Biomimetic Scaffolds for Craniofacial Bone Tissue Engineering: Understanding the Role of the Periosteum in Regeneration

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Abstract The role of the periosteum in bone tissue engineering is a new and exciting development. Although its regenerative capacity is known and its role in initiating wound healing is well-documented, a complete understanding of the underlying mechanisms and specific cues that cause healing induction is still unknown. Recently, a number of different studies have begun to explore how stimulating periosteal recruitment is involved in regeneration. In this chapter we review the importance of the periosteum as well as a number of different materials used to activate and initiate the healing process indicative of the periosteum. Our own work has focused on using electrospun chitosan/hydroxyapatite composite scaffolds in order to integrate the native periosteal tissue with our material and instigate the healing process in critical size calvarial bone defects. Critical size defects remain elusive and problematic in the clinic to date and tissue engineering is a promising candidate to alleviate such problems. In this chapter we will briefly review our material and its ability to induce osseointegration, osteoinduction and support the formation of new, mineralized tissue in a murine model. This material, along with others, reflect promising and auspicious developments in musculoskeletal tissue engineering and are helping to pave the way in understanding how the periosteum is involved in wound healing.

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1 Introduction

Regenerative bone tissue engineering encompasses a wide range of different strategies, materials and therapies aimed at repairing, restoring and regenerating tissue rather than replacing it. Since there are many different types of bones with different structures and diverse requirements for specific mechanical strengths, depending on the location and micro-scale composition/ arrangement of specific bones, there is no one "universal approach" to regenerative bone tissue engineering: Successful, tissue-engineered constructs for repairing bone after injury and/ or in the wake of the many bone disorders, will have to be tailored to the specifics of all of these different factors. For example, the Young's modulus in the longitudinal direction of a human femur can range from 15–20 GPa as determined from 3 point bending tests (Cuppone et al. 2004), whereas the Young's modulus for cranial bones is closer to 10 GPa (Motherway et al. 2009). Amongst the important features when engineering regenerative bone scaffolds are the mechanical properties at the onset of bone healing following a fracture. Regenerating bone is characterized by the presence of woven, or immature bone, with Young's moduli that range from ~30–1,000 MPa depending on the distance from the fracture point, with a median of ~130 MPa (Leong and Morgan 2008). This unique microenvironment harbors the osteoblasts that begin the healing process of bone repair. Understanding the mechanisms of bone development, maintenance and repair of specific bone types are crucial to developing successful, integrative materials and therapies.

An essential, yet often neglected component for successful regeneration of any injured bone is its outer living tissue envelope, called the periosteum. The outer fibrous layer of the periosteum contains mainly fibrous ECM proteins, mostly collagens and elastin, as well as fibroblasts and is highly vascularized, while the inner cambium layer is composed of osteoblasts and periosteal (stem-like) cells (Lin et al. 2014). The latter cells are multipotent cells that can differentiate into osteoblasts and chondrocytes (Hutmacher and Sittinger 2003; Lin et al. 2014). Sharpey's fibers are large bundles of collagen fibers that affix the periosteum to the outer layer of the cortical bone. During development, Sharpey's fibers are low in number, allowing the periosteum to move more freely, causing a much more highly activated layer of osteoprogenitor cells to induce tissue formation. Periosteum plays a large role in the initiation of bone regeneration during injury (Clark 2005; Clarke 2008; Zhang et al. 2008a; Rios et al. 2009). The inner layer of cortical bone, the endosteum, is a thin layer of osteoprogenitor cells, osteoblasts and connective tissue that attaches the cortical bone to the trabecular bone, as seen in Fig. 1 (Clark 2005).

The periosteum forms during the early stages of development during intramembranous ossification in flat bones, such as the skull. Mesenchymal stem cells (MSCs) from the neural crest proliferate and begin to differentiate into capillary forming

