

## INVITED MEDICAL REVIEW

**Bioengineering strategies for regeneration of craniofacial bone: a review of emerging technologies**BB Ward<sup>1</sup>, SE Brown<sup>2,3</sup>, PH Krebsbach<sup>2,3</sup>

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**Although advances in surgical techniques and bone grafting have significantly improved the functional and cosmetic restoration of craniofacial structures lost because of trauma or disease, there are still significant limitations in our ability to regenerate these tissues. The regeneration of oral and craniofacial tissues presents a formidable challenge that requires synthesis of basic science, clinical science, and engineering technology. Tissue engineering is an interdisciplinary field of study that addresses this challenge by applying the principles of engineering to biology and medicine toward the development of biological substitutes that restore, maintain, and improve normal function. This review will explore the impact of biomaterials design, stem cell biology and gene therapy on craniofacial tissue engineering.**

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**Introduction**

The craniofacial unit is a complex array of bone, cartilage, soft tissue, nerves and vasculature; giving rise to the most esthetically important component of the body. Damage to these structures, even when minimal, usually leads to noticeable deformity. When changes are extensive, difficult reconstructive challenges result. In addition, in the case of tumor treatment, the craniofacial unit is frequently subjected to radiation and a variety of chemotherapeutic strategies, creating a compromised wound bed for reconstructive surgery.

The bones of the craniofacial skeleton provide the foundation upon which other units are built and while soft tissue compensation is possible, adequate bony support is key to the return of both esthetics and function. For many years, bone grafts (autogenous, allogenic, and xenogenic) have been the mainstay for replacement. Current state of the art reconstruction of large bony maxillofacial defects involves free tissue transfer with microvascular reanastomosis of vascularized flaps from distant sites including fibula, iliac crest, scapula, and radius (Disa and Cordeiro, 2000; Emerick and Teknos, 2007). While these procedures have proven to be reliable and effective, they require extended hospitalization, and a secondary donor site with associated morbidity and complications. Furthermore, these techniques struggle to fully replicate normal form and function. As an alternative to the classic surgical approaches, developments in tissue engineering, gene therapy, and stem cell biology strive to utilize cells, biomaterial scaffolds and cell signaling factors to regenerate large skeletal defects with precise replication of normal body contours. A tissue engineering approach offers several potential benefits, including the lack of donor site morbidity, decrease in technical sensitivity of the repair, and most importantly, the ability to closely mimic the *in vivo* microenvironment in an attempt to recapitulate normal craniofacial development.

The optimal bone construct for repair would exactly replicate the lost structure, be fabricated with techniques generalizable to laboratories across the world, and would ultimately be replaced through the body's normal physiological processes of homeostasis over time. Furthermore, it would be reliable not only in standard tissue conditions but also in defects with compromised tissue beds following infection, radiation, chemotherapy and the scar of extensive trauma. Although no current technology meets all of these criteria, progress has been made in the preclinical arena that gives confidence to achieving this goal. The purpose of this review is to provide an overview and update on the current state of the art in bone reconstruction using new and evolving

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technologies. Discussion will be focused on the broad categories of tissue engineering, gene therapy, and stem cell biology with attention to concepts and the current state of art based on ongoing research. While human clinical applications are limited to date, great promise exists as technology and our understanding of these principles increases over time.

