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Bioengineered Teeth from Tooth Bud Cells

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Prospects for bioengineered dental tissue repair and regeneration therapies

The ability to obtain and manipulate postnatal tissues easily from individuals to generate biologic replacement tooth materials, such as dentin, enamel, and periodontal ligament, or, even better, replace teeth of predetermined size and shape entirely, is extremely valuable. Dental tissues exhibit little to no regenerative capabilities [1]. Small amounts of reparative dentin can be induced to form in response to subtle tooth injury [2–4], and cementum also exhibits limited regenerative capabilities [5]. In contrast, enamel exhibits no regenerative capacity, because progenitor dental epithelial cells that form enamel lose this ability well before tooth eruption [6]. Because individual teeth generally do not last the lifetime of individuals without requiring at least some repair—cavity filling, root canal, crown, or, at worst, extraction—the need for replacement teeth and dental tissue repair therapies is significant. As the close association between oral health, systemic health, and nutrition becomes more apparent [7], the necessity of proper oral health for long-term quality of life becomes more appreciated [8,9].

Exacerbating the nonregenerative nature of natural tooth tissues, a range of circumstances threatens the health and longevity of teeth on a regular

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basis. The risk for sustaining tooth injury is prevalent due to a variety of factors, including injuries obtained in sports and accident-related trauma [10], oral and other cancers, cancer treatment therapies in children and elderly populations [11,12], periodontal disease, and diabetes [13]. In addition, parafunctional habits, such as teeth grinding or clenching, and everyday chewing of foods, including soft foods, such as bread, and, in particular, hard foods, can result in chipped or cracked teeth. The high susceptibility of teeth to damage, combined with the nonregenerative nature of dental tissues, emphasizes the need for replacement tooth therapies. Until the present time, the fields of restorative dentistry and materials sciences have combined efforts to produce a variety of synthetic materials for use in the restoration of damaged dental hard tissues. Although these materials and therapies have proved effective, they do not exhibit the same mechanical and physical properties as naturally formed dentin and enamel. Differences in the physical properties of synthetic versus natural tooth tissues can result in uneven wear of synthetic and natural tooth tissues over time, resulting in unanticipated stresses on opposing and adjacent teeth. The somewhat incompatible physical properties of synthetic and natural tooth tissues can contribute to compromised oral health, which in turn can result in systemic health issues. Oral tissue infections and associated nutritional deficits can lead to imbalances in oral flora populations, eventually contributing to compromised overall health and reduced quality of life [14].

A tissue engineering approach to dental tissue regeneration

Based on recent reports indicating significant progress in bioengineering a variety of adult hard and soft tissues, the authors tested the ability to use a similar approach to bioengineering dental tissues. Using successful techniques of bioengineering neonatal intestine [15,16] and stomach [17], the authors used immature tooth bud tissue, enriched in dental progenitor cells, to seed biodegradable scaffolds that then were implanted in a host animal to provide sufficient vascularization of bioengineered tissues (Fig. 1). When the implants were harvested and analyzed after 25 to 30 weeks of growth, in many instances, the dissociated tooth bud cells had reorganized into what appeared to be small, anatomically correct tooth crowns with rudimentary tooth root structures (Fig. 2). Molecular and cellular analyses of bioengineered tooth tissues generated from pig [18] and rat tooth bud cells [19] demonstrate that developing bioengineered tooth crowns express the same genes and proteins found in naturally formed teeth [20]. The demonstration that a tissue engineering approach could be used to regenerate dental tissues is promising, suggesting that clinically relevant therapies based on this approach could be used to repair or regenerate dental tissues and whole teeth.