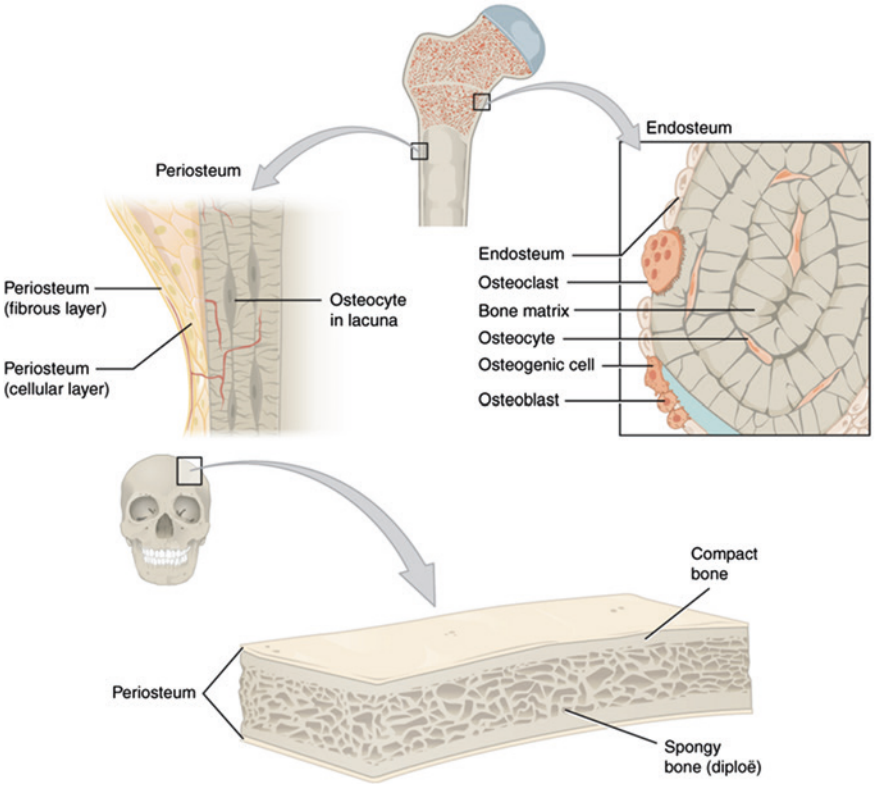

Fig. 1 Micro-scale bone anatomy. The top of the image depicts the hierarchical organization of bone tissue, with the periosteum surrounding the outer layer cortical bone, the presence of numerous cell types embedded in a calcified matrix and the inner endosteum separating the inner layer of the cortical bone from the trabecular bone. The bottom depicts the gross anatomy of cranial bone, showing the two outer layers of cortical bone and the inner trabecular bone, or diploe. Download for free at http://cnx.org/contents/9306de62-3f52-46f8-ab1a-94263c480eda@3

cells and osteoblasts. These osteoblasts begin depositing a collagen- and proteoglycan-rich microenvironment that later becomes mineralized. The early bone matrix (osteoid) becomes calcified through this mineralization process and matures into functional bone tissue. Osteoblasts and MSCs stay to the periphery of the calcified tissue and create new layers of bone, while osteoblasts that become entrapped in the matrix mature and differentiate into osteocytes. As the bone develops, dense groups of MSCs gather around the outer edges of the bone and form into the periosteum (Gilbert 2010). Upon complete maturity, the cranial bones contain two layers of cortical bone (outer and inner layers of the skull) which surround a thick layer of trabecular bone, called the diploe, as seen in Fig. 1 (Lynnerup et al. 2005).

2 The Role of the Periosteum in Bone Development and Regeneration

2.1 Periosteal Involvement in Wound Healing Initiation

The current gold standard for craniofacial reconstruction involves autografts due to the presence of an intact and functional periosteal layer (Allen et al. 2004; Zhang et al. 2008a). However, this introduces a secondary operative site which is often accompanied by surgical complications, donor morbidity/pain and a decreased quality of life. Methods for manufacturing bone grafts from either synthetic/natural materials or the use of cadaveric donor grafts are suboptimal due to the lack of a functional periosteum (Zhang et al. 2008a). Engineered materials typically lack the ability to successfully integrate with the host tissue and fail to induce osseointegration. Integration between the host and the graft is critical, since this integration will facilitate the migration of osteoprogenitor cells from the host into the graft and induce quicker, more regenerative responses and bone formation.

Focusing on craniofacial regenerative engineering, the inner layer of the periosteum in the skull harbors multipotent cells that have a fibroblast-like morphology and can differentiate towards either a chondrogenic or an osteogenic lineage (Zhang et al. 2005). The outer fibrous layer of the periosteum consists of fibroblasts and Sharpey's fibers, which are responsible for binding the cranial bones firmly, but at the same time allowing them to move and absorb shock or trauma. These fibers are most abundant where shock and force are common (Hutmacher and Sittinger 2003).

Cell labeling and tracking experiments have shown the pivotal contribution of the periosteum and endosteum to the initiation of bone healing, where other stromal cells from the marrow in trabecular bone are more involved in the later stages of wound healing (Hutmacher and Sittinger 2003). For example, the importance of the periosteum in bone callus formation was demonstrated by removing the periosteum from an autograft prior to implantation, which resulted in a substantial decrease in new bone formation as well as a 10-fold decrease in neovascularization (Tiyapatanaputi et al. 2004).

Using β-Galactosidase as a tag, Zhang et al. (2005) reported that the periosteal cells migrated from the host onto and localized on and around the graft, differentiating into osteoblasts, chondrocytes, osteocytes and perivascular vessel cells. This study demonstrated the multipotency of these cells and that they tend to remain on the surface of the graft rather than migrating into it (Zhang et al. 2005).

2.2 BMP Signaling

Although the molecular signaling involved in the initiation and morphogenesis of periosteal bone healing is not well defined, a number of molecules, such as proteins of the BMP (Sun et al. 2013), Hedgehog (Huang et al. 2014), and Wnt (Almeida et al. 2013) families, actively participate in this process.