## Tissue engineering

Tissue engineering is an interdisciplinary field of study that applies the principles of engineering to biology and medicine toward the development of biological substitutes that restore, maintain, and improve normal function (Langer and Vacanti, 1993; Scheller *et al*, 2009). Cells, designed biomimetic materials, and biochemical signals compose the tissue engineering triad, and the significant interdependence of all three elements is often used in concert to produce constructs suitable for replacement of damaged or diseased tissue. The multidisciplinary nature of craniofacial tissue engineering strategies stems from formidable surgical challenges faced in the clinical setting, where the need to regenerate tissue that restores function to oral and craniofacial structures has long been of great importance (Hollinger and Winn, 1999; Alsberg *et al*, 2001; Steiner *et al*, 2002). Specifically, craniofacial bony structures such as the alveolar ridge (Hibi *et al*, 2006), the maxillary sinus floor (Shayesteh *et al*, 2008), and the hard palate (Carstens *et al*, 2005) have been reported to be successfully regenerated using tissue engineering strategies in clinical studies. Additionally, calvarial bone (Tu *et al*, 2007) and the mandibular condyle of the temporomandibular joint (TMJ) (Alhadlaq and Mao, 2005; Lee *et al*, 2009) have been reconstructed successfully in preclinical animal models. However, given the developmental differences between the appendicular skeleton (mesodermal origin – undergoes endochondral ossification) and the cranial skeleton (neural crest and paraxial mesodermal origin – undergoes both endochondral and intramembranous ossification), significant advances in understanding the complex developmental patterns of the craniofacial unit must take place to reach the goal of using optimally designed biomaterials for human clinical applications (Jiang *et al*, 2002). Therefore, the engineering design principles and techniques needed to achieve the goal of controlling and regenerating components of an intricate biological tissue system such as the oral and craniofacial complex are diverse. The focus of this review will be to describe successful models currently applying these principles in the preclinical and clinical setting, although it is important to note that the preclinical findings may not always translate to the human experience.

In the bone tissue engineering paradigm, the purpose of the scaffold is to provide an osteoinductive extracellular matrix (ECM) analog to support initial cell adhesion, growth and development of new bone. Within the craniofacial tissue engineering field, the major classes of materials used are natural polymers, synthetic polymers, ceramics, composite materials, and electrospun nanofibers. In addition to selection of the

appropriate material, the scaffold should possess design characteristics that elicit a biological response from the microenvironment. Through optimizing design parameters and fabrication techniques, the ideal scaffold candidate is required to achieve the following: (i) to deliver progenitor cells or facilitate host cell recruitment via osteoconductive and osteoinductive material properties; (ii) to deliver important signaling molecules in a temporally and spatially controlled manner via growth factor incorporation and surface modification; (iii) to promote vascularization and tissue in-growth via changes in microporosity; (iv) to properly fit the shape of the anatomical defect via image and Computer-Aided Design (CAD) based scaffold design methods; (v) to provide initial plasticity while maintaining load-bearing stability via selection of the appropriate fabrication technique; and (vi) to degrade into biocompatible by-products at a rate that matches new tissue formation via selection of the optimal material composition (Giesen *et al*, 2004; Meinel *et al*, 2004; Hollister, 2005; Mao *et al*, 2006; Gersbach *et al*, 2007; Hutmacher and Cool, 2007; Hutmacher *et al*, 2007; Wang *et al*, 2007; Jones *et al*, 2009). To address the requirements of scaffolds specifically for craniofacial bone repair, one must consider the delicate balance of imparting sufficient porosity, permeability, and pore architecture to facilitate vascular invasion, mass transport, and cell survival in the scaffold interior, while maintaining the ability to withstand load bearing conditions that craniofacial bone sustains as a result of oral functions such as mastication, deglutition, and speech (Hollister, 2005; Hutmacher and Cool, 2007; Jones *et al*, 2007; Potier *et al*, 2007).

The requirement to include more of the above design criteria into a single biomaterial construct has spurred extensive investigation on the use of natural, synthetic, and composite materials. Natural scaffolds such as collagen type I, chitosan, calcium alginate, hyaluronic acid and composites have been shown to be osteoconductive, thus suitable for bone formation both *in vitro* and *in vivo* (Solchaga *et al*, 2002; Abbah *et al*, 2006; Chang *et al*, 2009; Chesnutt *et al*, 2009). However, a major disadvantage is the lack of mechanical integrity. Alternatives with structural stability have been investigated, including the use of synthetic polymers such as poly(lactic acid) (PLA), poly(glycolic acid) PGA, co-polymer polylactic-co-glycolic acid (PLGA), poly(methyl methacrylate) (PMMA) and poly(caprolactone) (PCL). These polymers exhibit the ability to support osteoblastic differentiation and bone tissue formation, and are therefore commonly used for oral and maxillofacial applications (Li *et al*, 2005; Petrie *et al*, 2008; Kretlow *et al*, 2009). Additionally, polymer/ceramic composite materials such as PLLA/HA have been used successfully as osteochondral constructs for TMJ engineering via the delivery of porcine chondrocytes in the polymer layer and fibroblasts transduced with adenovirus driving the expression of bone morphogenetic proteins (BMPs) (Schek *et al*, 2004, 2005).

