CRITICAL REVIEWS IN ORAL BIOLOGY & MEDICINE

Craniofacial Tissue Engineering by Stem Cells

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ABSTRACT

Craniofacial tissue engineering promises the regeneration or *de novo* formation of dental, oral, and craniofacial structures lost to congenital anomalies, trauma, and diseases. Virtually all craniofacial structures are derivatives of mesenchymal cells. Mesenchymal stem cells are the offspring of mesenchymal cells following asymmetrical division, and reside in various craniofacial structures in the adult. Cells with characteristics of adult stem cells have been isolated from the dental pulp, the deciduous tooth, and the periodontium. Several craniofacial structures—such as the mandibular condyle, calvarial bone, cranial suture, and subcutaneous adipose tissue—have been engineered from mesenchymal stem cells, growth factor, and/or gene therapy approaches. As a departure from the reliance of current clinical practice on durable materials such as amalgam, composites, and metallic alloys, biological therapies utilize mesenchymal stem cells, delivered or internally recruited, to generate craniofacial structures in temporary scaffolding biomaterials. Craniofacial tissue engineering is likely to be realized in the foreseeable future, and represents an opportunity that dentistry cannot afford to miss.

KEY WORDS: stem cells, tissue engineering, biomaterials, wound healing, regenerative medicine.

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(1) INTRODUCTION

The majority of craniofacial structures derive from mesenchymal cells (MCs). During development, MCs originating from the neural crest are known to migrate, differentiate, and subsequently participate in the morphogenesis of virtually all craniofacial structures, such as cartilage, bone, ligaments, cranial sutures, musculature, tendons, the periodontium, and the teeth. Once migrated, MCs work synergistically with mesodermal cells in the morphogenesis of craniofacial structures. Both mesenchymal cells and mesodermal cells are derivatives of embryonic stem cells, a few hundred cells of the inner cell mass of the blastocyst.

Mesenchymal cells undergo asymmetric division, with one offspring cell differentiating toward an end-stage cell, while the other replicates into an offspring mesenchymal cell. Residual offspring of mesenchymal cells, upon the completion of morphogenesis, continue to reside in various craniofacial tissues, and retain their status as stem cells. After birth, mesenchymal cells are called 'mesenchymal stem cells' (MSCs). In the adult, MSCs maintain physiologically necessary tissue turnover and, upon injury or disease, differentiate to launch tissue regeneration.

MSCs have been experimentally differentiated into all mesenchymal or connective tissue lineages (Caplan, 1991; Pittenger *et al.*, 1999) and, in many cases, have been used to engineer the craniofacial structures that their prenatal predecessors, mesenchymal cells, are capable of generating during development. The capacity of MSCs in the *de novo* formation and/or regeneration of craniofacial structures is too natural an endeavor to make one wonder why this potential has not been exploited substantially up until the past decade. As it turns out, our ability to grow human craniofacial tissues and organs is by no means a small task. Engineering craniofacial structures from stem cells was an insurmountable effort until advances from several seemingly unrelated disciplines—such as cell and molecular biology, polymer chemistry, molecular genetics, materials science, robotics, and mechanical engineering—converged into the self-assembling field of tissue engineering (Nerem, 1992; Langer and Vacanti, 1993). To engineer a functional biological structure, cells must be instructed to differentiate and receive positional cues, and to synthesize the appropriate extracellular matrix molecules in the overall shape and dimensions of the diseased or missing tissues/organs. Biomimetic scaffolds are frequently needed to enable cell growth and differentiation to occur in an environment that has been previously unfamiliar to either biologists or engineers. Craniofacial structures offer complex and, in many cases, unique challenges when they are being engineered.

Large-scale tissue-engineering research, craniofacial or otherwise, began to take place in the early 1990s and has grown exponentially ever since. The first overview of craniofacial tissue engineering appeared within the past few years and focused on primarily tissue-engineering principles (Hollinger and Winn, 1999; Alsberg *et al.*, 2001). As outlined below, substantial advances have been made in craniofacial tissue engineering:

- Stem cells have been isolated from several craniofacial tissues, with ongoing effort to purify and apply them in the tissue engineering of craniofacial structures.

- Several prototypes of the human-shaped temporomandibular joint condyle have been engineered with integrated cartilage and bone layers from a single population of mesenchymal stem cells.
- Various elements of the periodontium, including the periodontal ligament and cementum, have been engineered *via* cell-based or non-cell-based approaches.
- Craniofacial bone has been engineered from stem cells, growth factors, and/or biomaterials. A cranial suture-like structure has been engineered from cell- and growth-factor-based approaches.
- Adipose tissue has been engineered *in vivo* from mesenchymal stem cells, with potential applications in facial plastic and reconstructive surgeries.

The tissue-engineered craniofacial structures to date are undoubtedly prototypes that warrant further development and refinements, but nonetheless were not even available a few years ago (Hollinger and Winn, 1999; Alsberg *et al.*, 2001). Despite formidable challenges, this review offers the optimistic view that functional craniofacial tissues and organs can be grown *via in vitro* and/or *in vivo* approaches for ultimate therapeutic applications. This review is designed not only to serve as a timely and comprehensive synthesis of our current knowledge of craniofacial tissue engineering, but also to identify immediate challenges in this dynamic field. Craniofacial tissue engineering, like tissue engineering in general, is an interdisciplinary field by nature. A review such as this cannot possibly include some of the necessary technical details of those more individualized fields that have converged to form the foundation of craniofacial tissue engineering. The reader is referred to several recent in-depth reviews in the individual fields of stem cell biology, biomaterials, cell and molecular biology and genetics, and regenerative medicine (Drury and Mooney, 2003; Alhadlaq and Mao, 2004; Patel and Mikos, 2004; Atala, 2005: Gregory *et al.*, 2005; Hollister, 2005; Kyba, 2005; Mao, 2005a; Rahaman and Mao, 2005; Shi *et al.*, 2005).

(2) MESENCHYMAL STEM CELLS AND CRANIOFACIAL-DERIVED STEM CELLS

A stem cell is self-renewable and capable of differentiating into at least two distinctive cell types (Parker *et al.*, 2004). These two properties must both be satisfied for a cell to be defined as a stem cell. Self-renewal denotes that undifferentiated daughter cells are a precise replica and can further replicate many generations without losing their original characteristics (Caplan, 1991; Alhadlaq and Mao, 2004). Cells of an immortalized cell line can replicate many generations, but are generally incapable of multi-lineage differentiation. Thus, cell lines are not stem cells. Multi-lineage differentiation refers to the capacity of a single population of stem cells to differentiate into at least two distinctively different cell types (Caplan, 1991; Parker *et al.*, 2004). For example, a single population of MSCs can differentiate into both osteoblasts and chondrocytes. Pre-osteoblasts, a term frequently used in bone and dental literature, can differentiate into osteoblasts, but are incapable of differentiating into other mesenchymal lineages, such as chondrocytes or adipocytes, at least not without undergoing de-differentiation back toward MSCs (Caplan, 1991). Thus, pre-osteoblasts are not stem cells. It is convenient and often necessary to define a progenitor cell as one that is between a stem cell and an end-stage cell, although there are likely many unnamed cell differentiation stages between a stem cell and a progenitor cell, and then between a progenitor cell and an end-stage cell. For example, a pre-osteoblast is a progenitor cell between an MSC and an osteoblast or osteocyte.