Members of the FGF and IGF families are also upregulated in bone healing (Zhang et al. 2008a). There is a general consensus that wound healing shares some similarities with the natural fetal limb budding and normal bone development (Mariani 2010). During development, BMP-2, 4 and 7 are involved in the activation of core-binding factor α1 (CBFA1), a crucial transcription factor that induces osteogenesis in MSCs (Nishimura et al. 2002). Some studies suggest that BMP-2 is upregulated during the formation of the periosteal callus, which is the initiator to bone healing following cortical bone fracture (Bostrom et al. 1995). Knockout of BMP-2 during organogenesis disrupts the progression of healing following injury in BMP-$2^{-/-}$ mice, in spite of the presence of other osteogenic factors, indicating the pivotal role of this particular factor in fracture repair (Tsuji et al. 2006). BMP2 also plays an important role in angiogenesis and vascularization of the periosteum, as inferred from a decrease in VEGF levels and in specific MSC markers α-smooth actin, CD146 and angiopoietin-A, in a mouse model in which BMP-2 was selectively knocked in osteoblasts (Yang et al. 2013). Addition of BMP-2-transfected periosteal cells to an allogeneic implant yielded increased levels of ALP and accelerated wound defect healing in a rabbit mandibular injury model (Sun et al. 2013). As a caveat, BMPs induce bone formation and osteogenic differentiation in animal models, but in human studies BMPs fail to induce bone formation except at very high doses and following sustained release. BMPs have also had very little effect on non-union fractures (Aspenberg 2013).

2.3 Hedgehog Signaling

The hedgehog signaling pathway is a crucial signaling mechanism involved in development and injury repair. Recently, it has been shown to play a crucial role in stimulating periosteal healing initiation. Sonic hedgehog transfected periosteal cells showed significant increases in both osteogenic and chondrogenic differentiation of MSCs derived from autograft periosteum. Both Indian and sonic hedgehog were significantly upregulated in these cells, leading to a more developed, robust bone formation in vivo. Deletion of Smoothened, a receptor of the hedgehogs, resulted in a significant decrease in osteogenic differentiation and periosteal callus formation (Wang et al. 2010). Furthermore, osteophyte formation in osteoarthritis mouse models was significantly reduced by blocking Smoothened and inhibiting the hedgehog pathway (Ruiz-Heiland et al. 2012). Osteophytes are calcified bone formations in the subchondral regions of bone defects; hence, inhibiting their formation by blocking hedgehog is an indication for its role in bone tissue formation. Overexpressing sonic hedgehog in periosteal progenitor cells resulted in enhanced wound healing in a critical size mouse defect model. Seeding transfected periosteal-derived mesenchymal progenitor cells on scaffolds resulted in a marked increase in endothelial progenitors and microvessel formation (revascularization) and significantly enhanced donor site periosteal cell survival and migration into the construct (Huang et al. 2014).

2.4 Wnt Signaling

The Wnt signaling pathway is a ubiquitous and critical signaling pathway in a multitude of developmental process. In bone development and healing, the non-canonical Wnt/calcium pathway is pivotal for the induction of osteogenesis in the presence of calcium phosphate. Seeding of decalcified graft materials leads to a significant decrease in bone formation. Similarly, blocking of BMP and Wnt pathways using Noggin and Frizzled receptor antagonists also showed a comparable decrease in bone formation (Eyckmans et al. 2010). In the periosteum, down-regulation of the Wnt/β-catenin pathway by recombinant BMPs increased th levels of Sox9, a pro-chondrogenic marker, which ultimately led to chondrogenic, rather than to osteogenic differentiation of the periosteal progenitor cells (Minear et al. 2010). These studies not only demonstrate the importance of these factors for bone development and healing, but also show how they can be employed for as part of the strategy for the use of tissue engineered constructs.

2.5 Periosteal Cell Recruitment and Function

The main constituents of the periosteum responsible for healing are the periosteal cells. These adult stem-like progenitor cells are mainly responsible for instigating the healing process and are also indicative as to why in contrast to using functional autografts, cadaveric allografts lacking such a layer are inadequate for inducing appropriate healing (Allen et al. 2004). An engineered periosteal sleeve can be used to enhance the regenerative abilities of allografts. The three main prerequisite for engineering a periosteal sleeve around a graft material are (a) live osteogenic cells, (b) osteoinductive genes or factors and (c) an osteoconductive scaffolding material. In terms of cell sourcing, the most common choices are MSC derived from the bone marrow or adipose derived stem cells, as well as periosteal cells (Zhang et al. 2008a). These cell types offer a unique opportunity to avoid ethical issues involved with the use of embryonic stem cells as well as provide a renewable and autologous cell source. For example, Long and colleagues used MSCs cultured to form periosteal sheets to revitalize an allograft implant which then functioned like an autograft with an active periosteal layer (Long et al. 2014). These MSC-sheet wrapped allografts demonstrated superior periosteal callus formation, endochondral tissue formation around the periphery of the scaffolds and enhanced osseointegration.

