Craniofacial Tissue Engineering

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The craniofacial complex is composed of fundamental components such as blood vessels and nerves, and also a variety of specialized tissues such as craniofacial bones, cartilages, muscles, ligaments, and the highly specialized and unique organs, the teeth. Together, these structures provide many functions including speech, mastication, and aesthetics of the craniofacial complex. Craniofacial defects not only influence the structure and function of the jaws and face, but may also result in deleterious psychosocial issues, emphasizing the need for rapid and effective, precise, and aesthetic reconstruction of craniofacial tissues. In a broad sense, craniofacial tissue reconstructions share many of the same issues as noncraniofacial tissue reconstructions. Therefore, many concepts and therapies for general tissue engineering can and have been used for craniofacial tissue regeneration. Still, repair of craniofacial defects presents unique challenges, mainly because of their complex and unique 3D geometry.

The most common causes of craniofacial defects are congenital birth defects (1/700 live births), trauma, inflammation, and cancer surgeries (Miura et al. 2006). Among these, the most prevalent is acute trauma, including falls, assaults, sports injuries, and vehicle crashes (Rocchi et al. 2007; Grayson et al. 2015; Hunter et al. 2015). Significant facial trauma can also result from battlefield injuries, particularly when combined with an increased survivability of wounded soldiers because of improved battlefield medical care and body armor. A recent report indicated that craniomaxillofacial injuries can represent up to 26% of all battlefield injuries, as occurred in Operation Iraqi Freedom/Operation Enduring Freedom (Afghanistan) (Lew et al. 2010). Congenital anomalies (CAs) are major causes of infant mortality and childhood morbidity, affecting 2%–3% of all babies (Mossey and Castilla 2003). Genetic birth defects, environmental exposure, and folic acid deficiency are the main causes of CA.

Craniofacial bone reconstruction plays a crucial role in craniofacial repairs, because they provide support for adjacent soft tissues and anchorage for dental structures, thereby guiding the structural stability and appearance of the face (Fig. 1) (Petrovic et al. 2012). Especially for extensive craniofacial injuries, successful regeneration of craniofacial bone is necessary to restore normal function of the craniofacial complex (Genden 2010; Ward et al. 2010). With sufficient bone structure, it is comparatively easier to restore the soft tissues of the craniofacial complex to form the facial features. An extreme example of this is the world's first partial face transplant from a cadaver to a living human, which was performed on November

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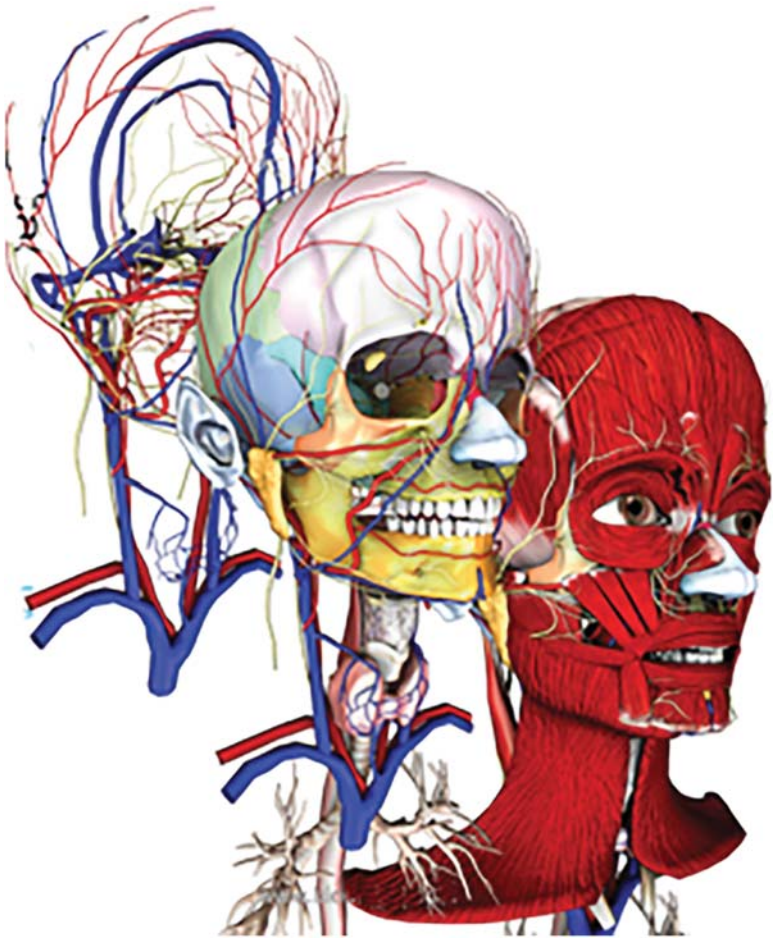

Figure 1. Schematic of the layered structure of craniofacial tissues. The skull and craniofacial bones provide the structural support for the muscle, vascular network, and skin (from catalog.biodigital.com/storeImages/detail/cranio_dvd.jpg).

27, 2005, and which has been performed globally since then (Petrini 2015). In addition, in March 2010, a team of 30 Spanish doctors performed the first full-face transplant in the world (Garrett et al. 2015). These accomplishments stress the importance of craniofacial bone defect reconstruction as a fundamental first step for successful craniofacial regeneration.