(2.1) Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells (MSCs) are self-renewable and can differentiate into all cell lineages that form mesenchymal and connective tissues (Conget and Minguell, 1999; Caplan, 1991; Pittenger *et al.*, 1999; Alhadlaq and Mao, 2004). In addition, MSCs have been reported to differentiate into hepatic (Petersen *et al.*, 1999), renal (Poulsom *et al.*, 2003), cardiac (Orlic *et al.*, 2001), and neural cells (Brazelton *et al.*, 2000; Mezey *et al.*, 2000).

The first successful isolation of bone marrow MSCs, then called colony-forming fibroblast-like cells, was described almost 4 decades ago (Friedenstein *et al.*, 1970). The isolation method was based on the adherence of marrow-derived MSCs to the plastic substrate of the cell culture plates, and a concomitant lack of adherence of marrow-derived hematopoietic stem cells. To date, this straightforward protocol is widely used for the isolation of MSCs in multiple vertebrate species (Alhadlaq and Mao, 2004).

Previous approaches, utilizing flow cytometry, have investigated the isolation of increasingly homogenous populations of MSCs based on differential cellular features within the bone marrow stroma. Further purification and cloning of MSCs are desirable for stem cell biology, similar to the development of cell lines. Recently, several relatively straightforward MSC culture protocols have been developed. Size-dependent sieving of a cell population from human bone marrow aspirates through a porous membrane resulted in a relatively homogeneous population that had the capacity of self-renewal and multi-lineage differentiation (Hung *et al.*, 2002). Positive selection of MSCs with microbeads, combined with fluorescence-activated cell-sorting (FACS) (Jones *et al.*, 2002) or magnetic-activated cell-sorting (MACS) (Kinnaird *et al.*, 2004), is an effective technique for the increasingly defined isolation and precise characterization of MSCs (Reese *et al.*, 1999; Lee *et al.*, 2001; Arbab *et al.*, 2004).

Whether highly purified or cloned MSC populations are necessarily needed for the engineering of craniofacial structures is not clear. First, stem cell populations that generate native craniofacial structures, such as the mandibular joint, are heterogeneous and likely include both mesenchymal and hematopoietic stem cells. The morphogenesis of the articular condyle requires stem cells, chondrocytes, and osteoblasts in addition to angiogenesis (Mao, 2005a). Second, host cell invasion and stem cell homing are likely inevitable in porous biomimetic scaffolds that are used as carriers for delivering stem cells and/or stem-cell-derived tissue-forming cells (Christopherson *et al.*, 2004; Hidalgo and Frenette, 2005).

(2.2) Dental Pulp Stem Cells

Although the regenerative capacity of the human dentin/pulp complex is not well-understood, it is known that, upon injury, reparative dentin is formed as a protective barrier for the pulp (Murray *et al.*, 2001). Accordingly, one might anticipate that dental pulp contains the dentinogenic progenitors that are responsible for dentin repair. Previous work has shown that

dental pulp contains proliferating cells that are analogous to bone cells, because they express osteogenic markers and respond to many growth factors for osteo/odontogenic differentiation (Hanks *et al.*, 1998; Unda *et al.*, 2000; Ueno *et al.*, 2001). In addition, dental pulp cells are capable of forming mineral deposits with distinctive dentin-like crystalline structures (About *et al.*, 2000; Couble *et al.*, 2000; Gronthos *et al.*, 2000). Recently, dental pulp stem cells (DPSCs) have been isolated from extracted human third molars (Gronthos *et al.*, 2000; Shi *et al.*, 2001).

To determine the colony-forming efficiency of the isolated DPSCs, investigators have prepared single-cell suspensions by collagenase/dispase treatment of pulp fragments filtered through a fine mesh to remove cell aggregates, and seeded at low plating densities (Gronthos *et al.*, 2000). Approximately 40 single-colony clusters can be retrieved from 10,000 cells in culture (Gronthos *et al.*, 2000, 2003). The clones from the initial primary culture demonstrated variable capacities for forming dentin-like structures following *in vivo* implantation (Shi and Gronthos, 2003). DPSCs showed a higher proliferation rate than bone-marrow-derived MSCs under the same culture conditions, potentially attributable to strong expression of cyclin-dependent kinase 6, a cell-cycle activator (Shi *et al.*, 2001). Expression of various perivascular markers—such as STRO-1, VCAM-1, MUC-18, and α-smooth-muscle actin—provides clues that DPSCs are a heterogeneous population of MSCs and likely located in the perivascular niche in the pulp (Gronthos *et al.*, 2000; Shi and Gronthos, 2003).

To elucidate the self-renewal ability, investigators isolated cells from DPSC implants at two months post-subcutaneous implantation, by enzymatic digestion and subsequent expansion *in vitro*. The isolated cells from the DPSC implants were sorted by fluorescent-activated cell-sorting (FACS) with human beta1-integrin monoclonal antibody. The isolated human cells were re-implanted into immunodeficient mice for two months. The recovered secondary implants yielded the same dentin/pulp-like structures as the primary implants. Human dentin sialophosphoprotein (DSPP) was expressed in dentin-like structures, confirming the human origin of the odontoblast/pulp cells in the secondary DPSC implants (Batouli *et al.*, 2003).

So that their multi-lineage differentiation capacity could be determined, DPSCs were cultured in adipogenic inductive agents. In a few weeks, DPSCs differentiated into adipocyte-like cells that were positive to Oil red O, and expressed PPARγ2 and lipoprotein lipase (LPL) (Batouli *et al.*, 2003). Furthermore, DPSCs were shown to differentiate into neuron-like and glial-like cells by expressing both nestin, an early marker of neural precursor cells, and glial fibrillary acid protein (GFAP), an antigen characteristic of glial cells (Gronthos *et al.*, 2000). These DPSC-derived cells developed long cytoplasmic processes, a departure from their usual bipolar fibroblastic appearance.

(2.3) Stem Cells from Human Exfoliated Deciduous Teeth (SHED)

The exfoliated deciduous tooth houses living pulp remnants consisting of connective tissue, blood vessels, and odontoblasts. We found that from 12 to 20 cells from each exfoliated incisor formed adherent colony clusters with extensive proliferative capacity (Miura *et al.*, 2003). *Ex vivo*-expanded SHED expressed STRO-1 and CD146 (MUC18), two early cell-surface markers for bone-marrow-derived MSCs (Shi and Gronthos, 2003). In addition, SHED expressed a variety of osteoblast/odontoblastic markers, including Runx2, alkaline phosphatase (ALP), matrix extracellular phosphoglycoprotein (MEPE), bone sialoprotein (BSP), and DSPP. After implantation into immunocompromised mice, with hydroxyapatite/tricalcium phosphate (HA/TCP) as a carrier, SHED differentiated into odontoblast-like cells that formed small dentin-like structures (Miura *et al.*, 2003). These results suggest that SHEDs are distinctive from DSPCs with respect to odontogenic differentiation and osteogenic induction (Miura *et al.*, 2003).