2.6 Vascularization and Extracellular Environment

Bone wound healing and repair requires proper and appropriate vascularization, which has been shown to have a reciprocal effect on osteogenesis. Angiogenic factors, such as VEGF and PDGF not only aid in vascularization, but also aid in bone

formation as well (van Gastel et al. 2012; Ferretti et al. 2012). Like wound healing in other tissues, initiation of bone healing also requires appropriate blood clotting, vessel and callus formation to stimulate the healing process. Periosteal cells are not only influential in the early steps leading to osteogenesis, but also in inducing angiogenesis (van Gastel et al. 2012; Ferretti et al. 2012). Further, incorporation of endothelial cells with MSCs seeded onto implants greatly enhances the initiation of wound healing and leads to healthy functional bone tissue long term (Zigdon-Giladi et al. 2013).

The microenvironment in which stem/progenitor cells reside is called a niche. The niche for bone/periosteal stem/progenitor cells is composed of nanofibrous extracellular matrix proteins, including collagens and elastin, and contains also other cell types, including fibroblasts and osteoblasts and sympathetic nerves/microvasculature (Lin et al. 2014). One of the goals of engineered regenerative tissue scaffolds is to confer biomimetic properties to these scaffolds. One of those properties is their nanofibrous structure, which can be obtained by diverse manufacturing processes, such as electrospinning (Frohbergh et al. 2012; Son et al. 2013), self-assembly (Kocabey et al. 2013; Cakmak et al. 2013) and phase separation (Hsu et al. 2013; Zhao et al. 2012). The goal is to create a tissue-specific environment that can emulate this niche and its unique components. Structure and mechanics are shown to be two of the main causes to induce context-dependent cellular instructions, like maintenance of stemness (Hashemi et al. 2011), proliferation (Li et al. 2013), or tissue-specific differentiation (Liu et al. 2014; Novotna et al. 2013).

3 Tissue Engineered Electrospun Hydroxyapatite Containing Chitosan Scaffolds

3.1 Key Features of Tissue Engineered Bone Scaffolds

Physical properties, such as elasticity, tensile strength, toughness, etc. also induce changes in bone patterning and morphogenesis during development, and these cues also aid in repair and remodeling (Hutmacher and Sittinger 2003). For example, incorporation of hydroxyapatite increases the mechanical properties (stiffness/Young's modulus) of poly-caprolactone (PCL) fibers and enhances osteogenic expression in vitro and new bone formation in vivo (Ba Linh et al. 2013). Bi-layer hydroxyapatite scaffolds have mechanical properties similar to mandibular trabecular bone as well as a porous architecture suitable for osseointegration (Guda et al. 2012).

In our own work we focused on periosteal regenerative engineering and aimed at developing a biomimetic/bioactive material that could be used to induce bone regeneration in critical size defects by stimulating/recruiting the cells from the periosteum of the surrounding tissue to initiate wound healing. Our biomaterial of choice was a composite scaffold generated by co-electrospinning pure chitosan and hydroxyapatite nanoparticles to mimic the biphasic nature of bone (Frohbergh et al. 2012). The nanofibrous ultrastructure of electrospun scaffolds closely mimics that

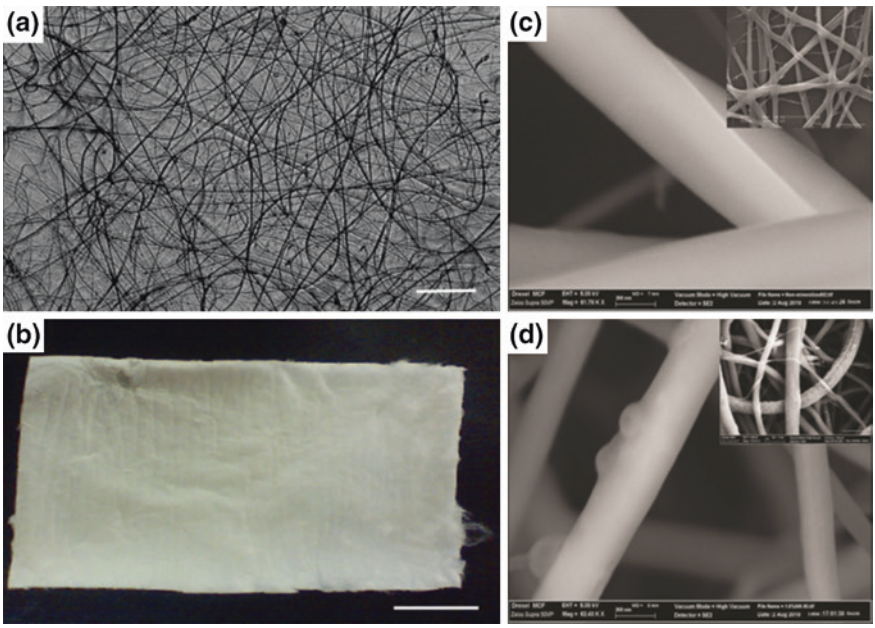

Fig. 2 Electrospun nanofiber morphology. **Panel a** microscopic view of the electrospun chitosan/hydroxyapatite/genipin fibers. **Panel b** gross macroscopic view of an electrospun scaffold. **Panels c** and **d** show the differences between the smooth surface of electrospun fibers without hydroxyapatite and the rougher surface of fibers studded with hydroxyapatite nanoparticles respectively (Frohbergh et al. 2012)