The current task is how to perfect tooth tissue engineering techniques, such that bioengineered dental tissues and whole teeth are integrated

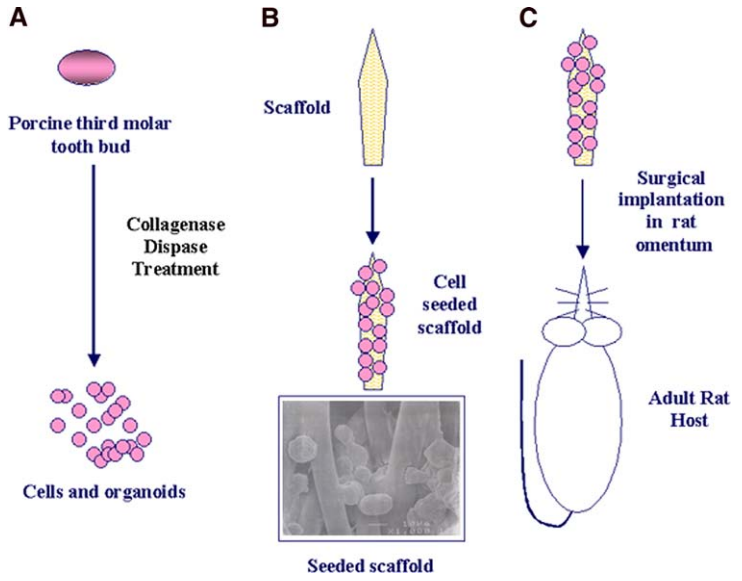

Fig. 1. Omental implant method schematic. Tooth bud cell-seeded scaffolds were implanted into the omenta of adult rat hosts, as diagrammed. (*A*) Tooth buds are dissociated into single-cell suspensions. (*B*) Cells are seeded onto biodegradable scaffolds. (*C*) Cell-seeded scaffolds are implanted into rat hosts.

physically and functionally with pre-existing dental tissues. Ideally, bioengineered dentin and enamel used to repair defects in pre-existing teeth can be integrated seamlessly with pre-existing naturally formed dentin and enamel crystals, eliminating the presence of interface sites susceptible to refracturing. Bioengineered whole teeth would be modeled to occlude with opposing and adjacent teeth properly and anchored to underlying alveolar bone via periodontal ligament tissue to transmit mechanical signals properly, allowing for orthodontic treatments as required. Biologic tooth substitutes would exhibit proper proprioception, facilitating the life of the implant and adjacent and opposing teeth.

Currently, with state-of-the-art techniques and materials for tissue engineering technologies, it is clear that these ambitious goals eventually can be achieved. Before tooth tissue engineering can become a widely practiced, clinically available therapy, however, impediments that are not insignificant must be overcome. Existing challenges in tooth tissue engineering can be classified broadly into two areas: (1) the identification and characterization of suitable dental progenitor cell populations that can be obtained easily and used for autologous tooth tissue engineering practices; and (2) the development of methods to reproducibly manipulate dental progenitor cells to bioengineer dental tissues and whole teeth of predetermined size and shape in a timely fashion. This article describes current efforts and future plans to facilitate the creation of clinically relevant tooth tissue engineering