(2.4) Periodontal Ligament Stem Cells (PDLSCs)

The periodontal ligament (PDL) connects the cementum to alveolar bone, and functions primarily to support the tooth in the alveolar socket. A recent report identified stem cells in human PDL (PDLSCs) and found that PDLSCs implanted into nude mice generated cementum/PDL-like structures that resemble the native PDL as a thin layer of cementum that interfaced with dense collagen fibers, similar to Sharpey's fibers (Seo *et al.*, 2004). After a three-week culture with an adipogenic-inductive cocktail, PDLSCs differentiated into Oil-red-O-positive, lipid-laden adipocytes (Seo *et al.*, 2004). Upon four-week osteo/odontogenic inductions, alizarin-red-positive nodules formed in the PDLSC cultures, similar to MSCs and DPSCs (Shi *et al.*, 2002; Seo *et al.*, 2004). Thus, the PDLSCs have the potential for forming periodontal structures, including the cementum and PDL.

(2.5) Challenges in Stem Cell Biology Related to Craniofacial Tissue Engineering

- The relationship between bone marrow MSCs and the newly identified stem cells from various craniofacial tissues needs to be defined. In many ways, the newly characterized craniofacial stem cells resemble bone marrow MSCs, especially in terms of their differentiation capacities (Shi *et al.*, 2005).
- Whether craniofacial-derived MSCs more effectively regenerate craniofacial structures than do appendicular MSCs needs to be explored. Whether craniofacial-derived MSCs are capable of healing non-craniofacial defects more effectively than are appendicular MSCs also warrants investigation.
- How mechanical stress modulates craniofacial morphogenesis and regeneration needs to be further explored (Carter *et al.*, 1998; Mao, 2005b).
- The extent to which tissue engineering should mimic or recapitulate the corresponding developmental events needs to be determined (Ferguson *et al.*, 1998).

(3) TISSUE ENGINEERING OF THE TEMPOROMANDIBULAR JOINT FROM STEM CELLS

Temporomandibular disorders (TMD) manifest as pain, myalgia, headaches, and structural destruction, collectively known as degenerative joint disease (Okeson, 1996). The temporomandibular joint (TMJ), like other synovial joints, is also prone to rheumatoid arthritis, injuries, and congenital anomalies (Stohler, 1999). The severe form of TMJ disorders necessitates surgical replacement of the mandibular condyle (Sarnat and Laskin, 1992).

In the past few years, we have reported the tissue

engineering of a mandibular condyle exhibiting the shape and dimensions of a human cadaver TMJ. The engineered mandibular condyle had stratified layers of cartilage and bone from a single population of bone-marrow-derived MSCs, and was molded into the shape and dimensions of a human cadaver mandibular condyle at 11 x 7 x 9 mm (l x w x h) (Alhadlaq and Mao, 2003, 2005; Alhadlaq *et al.*, 2004; Mao, 2005b).

(3.1) Cell Survival and Matrix Synthesis in the Tissue-engineered Mandibular Condyle

MSCs were isolated from femoral and tibial bone marrows of adult rats and exposed separately to either chondrogenic or osteogenic supplemented culture medium (Alhadlaq and Mao, 2003; Alhadlaq *et al.*, 2004). Poly(ethylene glycol) diacrylate (PEGDA) was dissolved in PBS with a biocompatible ultraviolet photoinitiator (Alhadlaq and Mao, 2003; Alhadlaq *et al.*, 2004). MSC-derived chondrogenic and osteogenic cells were encapsulated in PEGDA hydrogel into a negative mold of an adult human cadaver mandibular condyle in two stratified and yet integrated layers. The photopolymerized osteochondral construct was implanted into the dorsum of immunodeficient mice for up to 12 wks.

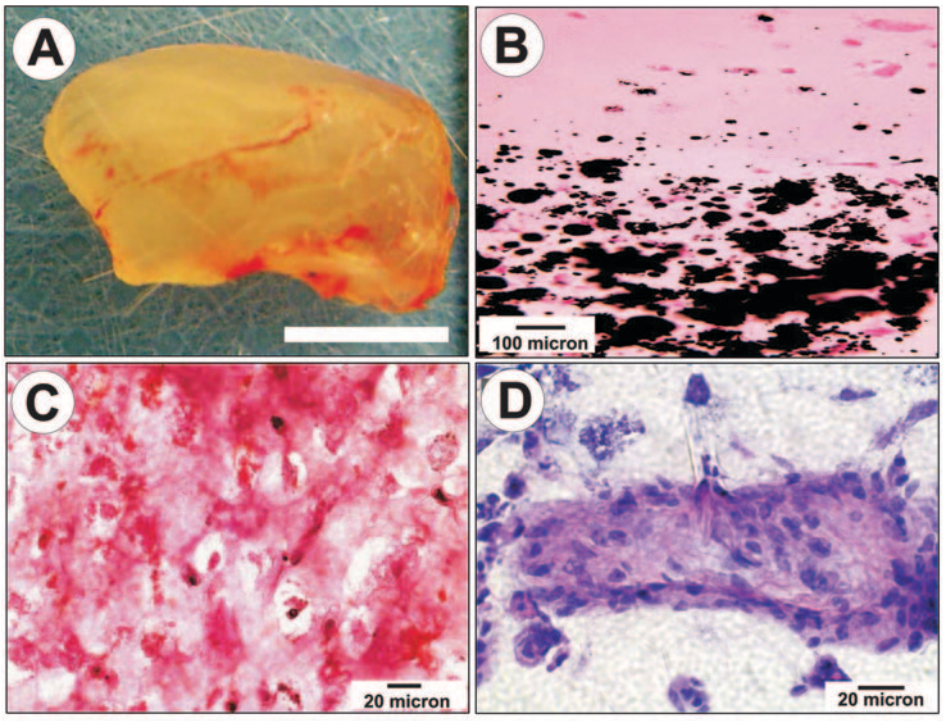

Figure 1. Engineered neogenesis of human-shaped mandibular condyle from mesenchymal stem cells. **(A)** Harvested osteochondral construct retained the shape and dimension of the cadaver human mandibular condyle after *in vivo* implantation. Scale bar: 5 mm. **(B)** Von Kossa-stained section showing the interface between stratified chondral and osseous layers. Multiple mineralization nodules are present in the osseous layer (lower half of the photomicrograph), but absent in the chondral layer. **(C)** Positive safranin O staining of the chondrogenic layer indicates the synthesis of abundant glycosaminoglycans. **(D)** H&E-stained section of the osteogenic layer showing a representative osseous island-like structure consisting of MSC-differentiated osteoblast-like cells on the surface and in the center. Reproduced with permission from Biomedical Engineering Society.

De novo formation of a structure having the same shape and dimensions as the cadaver human mandibular condyle was observed after 4 wks of *in vivo* implantation (Fig. 1). The tissue-engineered mandibular joint condyles retained the macroscopic shape and dimensions of the cadaver mandibular condyle (Fig. 1A). The interface between the upper-layer PEGDA hydrogel encapsulating MSC-derived chondrogenic cells and the lower-layer PEGDA hydrogel encapsulating MSC-derived osteogenic cells demonstrated distinctive microscopic characteristics (Figs. 1B-1D). The chondrogenic and osteogenic portions remained in their respective layers (*cf.* interface in Fig. 1B). The chondrogenic layer contained sparse chondrocyte-like cells surrounded by abundant intercellular matrix (Fig. 1C). The intercellular matrix of the chondrogenic layer showed strong, intense staining with safranin O (Fig. 1C), a cationic marker that binds to cartilage-related glycosaminoglycans (GAG), such as chondroitin sulfate and keratan sulfate. Some of the MSC-derived chondrogenic cells were surrounded by pericellular matrix, characteristic of native chondrocytes (Fig. 1C). In contrast, the osteogenic layer contained mineral nodules, as revealed by von Kossa staining (lower portion of Fig. 1B). The osteogenic layer also showed multiple island structures occupied by osteoblast-like cells (Fig. 1D).