of natural ECM in most tissues, including bone (Fig. 2). Inclusion of hydroxyapatite nanoparticles (in the absence of any fiber forming agents, such as ultrahigh molecular weight polyethylene oxide (Zhang et al. 2008b) in the electrospinning process not only simplifies the manufacturing process, but also instantly enhances both the mechanical properties as well as the bioactivity of our scaffolds. Crosslinking with a natural, non-toxic cross-linker genipin (Torricelli et al. 2014; Bavariya et al. 2013) resulted in a further increase in the Young's modulus and tensile strength of the scaffolds, reaching 147 ± 22 MPa, which is very similar value to the mechanical properties of the periosteum at the periphery of a wound callus., rendering our scaffolds suitable for craniofacial bone tissue engineering. Finally, the scaffolds supported adhesion and proliferation of 7F2 mouse osteoblast-like cells and enhanced their histiotypic differentiation (Fig. 3).

3.2 Electrospinning and Scaffold Fabrication

Electrospinning of natural biopolymers, such as collagen or chitosan may not necessarily be ideal manufacturing process for fracture healing in load-bearing bones, which require stiff and rigid scaffolds in order to provide for the mechanical

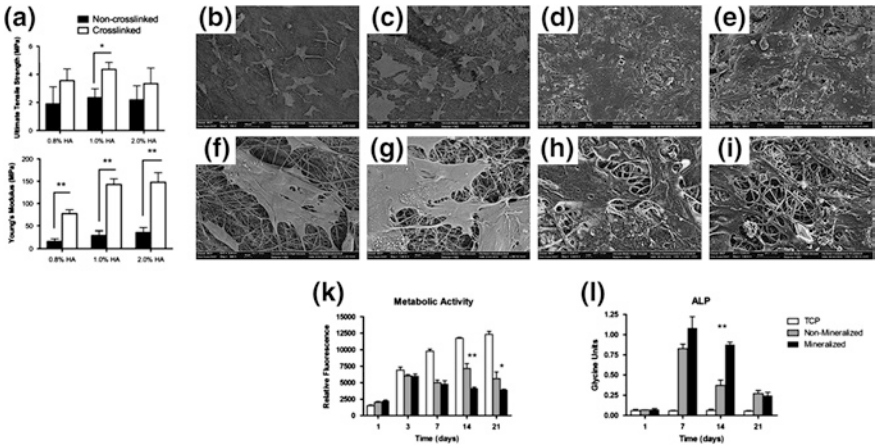

Fig. 3 In vitro characterization of 7F2 osteoblast-like cells on chitosan based scaffolds. Hydroxyapatite-containing scaffolds show mechanical properties similar to those of the periosteum at the formation of a wound callus in natural bone healing processes **a**. 7F2 cells attached and spread after 7 days of culture on both scaffolds without hydroxyapatite **b** and **f** and scaffolds with hydroxyapatite **c** and **g**. The cells remained viable for up to 21 days on both scaffolds without **d** and **h** and with **e** and **j** hydroxyapatite and proliferated on both scaffold types over a 21-day period **k**. ALP expression peaked at day 7 **l**

support required for movement and stability. However, in non-load bearing bones with critical size defects that will not heal spontaneously, scaffolds made of electrospun biomaterials may serve as bioactive "bridges" to cover the defects and induce healing. Mimicking the natural ECM fibrillar structure, electrospun nanofibers promote enhanced cell attachment and spreading and are easily tunable both mechanically (crosslinking) and structurally (coatings, fiber modifications, blended materials, etc.) (Bhardwaj and Kundu 2010; Chew et al. 2006; Huang et al. 2011; Ito et al. 2005; Li et al.; 2002, 2005). These integrative properties are exactly what most inert materials and cadaveric implants are lacking.