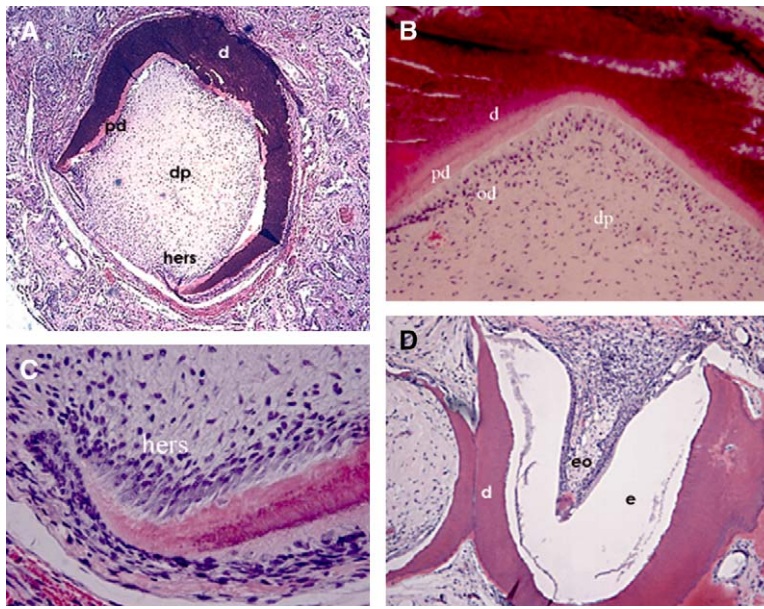

Fig. 2. Bioengineered dental tissues. (*A*) von Kossa staining of sectioned 20-week dental implant reveals bioengineered tooth crown exhibiting distinct pulp and predentin and dentin tissues. High magnification view of bioengineered tooth crown (*B*) and rudimentary Hertwig's epithelial root (hers) structure (*C*). (*D*) 30-week implant contains bioengineered teeth with significant amounts of dentin and enamel. d, dentin; dp, dental pulp; e, enamel; eo, enamel organ; hers, Hertwig's epithelial root sheath; od, odontoblasts; p, pulp; pd, predentin.

methodologies for the repair and regeneration of dental tissues from autologous adult tissues.

Characterization of tooth bud cell populations

The dental progenitor cells used in the authors' initial tooth tissue engineering studies were obtained from immature, unerupted tooth buds isolated from 6-month-old pigs [18] and 4-day postnatal rats [19]. The rationale for using pig and rat teeth at these developmental stages was to obtain tooth bud cell populations enriched in the two types of dental progenitor cells required to form all of the tissues in teeth and supporting periodontal ligament and alveolar bone tissues—epithelial and mesenchymal dental stem cells (DSC). Tooth bud tissues were dissociated enzymatically and mechanically and filtered to remove even small clumps of cells, generating single cell suspensions. The single cell suspensions were plated in vitro and cultured for approximately 1 week to eliminate differentiated cell types that do not survive long term in culture, resulting in enriched postnatal dental progenitor cell populations. Cultured cells then were harvested and seeded onto biodegradable polyester scaffolds, the purpose of which

was to provide a support onto which seeded dental progenitor cells—postnatal dental stem cells (PNDSC)—can adhere and orient themselves with respect to each other, allowing for requisite epithelial and mesenchymal dental cell interactions for tooth initiation and development. The cell-seeded scaffolds were grown in the omenta of host animals, an environment conducive to promote vascularization and growth of dental implant tissues.

Early results demonstrated that small tooth crowns formed in pig tooth bud cell-seeded implants grown for approximately 20 to 30 weeks [18] and in rat tooth bud cell-seeded implants grown for approximately 12 weeks [19]. The amount of time required to form bioengineered pig versus rat tooth crowns correlates with the amount of time required for naturally formed pig and rat teeth to develop and, therefore, likely reflects an endogenous developmental program for pig and rat PNDSC that is retained even when whole tooth buds are dissociated into single cell suspensions and cultured in vitro. The ability of PNDSC to retain a dental tissue differentiation program facilitates their use in dental tissue repair and whole tooth tissue engineering applications.

Histologic analyses of bioengineered dental implants reveal that small tooth crowns form throughout the implant. Tooth tissues form initially at the periphery and subsequently in the center of the implant, suggesting that PNDSC migrate from the periphery into the center of the scaffold over time before differentiating into dental tissues [20].

The fact that bioengineered dental tissue implants consist of many small bioengineered tooth crowns, with apparent random orientation, rather than one large bioengineered tooth adopting the size and shape of the scaffold onto which the PNDSC are seeded, reveals insight into certain properties of these cells. Because tooth formation depends on the interactions of two types of dental cells—epithelial and mesenchymal—it is logical to assume that each bioengineered tooth crown forms at sites within the scaffold to which both types of PNDSC are able to migrate, attain cell-cell contact, and initiate and maintain the reiterative and reciprocal growth factor signaling cascades leading to tooth development [21]. Another assumption is that only a subset of the total cell population seeded onto the scaffold is able to initiate or maintain a successful tooth development program, because small tooth crowns are scattered throughout the implant and generally form as discrete structures. These observations are consistent with the extensive literature documenting the heterogeneity of tooth bud tissues [22–24] and indicate the necessity of developing methods to sort tooth bud cell populations and to generate populations that are enriched in epithelial and mesenchymal PNDSC. Once purified homogeneous DSC populations are generated, the molecular and cellular properties of these cells can be assessed more easily, providing a molecular profile that can be manipulated for tooth tissue engineering applications.