(3.2) Cell Density Matters in the Tissue Engineering of Mandibular Condyle

A fundamental question posed above is to what extent the events of development should be recapitulated in tissue engineering. The following is an example that provides some clues to this question. A dissatisfactory feature in our previous work to engineer a human-shaped mandibular joint condyle (Alhadlaq and Mao, 2003; Alhadlaq *et al.*, 2004) was suboptimal tissue maturation. The straight line of osteochondral junction in the engineered synovial joint condyle (Fig. 1B) did not resemble the mutual infiltration of chondral and osseous tissues in the native osteochondral junction. We then attempted to improve tissue maturation and osteochondral integration by increasing the cell encapsulation density from the previous 5 x 10^6 cells/mL to 20 x 10^6 cells/mL.

At a density of 20 x 10^6 cells/mL, the tissue-engineered mandibular joint condyle again retained the pre-defined shape and dimensions of the human cadaver mandibular condyle (Alhadlaq and Mao, 2005). The cartilage layer was positively stained by safranin O, indicating the presence of cartilage-related glycosaminoglycans (Fig. 2A), and contained type II collagen (Fig. 2B). The deep portion of the cartilage layer, near the tissue-engineered osteochondral interface, contained chondrocyte-like cells with a hypertrophic appearance and characterized by the expression of type X collagen (data not shown, but *cf.* Alhadlaq

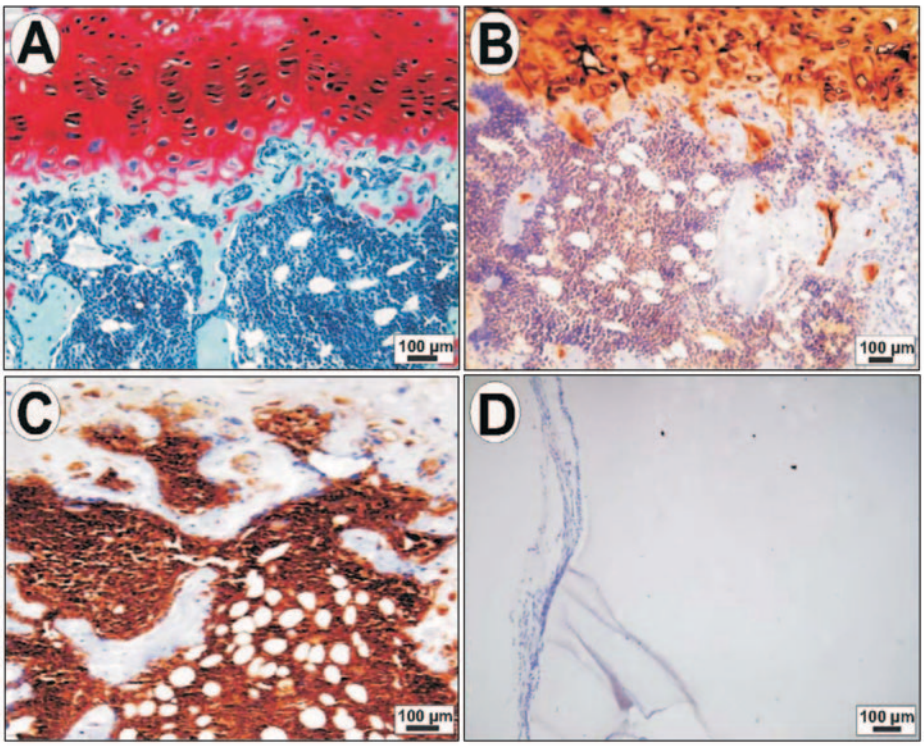

Figure 2. Histologic and immunohistochemical characterization of a human-shaped mandibular condyle engineered from mesenchymal stem cells after *in vivo* implantation. **(A)** Representative photomicrograph showing positive safranin O staining of the upper cartilage layer, indicating the presence of abundant glycosaminoglycans. In contrast, the osseous portion shows negative safranin O staining. **(B)** Positive immunohistochemical localization of type II collagen in the cartilage portion. The osseous portion was negative to type II collagen immunolocalization. **(C)** Positive immunolocalization of osteopontin within the osseous portion. By contrast, the cartilage portion lacks osteopontin expression. **(D)** Representative micrograph of hydrogel control cell-free construct showing host fibrous-tissue capsule surrounding the construct, but a lack of host cell invasion. Reproduced with permission from Mary Ann Liebert.

and Mao, 2005), a marker for hypertrophic and degenerating chondrocytes. In contrast, the osseous layer demonstrated immunolocalization of bone markers such as osteopontin (Fig. 2C) and osteonectin (*cf.* Alhadlaq and Mao, 2005). The chondrogenic layer lacked immunolocalization of bone markers, whereas the osseous layer lacked immunolocalization of cartilaginous markers (Figs. 2A-2C). There was mutual infiltration of the cartilaginous and osseous components into each other's territory (Figs. 2A, 2B). This mutual infiltration of chondral and osseous tissues, absent in our previous work, where we used a four-fold-lower cell encapsulation density (Alhadlaq and Mao, 2003; Alhadlaq *et al.*, 2004), resembles the osteochondral interface in age-matched native rat mandibular condyle (Alhadlaq and Mao, 2005). The cell-free PEGDA hydrogel scaffold showed an intact border surrounded by fibrous capsule without host cell invasion (Fig. 2D), further substantiating the conclusion that the engineered mandibular joint condyle is formed solely by MSC-derived chondrogenic cells and osteogenic cells, rather than by host cells.

(3.3) Functional *in vivo* Integration of the Engineered Mandibular Condyle

Ongoing research has begun to address the implantation of an engineered mandibular construct into a functional load-bearing model. Since both mass transport and mechanical properties depend upon 3D scaffold architecture, computational design techniques are needed to predict and ultimately optimize a microstructure to achieve the desired balance (Hollister, 2005). Architectural 3D scaffold design with the desired anatomical shape can be produced from image-based (Hollister *et al.*, 2000, 2002) or computer-aided-design (CAD)-based approaches (Hutmacher *et al.*, 2004). Scaffolds from these design approaches can then be built directly or indirectly by Solid Free-Form Fabrication (SFF) (Hutmacher *et al.*, 2004; Yeong *et al.*, 2004; Hollister, 2005), and have been applied in craniofacial reconstruction (Rohner *et al.*, 2003; Hollister *et al.*, 2005).

The ultimate goal is to use integrated design/fabricated scaffolds for functional mandibular condyle and other craniofacial reconstruction. Integrated design/fabrication methods not only make functional reconstruction possible, but also permit the testing of design hypotheses concerning scaffold/cell carrier combinations, eventually leading to optimal reconstruction methods. Our group has begun to test anatomically designed scaffolds for mandibular reconstruction in a Yucatan minipig model. We designed and fabricated a mandibular condyle scaffold directly from a minipig mandibular CT scan. The initial design was a shell into which autologous bone marrow was packed at the time of surgery (Figs. 3A-3C). Initial results at 1 and 3 months showed that the minipigs masticated normally, as documented by video, and, furthermore, that bone regenerated in the desired condyle shape, as shown by CT (Figs. 3D, 3E). Specimens will be processed for histology so that cartilage formation can be assessed. These results demonstrate that the shell scaffold design could support functional load-bearing as well as tissue regeneration. Further work will investigate how scaffold design, material, and biologic/carrier combinations affect regeneration.