Successfully engineered tissue constructs will mimic certain features of native tissues including their unique mechanical properties. While electrospun scaffolds made of "natural" biopolymers such as collagen or chitosan morphologically resemble the fibrous structure of the ECM, their mechanical properties make them less suitable for use as bone analogs. Although non-load bearing bones do not undergo much physical exertion, they still have the biphasic composite strength of bone, i.e., the mineralized collagen/hydroxyapatite ECM represents an organic/inorganic interface designed to withstand trauma. Achieving similar features in electrospun fiber scaffolds is crucial for the development of a suitable bone implant. Crosslinking can be used to enhance the mechanical properties of the constructs and fine-tune them to approximate the properties of bone ECM. Crosslinking can be physical, enzymatic or chemical. For our studies we used genipin as a natural, non-toxic chemical crosslinker (Bispo et al. 2010; Solorio et al. 2010; Zhang et al. 2010). Crosslinking with genipin increases the mechanical

properties (tensile strength) of electrospun chitosan fibers, as assessed for example by a suture pullout strength test (Norowski et al. 2012). While the complete mechanism of how genipin crosslinks chitosan is still not fully understood it is believed to involve a spontaneous reaction between genipin and the NH_2 subunits on the chitosan chain, creating partial covalent bonds and increased stability of the polymer chains (Austero et al. 2012), which in turn causes an increase in the scaffold stiffness. The Young's modulus of our scaffolds increased 4–5 fold upon cross-linking, while the ultimate tensile strength increased by 50 % (Frohbergh et al. 2012).

3.3 HA Containing Chitosan Scaffolds are Osteoinductive

In terms of functional tissue engineering, our aim was to fabricate a scaffold with structural and mechanical properties similar to those of non-load bearing bone and which emulates the regenerative capacity of periosteum. Specifically, our goal was to generate a bioactive scaffold capable of inducing/accelerating osteogenic differentiation in vivo similar to what occurs when osteoprogenitor cells from the periosteum migrate to damaged bone tissue. The osteogenic capacity of our fibrous scaffolds was assessed in vitro using 7F2 mouse osteoblast like cells. The cells attached to all of our scaffolds, mineralized or not, and proliferated over a 14-day period and covered the scaffold in a multilayered fashion. At the same time, the metabolic activity decreased over time, especially in cells cultured on hydroxyapatite-containing scaffolds, which is indicative of cells undergoing differentiation while ceasing proliferation (Moore and Lemischka 2006). Recently, (Venugopal et al. 2011) showed that mineralization of the electrospun scaffolds by inclusion of hydroxyapatite nanoparticles during the spinning process caused a significant increase in osteoblast mineralization and concluded that hydroxyapatite nanoparticles act as nucleation sites for osteogenic induction and maturation in vitro. Our recent in vitro studies yielded comparable results (Frohbergh et al. 2012).

These and similar studies suggest that electrospun composite materials can be considered osteoinductive in vitro by promoting the histiotypic differentiation of cultured osteoblasts or other progenitor cells towards functional osteocytes (Rajzer et al. 2014; Dong et al. 2014; Patlolla and Arinzeh 2013). In lieu of using allogeneic or autologous progenitor cells, periosteal osteoprogenitors would be an ideal cell source, however obtaining these cells is quite difficult and not practical in terms of the number of cells one would have to harvest for a suitable implant in a critical size defect. As an alternative, MSC can be isolated fairly easily from the bone marrow or adipose tissue and differentiated into osteoblasts by simple chemical differentiation protocols (Delorme and Charbord 2007; Frohlich et al. 2008; Giordano et al. 2007; Jaiswal et al. 1997). MSCs are lineage-restricted multipotent cells that are derived from the bone marrow, umbilical cord blood or adipose tissue and have the potential to differentiate into bone, cartilage and adipose (Delorme and Charbord 2007). Due to technical and ethical issues associated with ESCs and of induced pluripotent stem cells (iPSCs), especially their potential for

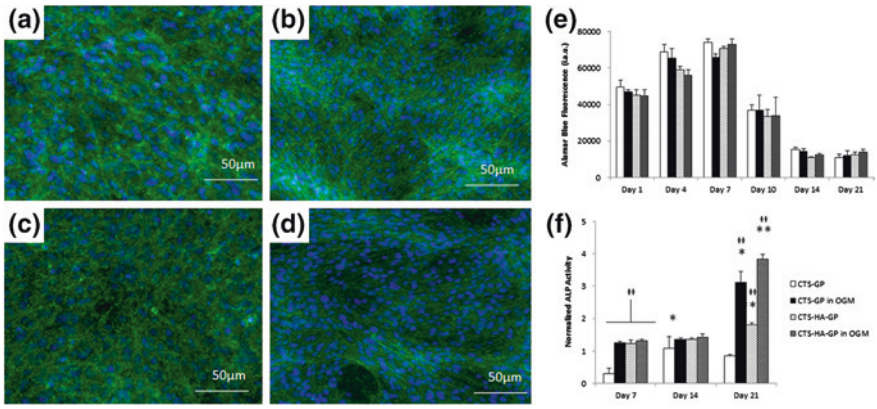

Fig. 4 In vitro assessment of osteogenic differentiation of mouse mesenchymal stem cells. Cell morphology was observed using DAPI/phalloidin (*blue*/*green*) staining and indicated formation of cellular multilayers on both scaffolds without hydroxyapatite **a** and **c** and with hydroxyapatite **b** and **d** at days 7 and 21 respectively. Reduction of Alamar blue activity between 14 and 21 days **e** coupled with an elevation in ALP activity at day 21 **f** is indicative of the mMSCs leaving the proliferative phase and entering the differentiation phase (Frohbergh et al. 2014)

immunogenicity and teratoma formation (Alvarez et al. 2012), lineage restricted, MSCs are preferentially used for bone tissue engineering (Ngiam et al. 2011).