CRANIOFACIAL BONE ENGINEERING

As described above, bone repair is a crucial and fundamental step of craniofacial reconstruction. Living bone is in a continuous state of dynamic equilibrium consisting of bone resorption, regeneration, and remodeling. Bone has an innate ability for a limited amount of self-repair following traumatic injury. In 1982, Enlow hypothesized that some areas of the face remodel by bony deposition, whereas others remodel by bony resorption (Enlow 1982).

Bone Reconstruction

Bone reconstruction has a long history. The earliest attempt to repair bone defects was reported in Edgar Smith papyrus, ca. 2000 BC, using metals (Frommelt 1987). And, in 1668, the first bone graft was performed by Van Meekren on a patient with a cranial defect, via a xenograft (Van Meek-

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ren 1682). Since then, a variety of techniques using different combinations of autogenous, allogeneic, and prosthetic materials have been used to achieve bone reconstruction. By far, autogenous bone grafting has generally yielded the most favorable results (Hokugo et al. 2004; Vogelin et al. 2005; Gimbel et al. 2007). Still, a better understanding of the basic biology of autogenous bone graft procedures will allow for a more educated and predictable utilization of bone reconstruction procedures in clinical practice. What is currently known is that shortly after bone graft transplantation, hematoma formation occurs around the graft. Next, an inflammatory reaction occurs around the graft lasting for 5–7 days, at which time the hematoma and surrounding tissue reorganizes into a dense fibrovascular stroma around the graft. Vascular invasion by the host brings cells with osteogenic potential into the graft, after 10–14 days. Infiltrating host cells subsequently differentiate into osteoblasts that deposit new bone, and into osteoclasts that resorb necrotic bone, facilitating bone graft penetration by host vascular tissue. Cortical grafts in the onlay position show only superficial revascularization in the first 10 ∼ 21 days, and central revascularization by 8 ∼ 16 weeks (Chen et al. 1994; Ozaki and Buchman 1998). Since the earliest vessels do not enter the graft until later, incompletely revascularized regions of necrotic graft may persist indefinitely, sealed off from the viable regions of the graft. Entrapped cores of dead bone can only be resorbed with appropriate revascularizaion. Therefore, rapid and efficient revascularization plays a key role in bone graft survival, and can be influenced by a variety of conditions including prior irradiation, immune response, and conditions at the recipient site such as the presence of necrotic bone, scarring, and infection (Lukash et al. 1984). Existing healthy bone can also serve to direct healthy matrix bone deposition.

Histologically, two types of bone can easily be distinguished: cancellous bone and cortical bone. Cortical bone is the compact bone that covers the surface of most bones. Cancellous bone, which makes up much of the enlarged ends (epiphyses) of the long bones and flat bones of the skull, is less dense than cortical bone, because of its higher surface area to mass ratio. Revascularization of cancellous bone grafts occurs more rapidly and completely than cortical bone grafts, because the large pores between the trabeculae in cancellous bone grafts permit the unobstructed invasion of vascular tissue, and rapid diffusion of nutrients from the host bed, thereby promoting osteogenic cell survival and increased osteogenesis (Pinholt et al. 1994). Cancellous bone grafts can be completely revascularized and ultimately replaced with new bone in several weeks to months, whereas cortical bone graft revascularization can proceed slowly and incompletely (Teng et al. 2013; Martuscelli et al. 2014). The dense lamellar structure of cortical bone limits vascular invasion, in which vasculature invasion is constrained to preexisting pathways and proceeds from the graft periphery to the interior of the graft. Furthermore, osteoclastic enlargement of the Haversian and Volkmann's canals must occur before blood vessels are able to penetrate the cortical bone graft, and vascular invasion is limited by the dense lamellar structure of cortical bone, where vasculature invasion is constrained to preexisting pores and proceeds from the graft periphery to the interior of the graft (Burchardt 1983). Therefore, cancellous bone grafts are well suited to treat bone gaps, because they revascularize quickly and stimulate significant new bone formation through osteoinduction. Conversely, cortical bone grafts are often used in cases of bone volume deficiency, where they can survive longer with limited resorption and retain a certain level of mechanical strength after transplantation (Neu 2000; Uhm et al. 2000).

The small population of cells present within autogenous bone grafts are what largely contribute to its superiority over other bone substitutes (Mulliken et al. 1984; Oklund et al. 1986). Osteogenic cells from the periosteum, endosteum, marrow, and intracortical elements of the graft contribute to graft viability and osteoid production (Burchardt 1987). And if the bone graft contains periosteum, the periosteum can provide blood supply and osteoprogenitor cells that further facilitate early vascularization and bone formation (Eyre-Brook 1984; Skawina and

Gorczyca 1984). Unfortunately, adult periosteum loses this osteogenic ability after being detached from the ordinal bone surface (Melcher and Accursi 1971; Weng et al. 2000). Shortly after transplantation, most of the osteocytes present within the graft undergo necrosis, thereby rendering the graft relatively inert. One of the approaches used in clinic to increase the survival rate of large bone grafts is to first implant the grafts into highly vascularized muscle tissue, to facilitate vascular ingrowth and to develop a vascular pedicle suitable for microsurgical anastomosis (Warnke et al. 2004; Mesimäki et al. 2009). This practice is somewhat limited because of donor-site morbidity and the amount of bone that can be harvested (Burchardt 1987).