Furthermore, bioengineered tooth crowns forming as discrete, very small, although anatomically correct, structures indicate the need to devise

strategies to guide the placement and interactions of epithelial and mesenchymal dental progenitor cells on the supporting scaffold in order to generate full-sized bioengineered dental tissues of predetermined size and shape.

Another property of PNDSC revealed by these studies is their slow growth, indicating that it likely is necessary to devise methods to hasten the formation of bioengineered replacement teeth. If human progenitor tooth cells exhibit similar properties to lower mammals, such as pigs and rats, which is likely, it can be estimated that human PNDSC require approximately 1 year or longer to generate bioengineered human teeth based on the growth rates of naturally formed human teeth. Because a year is a long time to wait for replacement teeth to grow, it is advantageous to devise methods to hasten the formation of bioengineered teeth if this approach is to attain widespread clinical application.

Generating enriched dental progenitor cell populations

Based on the need to generate enriched, homogenous epithelial and mesenchymal PNDSC populations for tooth tissue engineering applications, the authors use two approaches. The first is based on the ability to sort stem cells using antibodies that recognize the antigen, STRO-1, a carbohydrate moiety present on many types of stem cells [25,26]. Heterogeneous tooth bud cell populations can be incubated with magnetic beads to which anti–STRO-1 antibody is linked covalently. The cells that express STRO-1 become bound to the magnetic beads, whereas the STRO-1 negative non–stem cells are washed off. The STRO-1 expressing stem cell populations then are released from the magnetic beads, resulting in an enriched PNDSC population. Enriched epithelial and mesenchymal DSC populations are generated initially by dissecting the enamel organ (containing the epithelial DSC) away from the pulp organ (containing the mesenchymal DSC) of the starting tooth bud and then immunosorting the resulting epithelial and mesenchymal cell populations separately. This approach allows for determination and comparison of the characteristics of epithelial versus mesenchymal dental cell populations.

A second approach to generating enriched DSC populations is to perform Hoechst 33342 dye profiling, taking advantage of the ability of certain stem cells to exhibit the capacity to efflux the dye, whereas non–stem cells retain the dye [27]. After labeling, the cells are sorted by flow cytometry to generate enriched populations of Hoechst 33342 negative (stem) cells, termed side population (SP) cells, and Hoechst 33342 retaining (non–stem cell) populations. Once sorted, clonal epithelial and mesenchymal SP and non-SP cells can be expanded in vitro and tested in pairwise fashion (it takes epithelial and mesenchymal DSC to generate teeth) for their ability to initiate and maintain a developmental program for tooth development. Molecular and cellular profiling of identified DSC clones will provide insight into the

molecules that confer ''stemness'' onto DSC, providing a molecular map that can be manipulated to facilitate dental tissue bioengineering applications.

Examining dental cell-scaffold interactions

As discussed previously, the interactions of the scaffold with the PNDSC is of importance in guiding the size, shape, and differentiation of bioengineered dental tissues. Teeth are unique in that they are highly mineralized—enamel is the hardest substance in the body [28,29]. But teeth are organs, and early tooth development resembles that of soft tissue organs, such as the heart and liver, in that they are derived from the interactions of two cell types—epithelial and mesenchymal [30–33]. The size and shape of mature, highly mineralized teeth is determined early in development by the epithelial and mesenchymal interactions directing the morphology of the soft epithelial and mesenchymal tissues before any mineralization. Subsequent interactive signaling between the dental epithelium and mesenchyme results in dental tissue differentiation, including the induction of dentin-forming odontoblasts, enamel-secreting ameloblasts, and the placement of enamel knot signaling centers designating the locations of tooth cusps and tooth identity [34]. To master the task of generating bioengineered teeth of predetermined size and shape successfully, manipulating very early epithelial-mesenchymal cell interactions must be learned, to guide the eventual formation of the highly mineralized tooth tissues. Early dental cell and biodegradable scaffold interactions are key to this process.