In extending our *in vitro* studies, we tested the ability of our electrospun genipin-crosslinked scaffolds to promote osteogenic differentiation of murine MSCs (Frohbergh et al. 2014). As seen in Fig. 4, the scaffolds promote the assembly of multi-layer cell sheets on the surface, indicating appropriate adhesion of MSCs on the scaffold and the ability to form tissue-like structures on the scaffold surface (Fig. 4). They also induce initial osteogenic differentiation of MSCs which is further significantly enhanced in the presence of an osteogenic medium, indicating that the physicochemical cues from the material play a significant role in instigating MSC differentiation (Fig. 4).

3.4 HA Containing Chitosan Scaffolds are Osseointegrative/ Osteoconductive

Osteoconduction is an important and substantial finding, indicating that these scaffolds can support osteogenesis. However, it is equally, if not more important to ensure that engineered materials are also integrative with the host/patient and can promote substantial tissue/scaffold interactions to induce self-healing and regeneration. To show the osseointegrative capacity of our electrospun scaffolds, we used a cranial defect murine model induced by micro-drilling and removal of a section of the skull (Fig. 5). Scaffolds were implanted with and without naïve MSCs in order to compare the healing competence of the scaffolds alone and in the presence of cytokine signaling from implanted cells (Frohbergh et al. 2014).

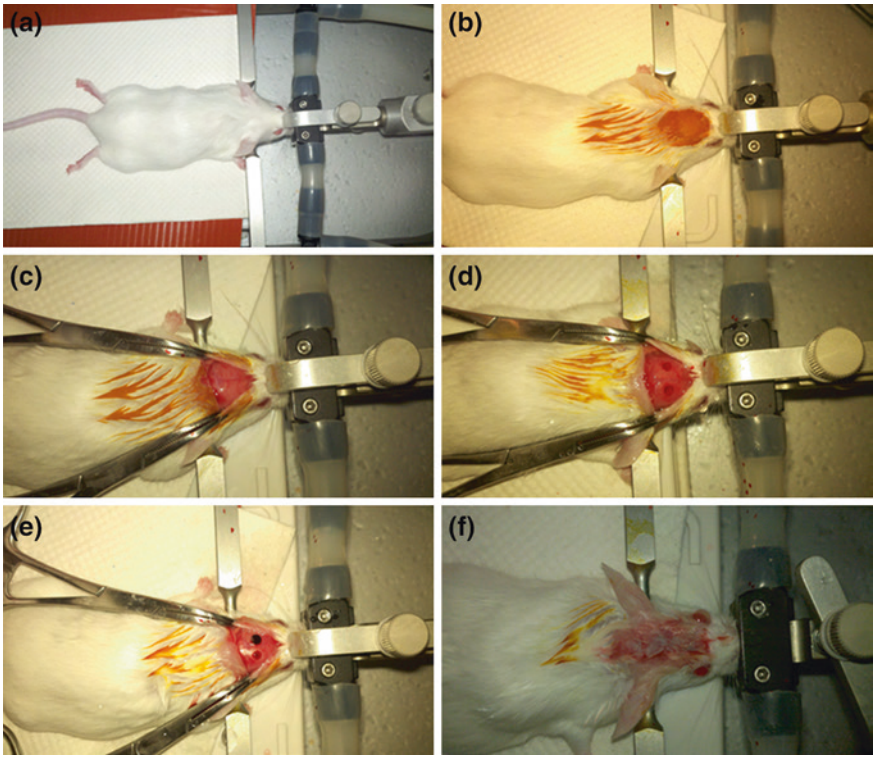

Fig. 5 Surgical Procedure to Generate Calvarial Defects. The animal was appropriately anesthetized and positioned in a stereotaxic fixture (**a**). The wound was shaved and sterilized (**b**). A distal incision was made exposing the parietal bones of the skull (**c**). Two critical size defects were drilled on either side of the sagittal suture, one for control (**d**) and the other fitted with a scaffold (**e**). Wounds were sutured and bio-glue was applied for extra stability (**f**)

Three month post-surgery, optimal osseointegration with the host tissue was provided by mineralized scaffolds that had been pre-seeded with MSCs, as inferred from both the presence of mineralized tissue in the defect area (microCT, Fig. 6 panel a) and of new, healthy tissue growing from the periphery of the wound onto the scaffold (histology, Fig. 6 panel d). In the absence of MSCs, the non-mineralized scaffold was essentially ineffective in inducing bone healing (Fig. 6 panel b), where as addition of MSCs to the non-mineralized scaffolds resulted in modest healing and bone regeneration (Fig. 6 panel c).