To overcome these issues, stem-cell-based bone-tissue engineering has been recognized as a promising alternative for bone reconstructions. As far back as 1968, Friedenstein's team first reported that fibroblast-like cells isolated from bone marrow not only had the ability to differentiate to haematopoietic cells, but also had the potential for osteogenic differentiation (Friedenstein et al. 1968; Friedenstein 1976). At that time, the cells were named "mechanocytes." Subsequent studies showed that these cells were chondrogenic and adipogenic (Owen and Friedenstein 1988). To date, these cells are commonly called mesenchymal stem cells (MSCs) (Beyer Nardi and da Silva Meirelles 2006). In addition to bone marrow, cells with MSC-like characteristics have been isolated from a variety of tissues including adult adipose, dental, and skeletal muscle tissues (Zuk et al. 2001; Qu-Petersen et al. 2002; Feisst et al. 2014). MSC-mediated bone regeneration has been widely tested in several clinical trials, demonstrating that local delivery of MSCs can enhance bone regeneration (Grayson et al. 2015).

Comparison of Craniofacial and Noncraniofacial Bone

Generally speaking, craniofacial bones are flat bones that share similar turnover and injury repair mechanisms of other skeletal bones, but with their own specific properties (Leucht et al. 2008). Whereas most of the knowledge gained from skeletal bone reconstruction can be applied to the craniofacial bone field, the unique features and properties of craniofacial bone regeneration require some additional considerations.

One of the major differences between craniofacial and noncraniofacial bones is their embryonic origin. The cranial neural crest gives rise to branchial arch–derived craniofacial bones and cartilages, whereas the axial skeleton is derived from the somites, and the lateral plate mesoderm forms the limb skeleton. Bone formation, or osteogenesis, is the transformation of preexisting mesenchymal tissue to calcified bone tissue. There are two major modes of bone formation: intramembranous and endochondral ossification. Intramembranous ossification is the direct conversion of mesenchymal tissue into bone, whereas endochondral ossification consists of mesenchymal cells that first differentiate to form a cartilage template that is later replaced by bone. Bones of the skull primarily form through intramembranous bone formation, with some contributions by endochondral ossification (Bilezikian et al. 2002). For example, Meckel's cartilage forms at the proximal end of the mandible then largely disappears as the mandible ossifies, thereby playing an important role in mandibular morphogenesis (Ramaesh and Bard 2003; Tsutsui et al. 2008). During intramembranous ossification of the skull, neural crest–derived mesenchymal cells proliferate into compact condensations. Some of these cells develop into capillaries, whereas others change their shape to become osteoblasts, which secrete a collagen–proteoglycan matrix that is able to bind calcium salts. As calcification proceeds, bony spicules radiate out from regions where ossification began. Eventually, the entire region of calcified spicules becomes surrounded by compact mesenchymal cells that form the periosteum. The cells on the inner surface of the periosteum also become osteoblasts and deposit osteoid matrix parallel to that of the existing spicules. In this manner, many layers of bone are formed.

Craniofacial and noncraniofacial bones also show different homeostatic mechanisms. Several publications showed that bone grafts from the

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craniofacial skeleton had better survival and longer volumetric maintenance as compared to bone harvested from the iliac crest, rib, and tibia (Peer 1951; Sullivan and Szwajkun 1991). In addition, reports have indicated that membranous bone grafts retained their volume better than endochondral bone grafts (Zins and Whitaker 1979, 1983). In 1999, Buchman and Ozaki's results suggested that bone volume maintenance may be the result of microarchitecture of the bone graft, based on the fact that intramembranous-derived craniofacial bone has a higher proportion of cortical bone as compared to endochondral bone (Buchman and Ozaki 1999). Evidence for this includes the fact that, under external stimuli such as ovariectomy and malnutrition, rat mandibular trabecular bone and bone mineral density loss occurs at a lower rate than in tibial primary spongiosa (Mavropoulos et al. 2007). Furthermore, the fact that certain bone diseases only affect the maxilla and mandible, such as bisphosphonate-related osteonecrosis of the jaw (BRONJ) (Price et al. 2004) and hyperparathyroid jaw tumor syndrome (Simonds et al. 2002), also suggest that different homeostatic mechanisms exist between craniofacial and noncraniofacial bones.

It remains ambiguous as to what causes these differences between craniofacial and skeletal bones, because osteoblast differentiation in both types of bone is regulated by the same key factors, including the transcription factors Runx2 and osterix (Ducy et al. 1997; Otto et al. 1997; Nakashima et al. 2002). Still, it has been shown that several growth factors, receptors, and associated signaling cascades play distinct roles in the craniofacial versus axial and appendicular skeleton (Abzhanov et al. 2007; De Coster et al. 2007; Kimmel et al. 2007).

CRANIOFACIAL BONE REGENERATION

Taken together, craniofacial and long bones are not only derived from different germ layers, but also show distinct characteristics. These results emphasize the need to take these differences into account when considering craniofacial bone–regeneration strategies.