Designing matrices suitable for the recruitment of osteoprogenitor/stem cells has been promoted by the approach of mimicking the composition, morphological

traits and mechanical function of the native bone ECM through the use of a wide range of electrospun bone regenerative nanofiber materials such as PLLA, PGA, PCL, silk fibroin, calcium phosphates, bioactive glass and glass ceramics (Jang *et al*, 2009). The nanofibrous structure is becoming increasingly useful and popular within the field of craniofacial tissue engineering because these materials are able to assist cells along an osteogenic pathway via nanoscale organization and novel nanocomposite systems (Kim *et al*, 2006).

The addition of osteoinductive factors is also essential to the success of craniofacial bone regeneration strategies. BMPs, primarily 2, 4 and 7, fulfill this role in the vast majority of applications. BMPs are a family of growth factors important to bone formation and regeneration which have been studied extensively (Reddi, 2005) and are now available in a recombinant form for human clinical application. One of the limiting factors in this methodology has been the duration of the BMP's presence at the site of action with one of the potential roles of the scaffolds being to assist in controlling growth factor release. Importantly, the balance of sustained release required for optimal effect while avoiding overly high concentrations leading to potential untoward effects must be managed.

Several preclinical models exist for the use of BMPs and recombinant human BMP (rhBMP) as a concentrate delivered on scaffolds, composites, and hydrogels. Defects studied have included animal models for ulna, radius, femur, alveolar cleft, spine, orbital floor, tibia, and calvarial defects (Bessa *et al*, 2008). At present, the only FDA-approved craniofacial application of rhBMP is for sinus augmentation. A recent report highlighting this use enrolled 160 subjects in a multi-center, randomized prospective trial using rhBMP on a collagen sponge versus autogenous bone graft for two-stage maxillary sinus floor augmentation. Outcomes measured included new bone formation, implant integration and functional loading at 6 months and 2 years. Success rates for both groups was 79% with significantly denser bone in the rhBMP-2 group. In addition, the autograft group was noted to have 17% long-term parasthesia, pain, or gait disturbance, highlighting the effects of donor site morbidity.

Limited clinical studies exist for tissue engineering concepts other than BMPs and platelet-derived growth factors (PDGF) in any body location. The majority of evidence is derived from small case series without controls and pertains to general orthopedic, rather than craniofacial applications. In one study, three patients with aneurysmal bone cyst, giant cell tumor, and fibrous dysplasia were treated with implants based on HA ceramics with multipotent stem cells induced toward the osteoblast lineage. Successful healing was reported although only based on plain radiographs and computerized tomography. There were no adverse reactions noted in this study with a limited number of patients (Morishita *et al*, 2006).

The preclinical and clinical studies reviewed here indicate that significant advancements are being made in the design of biomaterials to create replacements

for damaged or pathological tissues. As biomaterials become highly functionalized platforms upon which hybrid tissues can develop, craniofacial tissue engineering strategies hold significant promise for future clinical use.