The authors' initial tooth tissue engineering studies used biodegradable polyester scaffolds, fabricated from polyglycolic acid (or polyglycolide) (PGA) and polylactic acid (or polylactide) (PLA) [35]. The widespread use of these materials, alone and in combination with other materials, demonstrates their usefulness in bioengineering hard and soft tissues, including bone [36–38], skin [39] and intestine [15]. The authors' results suggest that the use of alternative scaffold materials or designs may facilitate the formation of bioengineered teeth of predetermined size and shape [20,40]. They are, therefore, investigating the use of alternative scaffold materials, including PGA/PLGA, collagen [41], silk [42], and combinations of these materials combined with modified scaffold designs. Recent progress in nanotechnology and 3-D imprinting–based scaffold fabrication and cell-seeding techniques [43] suggests the usefulness of these methods to guide the orientation and interactions of early dental epithelial and mesenchymal cell layers as they are seeded initially onto biodegradable scaffolds. In this way, the authors hope to guide the critical early dental cell proliferation and interactions that precede the morphologic events of tooth development, thereby defining the size and shape of teeth.

The authors also are working to define in vitro assays that can be used to screen for the early epithelial and mesenchymal dental cell interactions

characterizing tooth initiation, including the use of hydrogel materials and technologies to facilitate in vitro characterizations of PNDSC [44,45]. The ability to screen clonal epithelial and mesenchymal PNDSC lines rapidly for pairwise combinations whose interactions result in tooth induction will facilitate the identification of appropriate cell lines for future characterizations in tooth tissue engineering applications.

Current research goals

The authors' current research efforts in whole tooth tissue engineering are focused on three areas: (1) molecular profiling of epithelial and mesenchymal PNDSC to define the genes whose coordinated expression confers on these cells the ability to adopt dental cell differentiation fates; (2) defining methods of manipulating PNDSC via cell-cell and cell-scaffold interactions to generate bioengineered tooth tissues of predetermined size and shape that exhibit similar physical and mechanical properties to those exhibited by naturally formed dental tissues; and (3) promoting the formation of bioengineered tooth root structures, including cementum, periodontal ligament, and alveolar bone. Progress in each of these areas that will facilitate whole tooth tissue engineering efforts is described briefly.

Molecular profiling of epithelial and mesenchymal dental stem cells

It is the authors' hope that molecular profiling of epithelial and mesenchymal stem cells will provide important information. It is possible that the expression of certain growth factors, at discrete times and in discrete cell populations, may stimulate the initiation and maintenance of a tooth differentiation program that leads eventually to the formation of bioengineered dental tissues or even whole teeth. Once these gene profiles are determined, it may be possible to manipulate these signaling cascades to modify tooth development programs for dental tissue engineering purposes. For example, it may be possible to hasten replacement tooth development by overexpressing certain genes or prolonging or delaying the expression of other genes. In addition, it may be possible to induce nonodontogenic progenitor stem cells to adopt a tooth differentiation fate by inducing in them the expression of dental progenitor cell genes. An application for this is the ability to generate postnatal dental epithelial stem cell populations from alternative adult epithelial tissues. The absence of dental epithelial stem cells in adults is believed to be because once they form tooth crown enamel, they seem to lose their ability to self-renew and, instead, terminally differentiate to form tooth root tissues [46]. This phenomenon results in the absence of epithelial DSC populations in erupted teeth, precluding their use in tooth tissue engineering applications. It is possible that if the molecular profile of epithelial DSC can be determined (ie, if those genes can be identified whose combined

expression confers on epithelial cells the ability to form enamel-producing ameloblasts, for example, and to self-renew) and can be found to induce nondental epithelial tissues (such as cheek epithelium) to adopt an epithelial DSC fate, a continuous source of autologous epithelial DSC would be available for use in tooth tissue engineering applications.

Manipulating postnatal dental stem cells via cell-cell and cell-scaffold interactions

The ability to guide the formation of dental progenitor cells into bioengineered tooth tissues of predetermined size and shape rests on the ability to guide the early interactions and proliferation of epithelial and mesenchymal dental progenitor cells. The authors, therefore, are investigating the use of alternative scaffold materials and designs to guide early epithelial-mesenchymal cell interactions using the morphologic movements of naturally developing tooth tissues as a guide for these studies.