Craniofacial Bone Graft

Similar to other bone reconstructions, autogenous bone grafts are considered to be the gold standard for reconstructing craniofacial bone defects (Gruss et al. 1985; Manson et al. 1985). The iliac crest and rib bones, all derived from the mesoderm, are among the more commonly used donor sites for bone grafting in craniofacial surgeries because they can be readily harvested while minimally impacting the host. Other than the common shortcomings of bone grafts, such as limited harvest amount and donor site morbidity, the major concern with using these bones is that they do not show the characteristics of natural jawbone.

As previously discussed, lack of vascularization can lead to graft resorption with resultant loss of the geometric structure of the bone graft (Peer 1951). Periosteum preservation can improve graft survival in the craniofacial region (Thompson and Casson 1970) by facilitating early revascularization (Knize 1974). Conditions at the implant site also influence graft revascularization (Zins et al. 1984). Although muscle coverage results in increased bone graft revascularization (Ermis and Poole 1992), facial muscles generally originate from the surface of the skull and craniofacial bones, and craniofacial injuries are commonly associated with muscle damage, which negatively influences the ingrowth of blood vessels. Previously reports have shown that for mandibular defects small bony defects ($<$4 to 6 cm) can successfully be treated with nonvascularized cortical-cancellous bone grafts, whereas larger continuity defects require vascularized grafts (Hidalgo 1989; Pogrel et al. 1997).

Another issue is that most craniofacial bones show extremely complex 3D shapes as compared to long bones. It is, therefore, exceedingly difficult to select and reshape vascularized autologous iliac crest, fibula, or ribs to precisely fit craniofacial bone defects.

Previous publications have shown that rigid fixation can improve bone graft volume retention, by facilitating primary bone healing and rapid revascularization, especially in bones required for motion such as the femur (Phillips

and Rahn 1988; Lin et al. 1990). In contrast, most craniofacial bones show little motion, with the exception of the mandible. Even for the mandible, liquid food can be provided to restrain mandibular movement during the bone-healing process. While it may seem that rigid fixation would not significantly improve fixed craniofacial bone graft survival, a clinical study of 363 patients undergoing nasal reconstruction over a 14-year period showed that exceptional bone graft survival occurred when rigid interosseous stabilization was used (Jackson et al. 1998).

Craniofacial Bone Substitute

Stem Cells for Craniofacial Reconstruction

The main obstacles of using autologous bone for grafting is that it is only available in limited amounts (Neovius and Engstrand 2010). To overcome this critical shortcoming, and to create bone grafts of sufficient complex geometry, the field of bone-tissue engineering has been created as a practical approach to treat craniofacial skeletal defects by combining bioactive carriers, cells, and growth factors. Stem-cell-mediated bone repair has been used in clinical trials to regenerate large craniomaxillofacial defects to slow the process of bone degeneration in patients with osteonecrosis of the femoral head and for prophylactic treatment of distal tibial fractures (Grayson et al. 2015). To date, all of the MSC sources that have displayed promising osteogenic potential for bone regeneration also have been proposed as potential cell sources for craniofacial bone–tissue engineering (Cowan et al. 2004; Griffin et al. 2014).

The most well-characterized and used stem cells for bone regeneration are adult bone marrow stem cells (BMSCs). BMSCs are also the first type of cell tested for craniofacial bone regeneration. In 2001, augmented repair of cranial defects was observed by combining autologous sheep BMSCs with calcium alginate gel (Shang et al. 2001). Since then, MSCs from a variety of species, including mouse, rat, rabbit, canine, and porcine, have been confirmed as suitable cells for craniofacial bone repair (Abukawa et al. 2009; Zou et al. 2011; Lin et al. 2012). Successful craniofacial ossifications have been achieved using autologous BMSC-seeded bioscaffolds in several clinical studies (Kaigler et al. 2010; Behnia et al. 2012).

Adipose-derived mesenchymal cells (AMCs) are another type of commonly used cells for bone regeneration (Gimble et al. 2006; Sandor and Suuronen 2008). Recently, AMCs have emerged as a potential cell source for craniofacial tissue engineering (Zuk et al. 2001, 2002). Compared to BMCs, AMCs are more accessible and can be harvested in larger amounts. It has been shown that AMCs and BMCs isolated from the same donor showed similar growth kinetics and cell senescence (De Ugarte et al. 2003). In 2004, Longaker's group first showed that the AMCs promoted bone regeneration of critical-size calvarial defects, when seeded onto hydroxyapatite-coated poly-lactic-co-glycolic acid scaffolds (Cowan et al. 2004). Many subsequent publications have shown that AMCs are a promising cell source for craniofacial bone regeneration when combined with a variety of scaffold materials (Gomes et al. 2012; Azevedo-Neto et al. 2013; Jin et al. 2014).

Certain types of perinatal stem cells, including umbilical cord–derived mesenchymal stem cells (Chen et al. 2013), amniotic epithelial cells (Barboni et al. 2013), and amniotic fluid mesenchymal cells (Berardinelli et al. 2013), have also shown osteogenic differentiation capacity and, therefore, the potential for craniofacial bone regeneration. One of the main advantages of these cells for promoting craniofacial bone regeneration is their ability to promote blood vessel formation (Maraldi et al. 2013).