## Stem cell biology

From an embryological perspective, the structures of the craniofacial region are predominantly derived from cells of neural crest mesenchymal origin. These cells, together with their synergistic partners, mesodermal cells, both arise from the embryonic stem cell (Couly *et al*, 1993; Jiang *et al*, 2002). Mesenchymal stem cells (MSCs) are adult cells from this lineage that persist with the ability to repair and regenerate tissue following insult. MSCs have the genetic capacity to differentiate into both mesenchymal and bone tissue lineage making them a useful tool and target for research in tissue regeneration (Krebsbach *et al*, 1997, 1999). Highlighting this potential is the fact that a single population of MSCs can differentiate into both chondrocytes and osteoblasts based on external factors to produce both bone and cartilage. MSCs are self renewable lineages which were first isolated and reported nearly four decades ago, and since then, techniques for isolation have become increasingly effective in regard to throughput and specificity with current use of fluorescence-activated, magnetic-activated cell-sorting, and genomic profiling (Friedenstein *et al*, 1966; Gronthos *et al*, 1999; Jones *et al*, 2002; Kinnaird *et al*, 2004). In addition, cloning technology can be harnessed to create large quantities of the desired cells. Given their properties, stem cells have been utilized in a variety of preclinical applications for regeneration of craniofacial bone.

One example of a complex bioengineering strategy for craniofacial bone, highlighting its potential, is the development of the mandibular condyle. It has been shown that the generation of (i) a cadaveric human mandibular condyle of bone and cartilage derived from adult rat bone marrow MSCs, exposed to osteogenic and chondrogenic conditions, and encapsulated in poly(ethylene glycol) diacrylate (PEDGA) hydrogels (Alhadlaq and Mao, 2003), and (ii) the vascularization and ectopic tissue formation of an anatomically shaped human tibia condyle made of a composite poly- $\epsilon$ -caprolactone and hydroxyapatite material seeded with osteogenically differentiated human MSCs after 6 weeks of subcutaneous implantation in athymic rats (Lee *et al*, 2009), are possible. This suggests that optimal cell types and local factors are required to make the possibility of an engineered condyle look promising for human application.

Adipose derived stem cells (ADSCs) are an alternative progenitor cell source, in that these cells are easily accessible through lipo aspirate and can be expanded *in vitro* to form adipocytes, chondrocytes and osteoblasts (Zuk *et al*, 2001). Additionally, ADSCs have been successfully applied to scaffolds and have undergone *de novo* formation of bone in subcutaneous tissue, calvarial defects, and critical sized bone defects in murine models (Lee *et al*, 2003; Yoon *et al*, 2007; Hao

*et al*, 2008). Translation of this technology to human application has been reported with ADSCs combined with iliac crest bone graft for a calvarial bone defect (Zuk *et al*, 2001; Shi *et al*, 2005).

In addition to MSCs and ADSCs, there has been extensive investigation into the capability of MSCs derived from various craniofacial tissues that form bone-like, dentin-like, and cementum-like structures. For instance, dental pulp stem cells have been isolated from dental pulp and in response to osteogenic conditions, have been shown to express osteogenic and odontogenic markers and form mineral deposits on dentin-like structures (Gronthos *et al*, 2000; Murray *et al*, 2001; Ueno *et al*, 2001). Periodontal ligament stem cells (PDLSCs) first isolated, sorted, and cultured from extracted third molars of human origin have been found to differentiate into adipocytes and osteoblasts *in vitro*, and generate cementum-like structures interfaced with dense collagen fibers *in vivo* (Shi *et al*, 2002; Seo *et al*, 2004). Lastly, *in vitro* expansion of human exfoliated deciduous teeth stem cells have been shown to have proliferative capacity, a surface antigen profile similar to MSCs, and have the ability to express osteogenic genes and form dentin-like structures *in vivo* when seeded onto HA/TCP scaffolds (Miura *et al*, 2003).

