone. This work is significant in expanding the scope of cell types potentially available with which tissue engineers can develop live implants. The use of molecular techniques to verify odontogenic induction of stem cells represents a new means of monitoring the efficacy of tooth engineering efforts.

Research efforts in adult stem cell biology offer additional approaches to the engineering of individual dental tissues, as well as whole teeth. For example, continued crown development stem cells in enamel organ epithelia have been identified within the cervical loop of the continuously growing mouse incisor. Mouse dental pulp tissue has been found to contain stem cells capable of regenerating a dentin-like tissue, and bone morphogenetic protein 2 (BMP2) has been found to direct pulp stem cell differentiation into odontoblasts. Such advancing knowledge of adult stem cell differentiation presents the clinical possibility of in vivo dental tissue formation or repair. In a similar fashion, it has been recognized that cells within the adult periodontal ligament (PDL) contain a population of stem cells that, when triggered appropriately, are capable of differentiating toward cementoblast/osteoblast-like cells and of secreting osteoblast-cementum-PDL-like tissues in vitro and in vivo.

Further, mesenchymal stem cells within the adult bone marrow have multilineage potential in vivo and in vitro, including differentiation along the osteoblast cell lineage. This knowledge prompted researchers and clinicians to focus on developing strategies for improving the success and predictability of “periodontal regenerative” therapy, defined as regeneration of cementum, bone, and a functional PDL. Some successes have been achieved in this area, including: a) excluding epithelial cell migration during wound-healing; b) use of graft materials, and c) use of growth factors and enamel matrix derivatives. Clinical outcomes, however, are often disappointing. Studies using animal models of periodontal disease have shown some moderate success using gene therapy (BMPs; PDGF), cells (cementoblasts), and proteins incorporated into scaffolds. For reviews see Saygin et al., Cochran and Wozney, Bartold et al., Wikesjö and Selvig, Ripamonti and Reddi, and Lynch et al.

In summary, there have been positive outcomes, and in fact greater clinical success, with therapies targeted at regeneration of periodontal tissues versus enamel and dentin. Similarly, at the research level, using cells in vitro and animal models in vivo, impressive data exist demonstrating the potential for regeneration of enamel, dentin, cementum, bone, and ligaments; however, there has been limited success with regard to forming the “complete tooth complex.” Possible reasons for limited success to date and strategies for forming the complete tooth complex are the subject of the next section.

**Why No Root?**

While many exciting successes have been realized in the area of de novo tooth building in vitro and in vivo, there has yet to be a report of successful
root formation in these experiments. Here, we would like to address the issues associated with the failure to form a fully functional tooth, that is, one with a completely formed periodontal support system, and then provide some potential solutions to this dilemma, that is, “rooting the crown.”

Efforts to bioengineer root structures have generally not been reported, although attempts at cultivating various periodontal tissues in vitro have met with some success and have shed some light on what ingredients may be missing from studies achieving crown but not root structures. Thomas and Kollar combined murine ERS cells with differently aged dental papilla and showed the ability of the ERS to induce papilla cells to differentiate to odontoblasts and form dentin.78 The authors further demonstrated that day 18 (from vagina plug date) papillae were required for root dentin formation; any younger tissue produced bony mineral instead, indicating that before d 18 the cells were not primed to produce dentin. MacNeil and Thomas, in a series of elegant experiments, showed clearly that cells from dental follicle tissue could produce cementum-like mineralized tissue on the surface of dentin fragments.77 The presence of a basement membrane on dentin was a prerequisite for the formation of an adherent cementum and that epithelial cells may undergo a mesenchymal transformation to become cementoblasts.38,39

Results from in vitro studies demonstrate the potential for dental follicle-derived cells to differentiate to the required cells for periodontal formation. We and others have shown that murine cementoblasts that promote mineral formation in vitro can correlate to in vivo mineralization, including some limited success in forming cementum on root surfaces, when added back to mice in a scaffold.79,81 Dental follicle cells, when harvested at the correct age or even into adulthood, may contain a stem cell population with potential to differentiate to PDL fibroblasts, cementoblasts, and osteoblasts of the nearby alveolar bone,34,42 and numerous in vivo and in vitro studies have supplied evidence of this mesenchymal lineage.82-84 A competing theory contends that ERS cells are responsible for acellular and possibly cellular cementum and that epithelial cells may undergo a mesenchymal transformation to become cementoblasts.38,39

These small successes in forming root tissues in vitro inspire continued efforts. They instruct that in the engineered tooth structures where crowns but no roots are formed, some crucial factor is missing or sufficiently altered to prevent roots from forming. We will discuss three possibilities for this failure: 1) inappropriate or lack of cell signaling factors; 2) altered developmental timing; and 3) inappropriate spatial relationship or organization of cells/tissues (Figure 3).