(3.4) Challenges of the Tissue Engineering of the Temporomandibular Joint

- To promote matrix synthesis and tissue maturation of stem-cell-derived chondrogenic and osteogenic cells encapsulated in biocompatible and bioactive scaffolds. It may be necessary to incorporate an array of growth factors and/or transcription factors separately for tissue-engineered chondrogenesis and osteogenesis.
- To enhance the mechanical properties of a tissue-engineered mandibular condyle for ultimate *in situ* implantation into the human temporomandibular joint.

- To facilitate the remodeling potential of a tissue-engineered mandibular condyle.

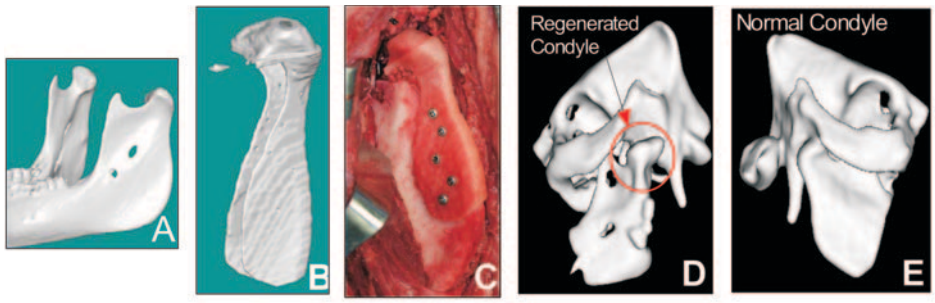

Figure 3. Design and engineering of minipig mandibular condyle. **(A)** Original Computed Tomography (CT) scan of minipig mandible. **(B)** Image-based design of condyle scaffold. **(C)** PCL (polycaprolactone) degradable polymer scaffold fabricated with SLS (Selective Laser Sintering) attached to the ramus. **(D)** Regrowth of condyle following 3 months' implantation (new condyle shown in red circle). **(E)** Comparison with normal condyle from contralateral side in Yucatan minipig.

(4) PERIODONTAL TISSUE ENGINEERING

A major goal in the reconstruction of periodontal bone defects is the simultaneous restoration of cementum, periodontal ligament, and alveolar bone structures in the face of the microbial assault and altered host immune response. Current periodontal therapies—such as tissue-banked bone allografts, cell-occlusive barriers, or enamel matrix proteins—result in only limited bone regeneration (radiographically < 50% and < 20% for vertical and horizontal bony defects, respectively) (Giannobile and Somerman, 2003; Murphy and Gunsolley, 2003; Reynolds *et al.*, 2003). Despite these constraints, the field of periodontal tissue engineering has witnessed several major incremental advances, as discussed below.

(4.1) Microbial Influences on Periodontal Tissue Engineering

Periodontal infection is initiated by invasive oral pathogens that colonize dental plaque biofilms on the tooth root surface. This chronic challenge of virulent micro-organisms leads to the destruction of tooth-supporting soft and hard tissues, including alveolar bone, tooth root cementum, and the PDL. Thus, tissue-engineered constructs for periodontal reconstruction will likely face constant exposure to the oral bacterial flora, which contains 10^6-10^7 colony-forming units *per* milliliter (CFU/mL) of saliva (Motisuki *et al.*, 2005). Even in the healthy oral cavity, the mucosal surfaces are colonized by approximately 90% anaerobic and 10% aerobic bacteria (Weber, 1997; Callender, 1999). Although the incidence of infection in oral surgical procedures is between 1 and 5% (Loukota, 1991), the consequences will, at least conceptually, compromise the outcome of periodontal tissue engineering.

(4.2) Growth and Amelogenin Factors in Periodontal Regeneration

Over the past decade, numerous polypeptide growth and amelogenin-like factors have been used in the repair of periodontal defects. Pre-clinical investigations have revealed encouraging results with respect to the repair of periodontal defects with growth factors such as platelet-derived growth factor (PDGF) (Lynch *et al.*, 1991; Rutherford *et al.*, 1992; Giannobile *et al.*, 1994; Giannobile, 1996), bone morphogenetic proteins (BMPs) (Sigurdsson *et al.*, 1995; Giannobile *et al.*, 1998), and fibroblast growth factor-2 (Rossa *et al.*, 2000; Takayama *et al.*, 2001; Nakahara *et al.*, 2003). PDGF has demonstrated the promise to regenerate bone in periodontal osseous defects, as shown in human clinical trials when used alone (Camelo *et al.*, 2003; Nevins *et al.*, 2003, 2005) or in combination with IGF-1 (Howell *et al.*, 1997b). BMPs have been shown to induce bone regeneration in peri-implant and maxillary sinus-floor augmentation procedures (Boyne, 1996; Boyne *et al.*, 1997; Hanisch *et al.*, 1997), extraction socket defects (Howell *et al.*, 1997a; Cochran *et al.*, 2000; Fiorellini *et al.*, 2005), and mandibular discontinuity defects (Boyne *et al.*, 1999; Warnke *et al.*, 2004). The use of BMPs for oral and craniofacial reconstruction has recently been reviewed (Nakashima and Reddi, 2003; Wikesjö *et al.*, 2005).

Enamel matrix derivative (EMD) appears to promote periodontal regeneration by mimicking corresponding developmental events. EMD is composed of amelogenins with metallo-endoprotease and serine protease activity. Although their roles in epithelial-mesenchymal interactions remain to be clarified, EMDs have been shown to promote periodontal regeneration in multiple controlled human clinical trials (Heijl *et al.*, 1997; Zetterström *et al.*, 1997; Silvestri *et al.*, 2000; Tonetti *et al.*, 2002). In addition to EMD, a product that utilizes a biomimetic RGD cell-binding peptide fused to anorganic bone matrix has demonstrated efficacy in promoting periodontal bone repair in human clinical trials (Yukna *et al.*, 2000, 2002).