Although the cell types discussed have potential for great use for bone repair, given the complex nature of craniofacial bone, the use of human embryonic stem cells (hESCs) might provide a repository of cells that can be isolated, manipulated, and utilized for future cell-based engineering strategies (Thomson *et al*, 1998). One goal for hESC research is the controlled differentiation into specific progenitor cells for the purpose of replacing or regenerating damaged tissue. Therefore, the ability to obtain large quantities of multipotent cells from hESCs represents a challenge for cell based therapy and tissue engineering strategies that currently rely on human bone marrow stromal cells (hBMSCs). Recent studies show that mesenchymal precursors have been derived from hESCs (hES-MSCs) via various isolation methods, and the generation of osteoblasts has been achieved in the presence of known osteogenic supplements and co-culture with primary bone derived cells (Sotille *et al*, 2003; Bielby *et al*, 2004; Barberi *et al*, 2005; Cao *et al*, 2005; Ahn *et al*, 2006, Karp *et al*, 2006; Olivier *et al*, 2006; Duplomb *et al*, 2007; Karner *et al*, 2007; Tong *et al*, 2007; Arpornmaeklong *et al*, 2009; Brown *et al*, 2009). However, the identification and characterization of a pure osteoprogenitor cell population has yet to be achieved. Osteoprogenitor cells derived from hESCs have tremendous potential, as they can serve as a tool through which one can not only characterize early bone development and cellular behavior on bone-related biomaterials but also have application specifically in craniofacial bone repair.

To enhance the understanding of the differentiation pattern and bone formation capacity of hESCs in the skeletal defect, investigators have studied the complete temporal pattern of osteoblastic differentiation of hES-MSCs in a long-term culture, as well as the influence of the three-dimensional matrix on the osteogenic differ-

entiation and bone formation capacity of hES-MSCs in the calvarial defect. It was found that incubation of hES-MSCs in osteogenic medium induced osteoblastic differentiation in a similar chronological pattern to previously reported human bone marrow stromal cells (hBMSCs) and primary osteoblasts. Furthermore, it was also demonstrated that differentiation was enhanced by three-dimensional matrix of collagen scaffolds (Arpornmaeklong *et al*, 2009). The fate of transplanted cells in the bone formation process was verified by identifying the presence of human cells in the matrix of the newly formed bone, suggesting that hES-MSCs represent an osteoprogenitor population that can be sorted, enriched and manipulated for use in craniofacial engineering strategies. Of particular importance is the fact that the lineage progression through both mesodermal and neural crest lineages can be controlled for hESCs, addressing one of the major challenges unique to healing the cranial skeletal given the dual embryonic origins (Jiang *et al*, 2002; Mao *et al*, 2006; Lian *et al*, 2007; Brown *et al*, 2009; Goldstein *et al*, 2010).

### Gene therapy

Gene therapy depends on the transfer of genetic material into living cells for the purposes of treating a disease process or regenerating tissues. Both viral and non-viral vectors for gene transfer have been developed and applied to pre-clinical and limited clinical settings (Scheller and Krebsbach, 2009). Non-viral vectors have advantages attributable to safety and the ability to introduce large segments of DNA; however, viral vectors remain the mainstay of therapy as a result of their efficacy in transmitting the genetic material to the host. Compared with the focus of gene therapy on treatment of disease, the use of gene therapy for the regeneration of craniofacial bone has been somewhat limited. However, animal craniofacial models for gene therapy do exist not only for bone (Krebsbach *et al*, 2000; Dunn *et al*, 2005), but also for cartilage (Palmer *et al*, 2002) and periodontal ligament (Jin *et al*, 2004) as well as complex multiunit structures such as the TMJ (Nakashima *et al*, 2006; Rabie *et al*, 2007) and salivary glands (Cotrim *et al*, 2006; Voutetakis *et al*, 2008).

A number of applications are currently evolving with promise for future augmentation of the bone healing process or replacement of missing bone. BMPs have maintained a prominent role in this model with potential advantages of gene therapy conveying the ability to maintain protein expression at clinically effective levels over longer periods of time compared with direct delivery of protein. In addition, because of the sustained presence, gene delivery techniques are one of the few modalities studied for potential application in irradiated fields (Nussenbaum *et al*, 2003; Hu *et al*, 2010).