As described above, most craniofacial tissues are derived from the neural crest. Paraxial mesoderm–derived iliac crest bone and osteoblasts show distinctly different properties than neural crest cell (NCC)-derived craniofacial bones and osteoblasts (Chai and Maxson 2006; Oppenheimer et al. 2008; Aghaloo et al. 2010), making them less desirable for craniofacial reparative bone applications. Therefore, a variety of cell populations harvested from the craniofacial complex have been examined for utility in craniofacial bone regeneration (Zhao and Chai 2015).

These studies showed that, similar to long bones, the periosteum of craniofacial bones contains progenitor cells that support craniofacial bone repair (Ochareon and Herring 2011). Craniofacial bone marrow cells have also been well characterized. A recent study using labeled NCC and mesoderm-derived bone marrow cells showed bone defect healing via the selective recruitment of cells from their respective tissues embryonic origin (Leucht et al. 2008), thereby reinforcing the presence of embryonic site-specific differences in craniofacial versus appendicular skeleton–derived BMSCs. A rat study showed that in vitro cultured mandible–derived BMSCs showed higher alkaline phosphatase activity, mineralization, and osteoblast gene expression, and formed 70% larger bone nodules containing threefold more mineralized bone, as compared to long-bone BMSCs (Aghaloo et al. 2010). It has also been shown that human stromal cells isolated from mandibular or maxillary marrow showed increased cell proliferation, stronger expression of osteoblastic markers, and delayed senescence as compared to iliac crest–derived marrow cells harvested from the same patient (Akintoye et al. 2006). But the relatively small size and anatomic complexity of the maxilla and mandible render the efficient isolation of bone marrow stem cells a difficult process (Yang et al. 2014).

Recently, a new type of BMSC, isolated from calvarial bone sutures, has shown utility for craniofacial bone regeneration (Zhao et al. 2015). In 2015, Chai's group identified $Gli1^{+}$ cells present within the calvarial suture mesenchyme of adult mice that can give rise to both an osteogenic front, periosteum, dura, and craniofacial bones. In humans, cranial sutures normally fuse between 20 and 30 years of age, whereas facial sutures fuse after ~50 years of age (Senarath-Yapa et al. 2012; Badve et al. 2013), suggesting the possibility of isolating these cells for use in craniofacial bone regeneration. Still, the limited amount of suture mesenchyme prevents harvest of large numbers of cells.

All of the tissues found in teeth originate from the neural crest. Stem cells isolated from adult dental tissues, including dental pulp stem cells (DPSCs) (Gronthos et al. 2000; Papaccio et al. 2006; Zhang et al. 2008), stem cells from human exfoliated deciduous teeth (SHED) (Miura et al. 2003), periodontal ligament stem cells (PDLSCs) (Seo et al. 2004), and periapical stem cells (PSCs) (Han et al. 2010), all showed potential for osteo/odontogenic differentiation and the ability to form calcified dentin or osteodentin-like tissue. Therefore, these cell populations can be used not only to regenerate these same dental tissues, but also for craniofacial and noncraniofacial bone–tissue regeneration. In particular, DPSCs, which can be easily isolated from extracted wisdom teeth, showed a higher proliferation rate as compared to bone marrow–derived MSCs under the same culture conditions, potentially attributable to the strong expression of the cell-cycle activator, cyclin-dependent kinase 6 (Shi et al. 2001).

Widespread use of dental tissue–derived stem cells is somewhat limited by the size of the available tissues that can be used. Comparatively, it may be easier to harvest gingiva-derived mesenchymal stem cells (GMSCs), which also share the multilineage differentiation potential (Zhang et al. 2009). It has been shown that human GMSCs can support new bone formation in both mandibular and calvarial defects at 2 months in rat postsurgical reconstructions (Wang et al. 2011).

Cleft lip and palate (CL/P) are among the most common congenital malformations, occurring in 1/700 live births (Tolarova and Cervenka 1998). Standard surgical treatment of CL/P patients involves the removal of small pieces of orbicular oris muscle (Shkoukani et al. 2013). Bone reconstructive repair of a critical-size cranial defect was successfully achieved using muscle cells derived from these discarded tissues (Bueno et al. 2009).

Stem cells and/or scaffolds loaded with active growth factors, which stimulate osteoprogenitor cells to differentiate toward osteogenic support of the new bone formation, are also commonly used for bone-tissue engineering (Friedlaender et al. 2001; Terheyden et al. 2004). One of the most commonly used growth factors for craniofacial bone regeneration is bone morphogenic protein (BMP) (Terheyden et al. 2001a,b; Warnke and Coren 2003; Warnke et al. 2004).

Challenges for the Use of Stem Cells in Craniofacial Tissue–Engineering Applications

One of the main obstacles in translating experimental observations into clinical practice is the relatively poor mechanistic understanding of stem-cell-mediated therapies. In vivo stem-cell therapy requires large numbers of cells; insufficient cell numbers will not provide positive outcomes (Habisch et al. 2007). Despite the obvious benefits associated with cell delivery for bone regeneration, very few transplanted cells survive long term following transplantation (Dupont et al. 2010). The current lack of reliable methods to control MSC fate, especially in the in vivo condition, is one of the main problems that needs to be addressed. Better understanding of methods to optimize regenerative approaches is required before stem-cell-mediated therapies can be implemented as standard care in regenerative medicine.