**Cell-Signaling Factors**

While many signaling molecules for crown development have been identified,8 relatively few regulatory signals for root development are currently known. As described above, crown development relies on reciprocal and reiterated molecular signals between epithelium and mesenchyme to control morphogenesis and development.27 Epithelial signaling centers, such as the dental placode and enamel knot(s), appear at key times to deliver the correct cues to advance morphological development. If epithelial-mesenchymal (E-M) interactions play a role in forming the tooth root, then ERS is one likely source for epithelial signaling. An apical extension of the epithelium, ERS is a structure composed of inner and outer enamel epithelium that undergoes a morphological change by bending inward during root formation, and then it disintegrates when root formation is completed.85 Recent studies have begun to identify potential molecular signals (BMP2, BMP4, Msx2) originating from ERS that may influence root formation, using immortalized cells and in vitro culture systems.86,87 While BMP and Msx signaling seem to operate differently in root versus crown formation, there is some suggestion that Msx2 and BMP4 interaction may be involved in regulation of root morphogenesis.88

In support of accumulating evidence for E-M interactions in root development, amelogenins and amelogenin-like molecules have been reported to have signaling properties for cells of the periodontium and also pulp cells.89-93 In addition to their suggested role as signaling molecules, it is well established that, during tooth development, meloblast matrix proteins, including amelogenins and their splice products, play major roles in regulating crystal growth.11,15 Following enamel maturation, they constitute only about 1 percent of this tissue. While there has been a long-standing hypothesis of amelogenin as a signaling molecule in development
of the periodontium, confirmatory data are needed. Interestingly, a clinical treatment for regenerating tissues lost to periodontal disease, Emdogain (Straumann, Sweden), presumes this epithelial-mesenchymal crosstalk and utilizes an amelogenin-rich porcine protein slurry with some degree of success in regenerating or repairing damaged periodontal tissues in adults.95 Another consideration for signaling is that expression of amelogenins may not be limited to ameloblasts, because there has been some evidence for transitory expression in odontoblasts during crown development.94,96 Problem: A signaling relationship between ERS and/or odontoblasts and periodontal progenitor cells may be necessary for root development to proceed, but the models being used in attempts to develop a whole tooth are not providing the required signals. Potential Solution: Identify putative signaling factors, temporally and spatially, and test in existing models.

In the studies referenced above, putative ERS formation was observed in the bioengineered tooth tissues.95 However, if one accepts ERS to be the epithelial signaling center, its mere presence may not be enough to actually facilitate proper signaling and response by cells of the follicle. During the time just prior to initiation of root formation, the ERS tissue becomes interrupted and eventually disintegrates completely, leaving only small epithelial cell rests (of

Figure 3. Crown meets root

Successes in tooth crown and root tissue engineering and potential problems thwarting the formation of crown and tooth together in engineering experiments:

I. Crown Success. As described in the text, there have been some successes in using mixed cell populations (epithelial and mesenchymal cells), as well as tooth buds in various model systems to regenerate crowns. The insert is an enlargement of the ERS region and depicts the various aspects of the ERS region during active crown development. Thesleff et al.97,98 have proposed that this region contains the “epithelial stem cell niche,” allowing for continuous formation of crowns noted in rodent incisors and molars of the vole. It is possible that failure to observe root formation under current attempts to build a “complete” tooth are related to inappropriate disintegration of this region and subsequent turning-off and/or switching to the required signals for root development (note “?” in figure).