Enhanced fracture repair and bone formation have been achieved using viral-mediated gene delivery. A number of studies (Peng *et al*, 2004a; Shen *et al*, 2004a,b) using muscle-derived cells for *ex vivo* transduction of bone resulted in fracture repair when placed into femur critical sized defects. Transduction with

BMP-4 leads to radiographic and histological healing with bone, which was  $77 \pm 28\%$  the strength of normal control femur. Other work has demonstrated that the addition of VEGF to BMP-4 enhances this process. Likewise, other groups have reported a number of studies using a similar approach with BMP-2 (Betz *et al*, 2010). In this model, an expedited *ex vivo* approach where muscle or fat fragments were transduced with BMP-2 and incubated in tissue culture only 24 h prior to implantation. Moreover, the femoral critical size defect model was used with radiographic and histological evaluation. Bone volumes in the transduced sites showed statistically significant increases in bone formation with 100% of bone defects being bridged in the study animals. BMPs 6, 7 and 9 have also been utilized in gene therapy bone repair models (Alden *et al*, 2000; Jane *et al*, 2002) with similar abilities to induce bone formation.

While BMPs have been the most frequently investigated factors for gene delivery, alternatives have been described. Sonic hedgehog (Shh), PDGF-B, and constitutively active activin-like kinase 2 (caALK2) have all been investigated. Shh was studied in a craniofacial model using rabbit cranial defects (Edwards 2005). Human Shh cDNA was isolated from fetal lung tissue and cloned into the replication-incompetent retroviral expression vector LNCX. Alginate/type I collagen constructs with Shh transduced cells were placed in cranial defects and examined grossly, radiographically, and histologically at 6 weeks. Shh treated animals had statistically significant bone healing compared with controls and no treatment related side effects were noted. Interestingly, caALK2 has been investigated using an allograft model for enhanced healing. Allografts were coated with AAV-caALK2 vector which mediated *in vivo* gene transfer. The AAV vector was capable of transducing osteoblasts in the fracture callus and directly inducing bone formation (Anusaksathien *et al*, 2004; Ulrich-Vinther, 2007).

In an effort to determine if regenerative gene therapy methods could overcome the negative effects of radiation therapy, animal models that more directly mimic the compromised wound beds of irradiated cancer patients have been used for testing *ex vivo* (Nussenbaum *et al*, 2003) and *in vivo* (Hu *et al*, 2010) gene therapy. In a critical size calvarial bone defect animal model, bone defects were treated with an inlay calvarial bone graft or transduced dermal fibroblasts. Two weeks postoperatively animals were randomized to receive 12-Gy radiation or no radiation control. While radiation treatment decreased the amount of bone formation from 87% to 65% bone regeneration was still successful giving promise to the possibilities of treating ablative defects prior to radiation therapy with these treatment modalities (Nussenbaum *et al*, 2003).

An alternative approach to gain more control over the timing of expression of transferred genes has been the use of inducible vector systems. An example of this is the doxycycline inducible 'tetON' promoter system with selective induction of BMP-2 or BMP-4. In bone regeneration models, only vector containing sites

induced bone following administration of oral doxycycline (Gafni *et al*, 2004; Peng *et al*, 2004b). Taken together, these examples illustrate the potential benefits of gene therapy over other bioengineering approaches and will undoubtedly continue its progress as a leading candidate for future clinical applications.

## Conclusion

Craniofacial tissue engineering research has tremendously developed over the last two decades as a result of the individual strides made in the fields of developmental biology, stem cell biology, polymer chemistry, mechanical engineering, and biomedical engineering. The unique challenges faced by maxillofacial surgeons in the clinical setting has spurred investigation of effective tissue engineering strategies that involve isolated and enriched progenitor cells, sophisticated gene delivery methods, and complex biomaterial scaffold fabrication and design techniques. As we begin to understand how biomaterial properties influence cellular behavior, we will progress toward the development of biomimetic scaffolds that contain incorporated signaling cues that induce cellular differentiation and ECM deposition, possess composite material properties that have the ability to generate hybrid tissue, and have tunable three-dimensional geometrical architecture that appropriately restores form and function to craniofacial bone defects.

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