(4.3) Gene Delivery in Periodontal Tissue Engineering

Gene transfer offers significant potential to improve growth factor delivery to tooth-supporting defects (Baum *et al.*, 2002). Gene therapy has demonstrated strong potential to target, deliver, and improve the bioavailability of growth factors (such as BMPs and PDGFs) to stimulate the tissue engineering of periodontal defects (Fig. 4). Delivery of PDGF by gene transfer stimulates remarkable mitogenesis and proliferation of gingival fibroblasts, PDL, and cementoblasts, in comparison with continuous PDGF administration *in vitro* (Zhu *et al.*, 2001; Chen and Giannobile, 2002; Jin *et al.*, 2004a). Adenovirus-mediated PDGF-B gene transfer accelerated gingival wound healing in an *ex vivo* wound repair model (Anusaksathien *et al.*, 2003). Surgically created alveolar bone wounds treated with AdPDGF-B revealed significant regeneration of cementum and PDL, along with extended growth factor expression, as demonstrated by *in vivo* biodistribution (Jin *et al.*, 2004a). Application of a dominant-negative mutant of the PDGF-A gene (PDGF-1308) directly to periodontal osseous defects or to cementoblasts transplanted *ex vivo* in polymer scaffolds led to impaired periodontal regeneration (Anusaksathien *et al.*, 2004; Jin *et al.*, 2004b). BMP viral gene delivery has showed remarkable potential in the regeneration of both long bones and craniofacial bones (Lieberman *et al.*, 1998, 1999; Alden *et al.*, 2000; Baltzer *et al.*, 2000; Krebsbach *et al.*, 2000; Shen *et al.*,

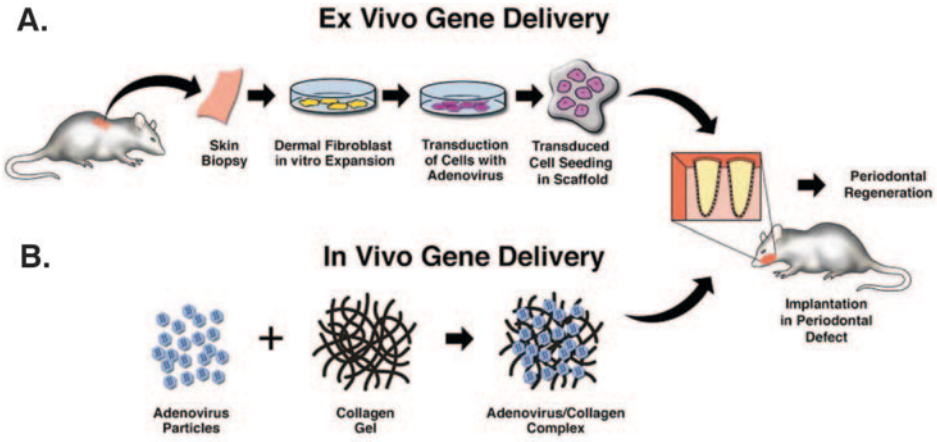

Figure 4. Delivery approaches for periodontal bioengineering. *Ex vivo* gene therapy involves the harvesting of tissue biopsies, expansion of cell populations, genetic manipulations of cells, and subsequent transplantation to periodontal osseous defects **(A)**, while the *in vivo* gene transfer approach involves the direct delivery of growth factor transgenes to the periodontal osseous defects **(B)**.

2004a,b; Dunn *et al.*, 2005). *Ex vivo* gene delivery of BMP7-transduced fibroblasts has regenerated alveolar bone and cementum, whereas local knock-out of BMP bioactivity by Noggin, a BMP-antagonist, blocks cementogenesis of tissue-engineered cementum (Jin *et al.*, 2003a, 2004b).

(4.4) Cell-based Therapies in Periodontal Tissue Engineering

The transplantation of periodontal stem cells or their derivatives has been examined for their potential to reconstruct periodontal tissue defects (for reviews, see Thesleff and Tummers, 2003; Risbud and Shapiro, 2005). Transplantation of cells derived from the periodontal ligament has showed the potential to regenerate periodontal attachment structures *in vivo* (Lekic *et al.*, 2001; Dogan *et al.*, 2003; Nakahara *et al.*, 2004; Akizuki *et al.*, 2005; Hasegawa *et al.*, 2005). Cementoblasts or tooth-lining cells have a marked ability to induce mineralization in an *ex vivo* model (Jin *et al.*, 2003b) and *in vivo* in periodontal wounds (Zhao *et al.*, 2004). However, when less-differentiated dental follicle cells are delivered in a similar fashion, these cells inhibit periodontal healing (Zhao *et al.*, 2004). As described above, periodontal stem cells promote the formation of cementum-like mineralized tissue *ex vivo* (Seo *et al.*, 2004; Trubiani *et al.*, 2005). Allogeneic foreskin fibroblasts have recently been shown to promote new attachment of gingival recession defects (McGuire and Nunn, 2005).

(4.5) Challenges of Periodontal Tissue Engineering

- A major unmet challenge is the modulation of the exuberant host response to microbial contamination that plagues the periodontal wound. Dual delivery of host modifiers and anti-infective agents is likely necessary to optimize periodontal regeneration.
- The necessary interactions of multiple cell lineages should be clarified. These include cementogenic cells, fibroblasts, and osteogenic cells.
- Despite the important role of exogenously delivered cells in the regeneration of severe periodontal defects, it is advantageous to attract endogenous periodontal tissue-forming cells by growth and/or trophic factors.

(5) TISSUE ENGINEERING OF CRANIOFACIAL BONE

Current surgical approaches for reconstructing craniofacial defects include autogenous bone grafts, allogeneic materials, and prosthetic compounds such as metals and plastics (Marchac, 1982; Shenaq, 1988; Goodrich *et al.*, 1992; Nicholson, 1998; Rah, 2000; Bruens *et al.*, 2003; Cowan *et al.*, 2004). Despite certain levels of clinical success, each of these strategies has limitations. For example, autogenous bone grafts, the gold standard for craniofacial bony reconstruction, necessitate donor site morbidity (Silber *et al.*, 2003). Prosthetic materials carry the risk of loosening and infection (Rah, 2000). Distraction osteogenesis, while increasingly utilized as endogenous bone tissue engineering, also has complications in upward of 35% cases, such as pin-tract and soft-tissue infections, scarring, device failure, and non-union (McCarthy *et al.*, 1992; Mofid *et al.*, 2001). Accordingly, stem-cell-based strategies for craniofacial reconstruction may overcome these deficiencies.

(5.1) Adipose-derived Stem Cells Heal Calvarial Defects

Adipose-derived mesenchymal cells (AMCs) are readily obtained *via* lipo-aspirate, and can be expanded in culture (Zuk *et al.*, 2001, 2002; De Ugarte *et al.*, 2003). Several groups have reported that AMCs are multipotent and capable of differentiation into muscle, bone, and cartilage cells (Zuk *et al.*, 2001, 2002; Gimble and Guilak, 2003; Hicok *et al.*, 2004). Bone tissue engineering is perhaps the most widely investigated application of AMCs. For example, rat AMCs seeded in polyglycolic acid scaffolds and implanted subcutaneously have led to bone formation (Lee *et al.*, 2003). Similar findings have been noted with human AMCs using HA-TCP scaffolds in immunodeficient mice (Hicok *et al.*, 2004). Recently, human AMCs manipulated *via* BMP-2-mediated gene therapy in collagen I scaffolds induced significant bone formation in the hindlimb of immunodeficient mice (Dragoo *et al.*, 2003, 2005). Finally, AMCs were used as a potential source for cell-based therapies for healing calvarial defects (Fig. 5, from Cowan *et al.*, 2004). The adipose-derived stem cells were seeded in apatite-coated PLGA scaffolds and implanted into the surgically created, critical-size calvarial defects (Cowan *et al.*, 2004). AMCs induced new bone formation similar in amount to that induced by either bone marrow MSCs or osteoblasts (Cowan *et al.*, 2004).