MSC isolation and selection is another challenge. For most clinical applications, only a limited amount of viable tissue is available for stem-cell isolation (Sodek and McKee 2000). In addition, MSC selection and enrichment is hampered by lack of a single definitive stem-cell marker (Gronthos et al. 2003). Current isolation methods can achieve heterogeneous cell populations at best, and the proportions of MSC progenitors in these enriched MSC populations can vary even when the samples are obtained from the same donor at the same time (Digirolamo et al. 1999). Previous reports have indicated that only ~30% of the initial adherent bone marrow–derived MSC clones showed trilineage (osteo/adipo/chondro) differentiation potential (Pittenger et al. 1999; Muraglia et al. 2000). Furthermore, only half of the single colony–derived clones transplanted with hydroxyapatite-tricalcium phosphate ceramic scaffolds showed bone formation in vivo (Kuznetsov et al. 1997). Although current clinical trials have shown that it is not necessary to generate purified cell populations to achieve positive results in the clinic (Prockop 2007), it is possible that more purified MSC populations will help to achieve more reliable clinical outcomes.

There also is a need for optimized methods to store and expand enriched MSC populations while maintaining the "stemness" of the MSCs, as required for advanced clinical application. This is because MSCs tend to spontaneously differentiate into various cell types over time in culture (Banfi et al. 2000; Izadpanah et al. 2008) and MSCs lose their multipotentiality after six or seven passages in in vitro cell culture (Colter et al. 2000; Sekiya et al. 2002). One of the most promising solutions is to create 3D culture conditions that resemble the in vivo stem-cell niche (Bara et al. 2015; Xiong et al. 2015). To date, no standardized methods have been achieved for this approach, despite many diverse attempts (Chow et al. 2001a,b). For example, a variety of tissue culture media have been tested, including those with low serum, various substitutes for fetal bovine serum (FBS) such as autologous serum, fresh frozen plasma, and human platelet lysates (Lange et al. 2007; Le Blanc et al. 2007), and a variety of serum-free media (Sekiya et al. 2002; Müller et al. 2006; Agata et al. 2009; Lindroos et al. 2009). A variety of 3D scaffold tissue culture methods have also been tested as in vitro 3D stem-cell niche systems, including the 3D hanging drop model (Schmal et al. 2013), bioreactor (Santos et al. 2016), and 3D printing (Alessandri et al. 2016).

CRANIOFACIAL RECONSTRUCTION

Craniofacial reconstruction involves the regeneration of multiple hard and soft tissues that are well vascularized and innervated. For better outcomes, it is ideal to regenerate the craniofacial tissues "all-in-one," including both soft and hard tissues. A concept of the "biological boundary" was introduced in 1989 (Whitaker 1989), which emphasized that the soft tissue envelope has a genetically predetermined shape that is inclined to remain constant. However, soft connective tissues show faster growth rates as compared to hard tissues, because of the fact that soft tissue cellular components show higher rates of migration. Therefore, one of the main challenges for successful bone healing is preventing the ingrowth of connective tissue into the bone space, especially for large and complex

craniofacial bone repair. To achieve this, the concept of guided tissue regeneration, which can be achieved by placing an inert membrane barrier over the defect to block the ingrowth of connective tissue, has been widely used for successful craniofacial reconstruction (Dahlin et al. 1988, 1990).

Computer-Aided Design (CAD)

One central challenge for successful craniofacial reconstruction is how to mimic the complex 3D structure and multicellular interactions that naturally occur in complex craniofacial structures (Thesleff et al. 2011). Resorption of bone grafts or regenerated bones often occurs after bone reconstruction. Minor bone resorption may not significantly influence the successful repair of long bones, but can seriously affect craniofacial reconstruction outcomes, again because of the complex geometry and small sizes of many craniofacial bones. Craniofacial reconstruction requires more accurate regenerative approaches to achieve desirable outcomes.

Currently, with the development of 3D computer tomography (CT) and CAD techniques, CT/CAD-based scaffold design can greatly benefit the accurate regeneration of craniofacial bones. One of the most commonly used strategies for accurate craniofacial reconstruction is the use of titanium scaffolds manufactured by the CT/CAD technique, based on the fact that nonabsorbable titanium scaffolds can achieve appropriately detailed complex craniofacial anatomical geometry. Terheyden's group has used a titanium mesh cage filled with bone mineral blocks infiltrated with 7 mg recombinant hBMP7 and 20 ml of the patient's bone marrow to successfully restore mandibular defects greater than 7 cm (Warnke et al. 2004). The titanium mesh cage was first implanted into the patient's right latissimus dorsi muscle for 7 weeks before being transplanted as a free bone muscle flap. Eventually, the reconstructed mandible achieved the desired aesthetic and masticative functional levels. In another case, a titanium mesh was prefabricated and filled with β tricalcium phosphate (β-TCP) granules that were soaked in rhBMP-2 for 48 h before infiltrating with cultured adipose-derived stem cells (ASCs). In this instance, sufficient new bone developed to support dental implants in 10 months (Sandor et al. 2013). A combination of milled bone from the iliac crest, ASCs isolated from fat tissue harvested from the gluteal region, and autologous fibrin glue have also been used to treat multiple fractures (Lendeckel et al. 2004). Bone regeneration was observed within 3 months of surgery in these cases. Functional and anatomically correct repair of a maxillary defect was achieved using a microvascular custom-made ectopic bone flap developed from a titanium cage filled with β-TCP granules mixed autologous ASCs (Thesleff et al. 2011). To date, titanium cages filled with granules, cancellous bone chips, or bone blocks have been shown by far to be the best strategy for bone restoration of large craniofacial defects.