Bioengineered tooth root formation

Results to date indicate that although the anatomy of bioengineered tooth crowns closely resembles that of naturally formed tooth crowns, bioengineered tooth root structures are relatively undeveloped. The presence of Hertwig's epithelial root sheath structures in bioengineered teeth, rudimentary tooth root structures that precede the formation of mineralized tooth root tissues [6], suggests that tooth root development is initiated but does not continue to develop into functional tooth roots containing cementum, periodontal ligament, and alveolar bone, as found in naturally formed teeth. There are several plausible explanations as to why functional tooth roots have not developed in the bioengineered tooth tissues analyzed to date. One is that the bioengineered dental implants were not allowed to develop for long enough. It is possible that if the implants were allowed to grow for longer periods of time, more developed tooth root tissues would form. Another possibility is that the environment of the omentum is not conducive to tooth root development. This is a logical assumption based on the link of natural tooth root formation to tooth eruption, where mechanical forces imposed on teeth as they erupt through overlying alveolar bone are believed to facilitate tooth root formation [6,47]. It also is likely that the mandible provides an environment enriched in growth factors and additional signals that are not present in the omentum. The authors have performed bioengineered bone/tooth coculture experiments in an attempt to obtain more developed tooth root tissues in bioengineered teeth [25]. The results of these experiments, although preliminary, suggest that cobioengineered tooth/bone constructs exhibit tooth root structures that are more developed than those in bioengineered tooth constructs alone. Recent reports of bioengineered bone formation in the rabbit model [48] suggest that the growth of

bioengineered teeth in the jaw may require more sophisticated preparation of the implant site to promote tooth formation. In summary, these promising results bring closer the eventual goal of bioengineering replacement teeth in the jaw at the site of lost or missing teeth.

Summary

Although in its infancy, this research is highly significant in that it suggests that a tissue engineering approach can be used to bioengineer highly mineralized, anatomically correct replacement tooth tissues. The widespread interest in this research reflects the need for alternative therapies to treat a wide variety of dental repair needs [49,50].

The significance of this research is manifold. The authors' preliminary results indicate that it eventually will be possible to devise clinically relevant therapies to replace damaged or lost dental tissues or teeth with biologic dental materials as a viable alternative to synthetic dental materials. This research provides a model to study dental cell interactions in a way previously not possible. Currently available technologies to generate and characterize clonal DSC populations and the ability to study the behaviors of individual cell populations, and even individual cells, on diverse types of synthetic or nanofabricated materials [51], combined with sequential growth factor delivery techniques [52,53], are unprecedented.

This research also will provide intermediate products that can be used to augment existing synthetic dental repair materials. For example, before bioengineered whole tooth therapies are available clinically, it may be possible to use bioengineered dental materials to improve the function and duration of currently used titanium implants. The ability to secure titanium implants to underlying alveolar bone via autologous bioengineered periodontal ligament tissues would be a significant improvement over current methods, which secure the implant with direct embedment into the bone. The ability of periodontal ligament to transmit mechanical forces of mastication from the implant to the underlying bone would stimulate and maintain bone, likely improving the stability and longevity of the implant. Bioengineered periodontal ligament would provide a more natural environment for synthetic implants and a means to perform orthodontic treatments as required. A recent report using modified implantation method to induce the growth of periodontal ligament onto an artificial implant [54] suggests that a hybrid synthetic and biologic tissue approach may exhibit significant clinical relevance in the foreseeable future.

We have entered an exciting era where the diverse fields of tissue engineering, material science, nanotechnology, and stem cell biology have converged synergistically to provide unprecedented opportunities to characterize and manipulate signaling cascades regulating tissue and organ regeneration. These opportunities likely will lead to discoveries that will facilitate the creation of therapies to improve the health and well being of

many people. The field of tooth tissue engineering is one of many areas likely to see significant progress in the next decade.

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