While these experimental reports from investigations with rodents are exciting, the real question relates to the translation of experimental work into clinical practice. In other words, how close are we to engineering bone using human-derived AMCs? Fortunately, this question has recently been addressed by a case report from Germany. Human-derived AMCs were combined with bone chips from the iliac crest and used to regenerate a large calvarial defect to near-complete continuity in a seven-year-old patient (Lendeckel *et al.*, 2004). This important report provides a proof of principle that AMCs can be utilized for bone tissue engineering. Future strategies will no doubt focus

on the enrichment of osteoprogenitors within this heterogeneous population and continued optimization of scaffolds that promote osteogenesis.

(5.2) Special Considerations of Cranial Skeletal Repair

The skeleton of the head has a complex developmental history. Whereas the appendicular skeleton is derived solely from the mesoderm and forms bone through endochondral ossification, the cranial skeleton is a patchwork of bony elements derived from the cranial neural crest and the paraxial mesoderm, which forms bone through both endochondral and intramembranous ossification (Le Lièvre and Douarin, 1975; Noden, 1988; Couly *et al.*, 1993; Chai *et al.*, 2000; Jiang *et al.*, 2002). If adult tissue repair recapitulates fetal development, one might ask: Is cranial repair a different process from skeletal healing in other parts of the body?

Growing evidence indicates that at least one critical cellular player in the repair process—namely, the calvarial osteoblast—exhibits several distinctive characteristics, depending upon cellular derivation (for reviews, see Allen *et al.*, 2004; Barry and Murphy, 2004; Taichman, 2005). The first unique feature among osteoblasts from various parts of the body is their response to molecular signals: Both *in vitro* and *in vivo* responses to growth factors are different between calvarial osteoblasts and osteoblasts derived from the appendicular and axial skeletons (Anderson and Danylchuk, 1978; Uddstromer and Ritsila, 1979; Miller *et al.*, 1991; Sheng *et al.*, 1999). A second noticeable difference is the response of osteoblasts from different sites of the body to mechanical stimuli (Hock *et al.*, 1982; Allen *et al.*, 2004; for reviews, see de la Fuente and Helms, 2005; Mao, 2005b). Even within a single skeletal element, periosteal osteoblasts are dissimilar to endosteal osteoblasts, both in their response to molecular signals and in their response to mechanical stress (for reviews, see Epker and Frost, 1965; Jones *et al.*, 1991; Bord *et al.*, 2001; Midura *et al.*, 2003). Another difference is embryonic origin. The appendicular and axial osteoblasts are derived from mesoderm, whereas calvarial osteoblasts originate from the neural crest (Noden, 1988; Couly *et al.*, 1993, 2002; Chai *et al.*, 2000; Brault *et al.*, 2001; Jiang *et al.*, 2002; Ruhin *et al.*, 2003; Brewer *et al.*, 2004; Le Douarin *et al.*, 2004). Does any of these differences affect osteoblast behavior during bone repair? This fundamental question has rarely been systematically addressed. Therefore, we are left with the question: Does the embryonic origin of a bone have an effect on how it heals? Most of our knowledge of skeletal repair comes from the analysis of long-bone fractures. To understand if the repair of cranial defects is equivalent to, or distinctive from, the healing of long bones, one must compare the two healing processes directly. Variables to keep in mind include the dual origins of the cranial skeleton and the process by which individual bones form: *i.e.*, intramembranous *vs*. endochondral. The mechanical environment plays an indisputable role in long-bone healing (Rodriguez-Merchan and Forriol, 2004; Isaksson *et al.*, 2006) and most likely influences cranial healing as well, but this important aspect remains to be further characterized (Carter *et al.*, 1998; Mao, 2005b). Answers to these kinds of questions will have direct and profound implications for the treatment of skeletal injuries, diseases, and disfigurements.

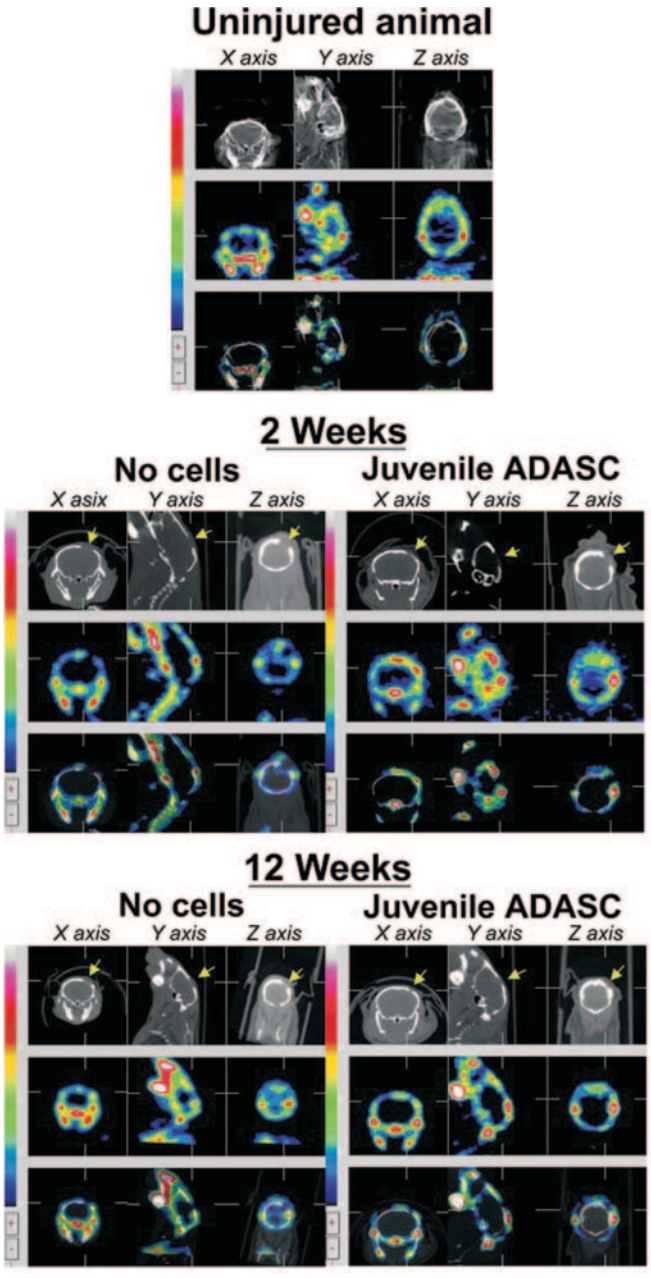

Figure 5. Bone metabolic activity of animals implanted with control (no cells) or adipose-derived adult stromal (ADS) cell-seeded scaffolds, as determined by radiolabeled methylene diphosphonate incorporation overlaid with micro-CT images. For each time-point, the top row displays the micro-CT scan, the middle row displays the metabolic activity, and the lower row displays the overlaid composite of metabolic activity and micro-CT scan. For all columns at each time-point, the left column is the x axis, the middle column is the y axis, and the right column is the z axis. For orientation, we have marked the defect with a yellow arrow for the three views of the micro-CT image. The location of the defect does not change between 2 and 12 weeks. Bone scan intensity is indicated in color on the left axis of the image, with white and red indicating the highest value and black and blue indicating lowest value. This Fig. originally appeared in Cowan *et al.* (2004) and is reproduced here with permission from the Nature Publishing Group (http://www.nature.com/).