While conventional regenerative strategies have failed to regenerate or mimic the 3D complexity of the multicellular interactions occurring in native craniofacial tissues, 3D cell culture in bioreactors combined with gene therapy or growth factors have shown promise for increased survival of bone substitutes (Scherberich et al. 2007; Cordonnier et al. 2010; Sohier et al. 2010).

3D Printing

3D bioprinting of tissue provides a promising approach to customize various combinations of biomaterials and cell sources to achieve complex 3D architectures. 3D printing is a method that fabricates objects by building consecutive layers of scaffold material plus cells layer by layer, until the desired 3D volumetric structures are achieved (Derby 2012). In this way, 3D printing techniques can provide precise spatial control of the functional components. For the purpose of tissue regeneration, it is possible to print cells directly, to print cell-laden biomaterials, or to print scaffold-free cell aggregates. Currently, there are three types of commercially available 3D printers for tissue engineering: inkjet printers (Klebe 1988), laser-based printers (Guillemot et al. 2010), and microextrusion printers (Fig. 2) (Cohen et al. 2006). Inkjet printers, the

most commonly used type of printer for both nonbiological and biological applications, print structures using a drop-on-demand process (Murphy and Atala 2014). Inkjet bioprinters have successfully been used to fabricate bone. This technique can greatly benefit craniofacial bone constructs because it provides fairly rapid and "customer-fit" reconstruction of complex surface bone (Saijo et al. 2009; Azuma et al. 2014). Inkjet bioprinting techniques have also been tested for functional in situ skin regeneration (Skardal et al. 2012). Microextrusion printers use pneumatic or mechanical (piston or screw) dispensing systems to extrude continuous beads of material and/or cells (Chang et al. 2008; Visser et al. 2013). Microextrusion bioprinters have been used to fabricate multiple tissue types, including branched vascular trees (Norotte et al. 2009), islet tissue (Xu et al. 2010), and aortic valves (Duan et al. 2013). Laser-based bioprinter design is based on a laser-induced forward transfer technique using a laser beam (Chrisey 2000). To date, laser-based bioprinters have been successfully used to print cellularized skin (Colina et al. 2005).

Current 3D techniques for craniofacial regeneration are largely limited to bone and cartilage tissues (Schek et al. 2005; Reichert et al. 2012). Hierarchical composite scaffolds, consisting of both soft and hard tissue components, have successfully been used for periodontal complex regeneration (Vaquette et al. 2012; Costa et al. 2014; Ivanovski et al. 2014). Laser-based bioprinters have been used to deposit nanohydroxyapatite in a mouse calvarial 3D defect model (Keriquel et al. 2010). Similarly, 3D-printed biphasic scaffolds containing poly-(epsilon)-caprolactone and hydroxyapatite in the size and shape of teeth have been tested, as well as 3D-printed dental epithelial (DE) and dental mesenchymal (DM) cell aggregates, to use as in vitro models for DE–DM cell interactions observed in natural tooth development (Kim et al. 2010).

The 3D printing techniques designed to achieve functional organ regeneration is still in its infancy (Ho et al. 2015). 3D-printed scaffolds, tissue analogs, and organs have been proposed as exciting alternatives to address some of these key challenges now facing the fields of regenerative medicine and dentistry (Derby 2012; Murphy and Atala 2014). This technique has the advantages of enabling the precise positioning of cells and biomaterials in 3D with finely tuned internal and external architectures, while being customizable to patient-specific needs (Obregon et al. 2015).

The craniofacial complex contains multiple types of highly integrated hard and soft tissues. It would be beneficial to regenerate composite hard and soft tissues at the same time, to achieve rapid functional recovery of regenerated craniofacial tissues. Teeth are somewhat unique components of the craniofacial complex. Since teeth are anchored in maxillary and mandibular jawbones, methods to generate jawbone together with dental tissues, in addition to 3D printing, are currently being investigated, with some success (Fig. 3) (Duailibi et al. 2008; Abukawa et al. 2009). A major obstacle in this approach is the design of methods and approaches that maintain the integrity of both the bone and tooth structures, while allowing them to be functionally integrated. Prior reports showed that bioengineered tooth constructs were surrounded by newly formed bone, precluding tooth eruption and proper function (Cowan et al. 2004). The clinical use of composite tissue regeneration, such as cartilage and bone and vascularized muscle, is still in its infancy, and further studies are needed to perfect these highly promising approaches.