Abbreviations: oee—outer enamel epithelium; iese—inner enamel epithelium, sr—stellate reticulum (and stratum intermedium)

II. Root Success. There have been some successes in developing roots or at least formation of new cementum, bone, and a functional PDL both in vitro and in vivo. Depicted here is the developmental model that yielded a successful root as reported by MacNeil and Thomas.77,78 They noted that without the influence of the basement membrane (i.e., epithelial signal) no PDL was present. Follicle cells were required for cementum formation.
Malassez). This breakup may be important in a spatial sense, as it allows access for the putative ectomesenchymal cementoblast precursors to contact the forming root dentin and lay down the early cementum layer. One possibility is that if ERS is improperly formed or not signaled to begin disintegration, then root progenitor cells may be refused the necessary access and root formation will be absent. In the earlier study by MacNeil and Thomas, ERS fragments were recombined with dentin, possibly allowing infiltration of cells from the overlying follicle tissue to come into contact with dentin surface and successfully form cementum- and PDL-like tissues.78

While we have been discussing ways in which root development may mimic crown development in the realm of cell signaling, an alternative but not necessarily mutually exclusive view emphasizes the differences between crown and root, that is, the absence of signals associated with crown development may be necessary for root development. Putative signals in this case have been identified as Notch and FGF10. These and other signals specifying continued crown development may need to be shut off in order to initiate root development. In essence, the epithelium may reach a juncture where it must pursue a dedicated “crown fate” or a “root fate.”97 It is unclear how significant this may be in tooth regeneration in humans, but provides an interesting lead for future studies. Problem: ERS in existing models does not disintegrate appropriately, leading to spatial/signaling alterations. Potential Solution: Further studies elucidating the signaling taking place within the ERS region in bioengineered tooth structures may supply clues as to whether signals necessary for initiation of root formation are disregulated or absent. A first step would be examining putative ERS and cementoblasts and any surrounding follicle tissue or bone (when formed) for signals (BMPs, Msx) and matrix proteins known to be expressed during root formation.

Developmental Timing

There are two aspects of developmental timing that we would like to approach. First, are bioengineered tissues grown long enough to form roots before they are harvested for histology? Tooth engineering studies have ranged from days to at least thirty weeks in length. While this explanation is a possibility, we feel it is not the most likely reason. A straightforward time course experiment would answer this question and indicate the minimum length of time for growing complete bioengineered teeth with roots.

The second aspect of timing has to do with the age and history of the tissues used. One lesson gained from crown development is that cells respond differently to the same signal at varying stages of development, and thus the history of the cell may determine its competence to receive and respond to new signals. So, a misstep along the way may render a cell unable to respond appropriately at the correct time. One example already discussed in this article is the experiment by Thomas and Kollar, in which the authors demonstrated that an age of eighteen days is required for papilla to be able to differentiate to odontoblasts.76 The various bioengineering studies have taken different approaches for selecting epithelial and mesenchymal tissues, and it is possible that tissues and cells collected may be too early to respond appropriately for initiation of root formation. Cells at the bell and cap stages, and similar mesenchymal and epithelial primordial tissue elements, theoretically should be able to signal and respond for root formation, just as they have successfully formed crown tissues in the studies outlined above. Problem: The lack of root formation may indicate some fundamental difference in root signaling vs. crown signaling, or may indicate that by isolation and recombination of tissues, some necessary signal for root formation is prevented or interrupted. In other words, the cell history has been interrupted. Potential Solution: Identify factors controlling root development and add these to existing models using appropriate temporal/spatial cues.

Spatial Relationship and Organization

A third category to contemplate overlaps somewhat with the previous two, but also adds to and integrates these ideas: the quality of organization of tissues in recombined and engineered teeth. In current models, the ERS, papilla, and follicle cells and/or their stage of maturation are not appropriate for root formation and/or the required epithelial components may not be available, or ERS may not disintegrate in a timely fashion. Earlier studies, while aiming at much simpler goals than recreation of a tooth, highlighted the importance of having the required correct cells/tissues, in the correct place. The orientation of dentin, ERS fragments, and dental follicle...
may have been key in successful formation of PDL and cementum-like material in the study of MacNeil and Thomas.77 Problem: Are all the necessary cells and tissues present and in the correct place? Potential Solution: The many questions that arise and cannot readily be answered on this subject emphasize the lack of knowledge about this aspect of tooth periodontal development. Tooth engineering studies could benefit greatly from more experiments like those of Kollar, MacNeil, and Thomas, focusing on what tissues are really required, what relationship they have, and how they must interact with one another to successfully form a functional periodontium.

With existing molecular tools now available to identify specific factors and signaling molecules, substantial advances toward defining the missing events/factors required for forming the “complete tooth” should be possible.

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REFERENCES

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