(5.3) Craniofacial Bone Regeneration after Ablative Cancer Surgery and Radiation Therapy

Oral cancer represents about 5% of all malignancies in the United States (Greenlee *et al.*, 2001). Despite advances in treatment strategies, the morbidity and mortality rates are significant, with a significant number of patients developing recurrence, distant metastasis, and second primary tumors

(Denham *et al.*, 2001; Myers *et al.*, 2001). Approximately 50% of all cancer patients receive radiation therapy. Radiation toxicity adversely affects bone development, remodeling, and fracture healing (Mitchell and Logan, 1998; Spear *et al.*, 1999). Radiation therapy also complicates reconstructive surgery, due to increased apoptosis and compromised vascularization (Okunieff *et al.*, 1998a,b). Irradiation of up to 8 Gy did not affect the BMP-2-induced osteoblast differentiation of C2C12 cells *in vitro* (Ikeda *et al.*, 2000). New bone was formed on BMP-2-treated hydroxyapatite disks implanted into a subperiosteal pocket in rabbits radiated pre-operatively with a fractionated dose of 20 Gy (Howard *et al.*, 1998). Despite these encouraging findings, 3-mm rat calvarial defects pre-operatively treated with a 12-Gy radiation dose, and subsequently with BMP-2, had successful bone regeneration, but incomplete healing of the defect (Wurzler *et al.*, 1998). In rat mandibular defects treated with a fractionated 45-Gy radiation dose, followed by transplantation of demineralized bone powder, only 39% of defects had more than 50% bone fill (Lorente *et al.*, 1992).

Combinatorial therapies appear to improve bone regeneration in irradiated sites. When a pedicle muscle flap was combined with BMP-3 treatment, complete healing of craniofacial defects occurred after a single pre-operative 15-Gy radiation dose (Khouri *et al.*, 1996). Treatment with no implant, with a microvascular muscle flap alone, or with BMP-3 alone did not heal the defect, suggesting the need for a well-vascularized recipient bed and an adequate population of responsive osteogenic cells, in addition to the delivering of an osteoinductive protein (Khouri *et al.*, 1996). The most effective bone regeneration for irradiated cranial sites appears to be a combination of cell transplantation and gene therapy approaches (Nussenbaum *et al.*, 2003, 2005). For example, it has been demonstrated that bone formation by transplanted bone marrow stromal cells could be greatly enhanced by the concurrent administration of anabolic doses of parathyroid hormone (Schneider *et al.*, 2003). However, it has yet to be established if such combinations of therapies would be effective in pre-operatively irradiated sites.

(6) IMPACT OF CRANIOFACIAL TISSUE ENGINEERING ON CLINICAL PRACTICE

Contemporary dental practice is largely based on conventional, non-cell-based therapies that rely on durable materials from outside the patient's body. Amalgam, composites, metallic implants, synthetic materials, and tissue grafts from human cadavers and other species have been the mainstream choices for the restoration of dental, oral, and craniofacial structures. Despite various levels of clinical success, conventional materials suffer from intrinsic limitations, such as potential immune rejection, transmission of pathogens from the donor, and the general inability of conventional materials to remodel with recipient tissues and organs. An often-preferred approach by surgeons to use autologous grafts, such as bone grafts, necessitates donor site morbidity. In contrast, tissue engineering relies on the principle that mesenchymal stem cells are capable of generating virtually all craniofacial structures, and temporary biomimetic scaffolds are necessary for accommodating cell growth and tissue genesis. Scaffolds provide the temporary structural framework for cells to synthesize extracellular matrices and other functional components in the intended shape and dimensions. Upon neogenesis of tissues or organs derived from stem cells, scaffolds should undergo degradation.

Decades of contemporary biomedical research have focused primarily on the understanding of the mechanisms of biological functions in health and disease. For example, our understanding of the mechanisms of dental caries has advanced tremendously. In contrast, the end goal of tissue engineering is to develop products capable of healing diseased or lost tissues and organs, thus representing a departure from conventional biomedical research, whose primary focus is an understanding of mechanisms. This does not imply that the understanding of mechanisms is unimportant in tissue engineering. Instead, an understanding of the mechanisms of interactions among cells, growth factors, and biomaterials undoubtedly will advance the end goal of developing cell-based therapies and off-the-shelf tissue-engineering products. However, an understanding of mechanisms is the means for tissue engineering, but not the end. A cadre of craniofacial tissue engineers with interdisciplinary skills in stem cell biology, molecular biology and genetics, polymer and materials science, and mechanical engineering, and a clinical knowledge of dental, oral, and craniofacial disorders is needed to advance the field of craniofacial tissue engineering.

Craniofacial tissue engineering is an opportunity that dentistry cannot afford to miss. This notion is based on both biological and strategic reasons. Biologically, mesenchymal cells are primarily responsible for the formation of virtually all dental, oral, and craniofacial structures. Mesenchymal stem cells, the reservoir of mesenchymal cells in the adult, have been demonstrated, in tissue engineering, to generate key dental, oral, and craniofacial structures. Many dental and craniofacial structures are readily accessible, thus presenting a convenient platform for biologists, bioengineers, and clinicians to test tissue-engineered prototypes (Hollinger and Winn, 1999; Alsberg *et al.*, 2001). Strategically, tissue-engineering technologies pioneered outside the dental community may have profound implications on dental practice.

The impact of craniofacial tissue engineering extends beyond dental practice. Several craniofacial structures engineered thus far serve as prototypes for the tissue engineering of non-craniofacial structures. Craniofacial-derived stem cells have potential implications in the tissue engineering of not only craniofacial structures, but also non-craniofacial tissues (Shi *et al.*, 2005; Sonoyama *et al.*, 2005). Tissue-engineered bone with customized shape and dimensions has the potential for the biological replacement of not only craniofacial bones, but also of segmental defects in the appendicular bones (Feinberg *et al.*, 2001; Chu *et al.*, 2002; Moore *et al.*, 2004). Calvarial bone defects are the most frequently used models for bone tissue engineering (Cowan *et al.*, 2004; Meinel *et al.*, 2005). The tissue-engineered mandibular condyle has served as a prototype for the engineering of other joints, such as the knee and hip (Alhadlaq and Mao, 2003, 2005; Alhadlaq *et al.*, 2004; Hollister, 2005; Hollister *et al.*, 2005; Mao, 2005a; Marion *et al.*, 2005). Tissue-engineered cranial sutures and periodontium offer clues for the fabrication of composite tissue constructs with multiple cell types and encapsulated in multiple materials (Giannobile and Somerman, 2003; Hong and Mao, 2004; Nakahara *et al.*, 2004; Rahaman and Mao, 2005; Moioli *et al.*, 2006). Several meritorious studies have led to the *in vitro*

fabrication of a TMJ disc from cell- and growth-factor-based approaches (Almarza and Athanasiou, 2004; Detamore and Athanasiou, 2005). Adipose tissue grafts engineered from stem cells with sustained shape and dimensions may offer applications in both facial reconstructive surgeries as well as augmentative and/or reconstructive procedures elsewhere (Alhadlaq *et al.*, 2005; Stosich and Mao, 2005, 2007). Conversely, craniofacial tissue engineering could not have advanced to the current stage without the incorporation of interdisciplinary skill sets of stem cell biology, bioengineering, polymer chemistry, mechanical engineering, robotics, etc. Thus, craniofacial tissue engineering and regenerative dental medicine are integral components of regenerative medicine.

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