One of the main obstacles of current 3D printing techniques is the size limit of the printout structure, mainly because hydrogel, the most commonly used injecting material, provides inadequate structural support (Chang et al. 2011). Recently, a new integrated tissue-organ printer (ITOP) has been developed to target the generation of stable human-scale tissue constructs (Kang et al. 2016). The key to this method is to print a sacrificial scaffold adjacent to the cell-laden hydrogel. The cell-laden hydrogel protects cell viability and supports cell expansion and differentiation. The sacrificial scaffolding material provides the initial structural and architectural integrity and, on removal, also provides diffusable microchannels to facilitate

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Figure 2. Components of inkjet, microextrusion, and laser-assisted bioprinters. (*A*) Thermal inkjet printers electrically heat the printhead to produce air-pressure pulses that force droplets from the nozzle, whereas acoustic printers use pulses formed by piezoelectric or ultrasound pressure. (*B*) Microextrusion printers use pneumatic or mechanical (piston or screw) dispensing systems to extrude continuous beads of material and/or cells. (*C*) Laser-assisted printers use lasers focused on an absorbing substrate to generate pressures that propel cell-containing materials onto a collector substrate. (From Murphy and Atala 2014; adapted, with permission, from Nature Publishing Group © 2014.)

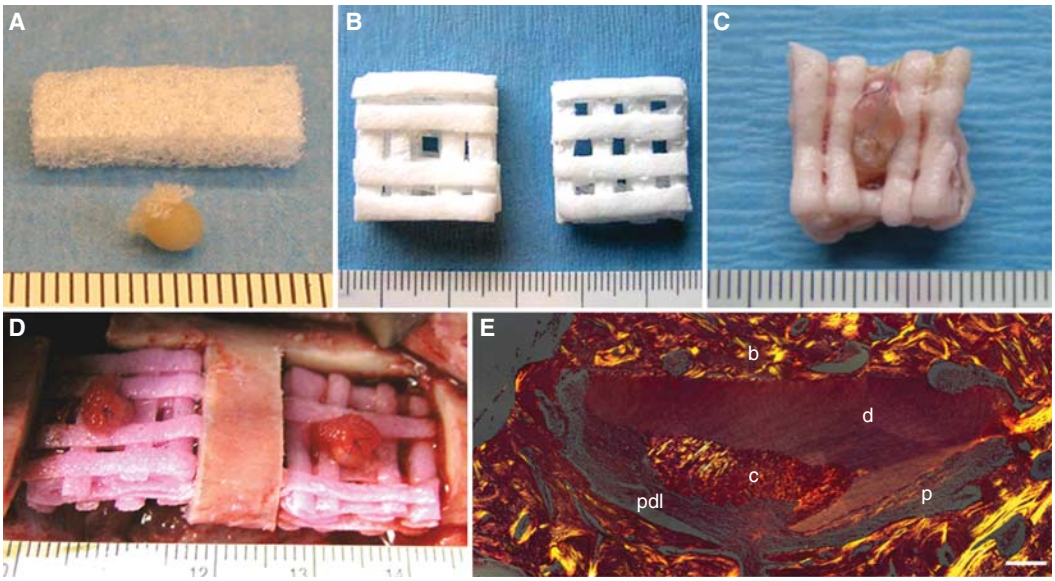

Figure 3. Composite bone–tooth constructs. (*A*) Tooth scaffolds composed of a dental mesenchymal (DM) cell seeded polyglycolide/poly-L-lactide (PGA/PLLA) scaffold sphere to mimic the dental papilla, and a dental epithelial (DE) cell seeded gelfoam strip to mimic the enamel organ. (*B*) Lattice bone scaffolds made by poly-DL-lactic-co-glycolic acid (PLGA), seeded with iliac crest–derived mesenchymal stem cells (MSCs) and grown in the rotational oxygen-permeable bioreactor system (ROBS) for 6 weeks. (*C*) Fabricated tooth–bone construct seeded with cells before implantation. (*D*) Surgical implant site before wound closure. (*E*) Bioengineered dental tissues that closely resembled those of naturally formed pig tooth tissues surrounded by alveolar bone. Scale bar = 100 μm. b, bone; bm, bone marrow; d, dentin; e, enamel; p, pulp; pdl, periodontal ligament.

nutrient perfusion. This technique has been used to fabricate human-scale mandibular bone, ear-shaped cartilage, and organized skeletal muscle.

Another major challenge of the 3D printing technique is how to precisely integrate biological components and gradients for composite tissue engineering. Compared to other tissue-regeneration methods, 3D printing can provide fairly accurate control of different tissues. Still, 3D printing has its own limitations, mainly because the formation of 3D structures is based on the accumulation of 2D structures. For example, accurately mimicking the branching patterns of the vascular tree has remained a big challenge of 3D printing (Kolesky et al. 2016).

CONCLUSIONS

Successful reconstruction of the craniofacial complex requires full restoration of both functional and aesthetic aspects of the face. Stem-cell-mediated tissue-regeneration approaches provide the potential for highly successful craniofacial tissue-regeneration. Despite increased knowledge and characterization of MSCs, and the mounting enthusiasm for the use of MSCs in regenerative therapies in humans, the detailed mechanisms of MSCs proliferation and differentiation are still not fully understood.

This lack of understanding has not permitted the full use of stromal cells to facilitate or enhance tissue repair in clinical practice because of fear of potential unwarranted deleterious behaviors of the transplanted cells (Grayson et al. 2015). Despite these concerns, stem-cell-mediated regenerative strategies, combined with precise CAD approaches, are anticipated to eventually provide many new and promising methods for successful craniofacial reparative therapies.

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