An ideal bioactive bone tissue scaffold will demonstrate two distinct properties: (1) the ability to induce host tissue migration and (2) minimize inflammation and immune rejection in the host. Crucial for the induction of bone tissue regeneration and healing is the migration osteoprogenitor cell from the periosteum (Allen et al. 2004; Hutmacher and Sittinger 2003; Zhang et al. 2008a; Zhang et al. 2005). Critical size defects in bone injuries do not effectively heal because there is no permissive tissue in the defect area for the osteoprogenitor cells to migrate onto in order

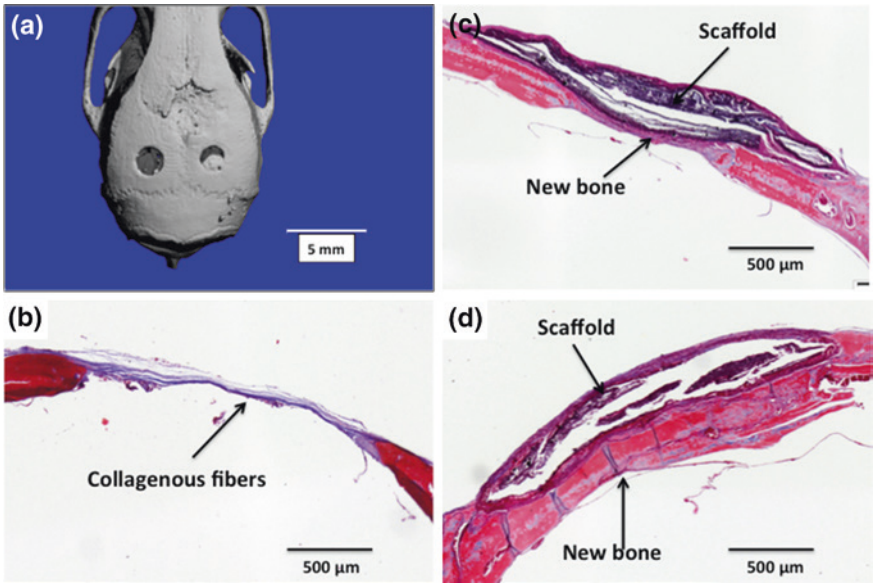

Fig. 6 Healing of critical size calvarial defects in a mouse model. Three months after surgery, microCT analysis shows significant formation of mineralized bone in critical size defects treated with mineralized genipin-crosslinked chitosan scaffolds (*right*) pre-seeded with murine mesenchymal stem cells (MSCs), but not in the untreated contralateral lesions (**a**). Panels B–D: Mason Trichrome staining of critical defects treated with non-mineralized scaffolds (**b**), non-mineralized scaffolds, pre-seeded with MSCs (**c**) and mineralized scaffolds pre-seeded with MSCs (**d**). Non-mineralized scaffolds failed to induce the healing process; the defects were covered with a collagenous matrix only (*blue*), as also seen with untreated samples (not shown). The MCSs had a minor beneficial effect in non-mineralized scaffolds. Note the significant enhancement of bone formation (*red*) in induced by MSCs when used in conjunction with mineralized scaffolds (Frohbergh et al. 2014)

to begin depositing matrix and initiate healing (Zhang et al. 2008a). Suitable biomaterials, such as genipin-cross-linked, mineralized chitosan, fulfill both the above requirements and can be used to bridge this gap and provide a template that will initiate and support the healing process to begin (Frohbergh 2013; Frohbergh et al. 2014).

In our studies untreated defects were covered by a thin acellular fibrous layer. In the absence of an appropriate scaffolding material, the critical size bone defect is will not heal on its own. The tissue growing on the scaffolds exhibits matrix formation and contain collagen type I, the main ECM component of newly forming bone tissue (Gentili and Cancedda 2009), as inferred from the Masson's Trichrome stain. Other studies have observed similar regenerative responses when using chitosan-based implants in vivo. For example, blended poly(vinyl alcohol)/N-methylene phosphonic chitosan scaffolds significantly increased ALP and collagen I levels in cultured MG-63 cells, a human osteosarcoma cell line and enhanced wound healing by 300 % when compared to untreated wounds in a rabbit tibia model (Datta et al. 2013). Liu and colleagues (2013) showed the ability of chitosan/hydroxyapatite/ultra-high molecular weight poly (ethylene oxide) scaffolds

to support MSC proliferation and osteogenic differentiation in vitro via the BMP/SMAD pathway. These authors also showed that their scaffolds promoted bone healing in a rat calvarial defect model more effectively than chitosan alone and chitosan/hydroxyapatite membranes (Liu et al. 2013).

Numerous preclinical studies demonstrated that implanting osteoinductive scaffolds seeded with naïve or pre-differentiated allogeneic or autologous progenitor cells results in enhanced regenerative capabilities of the cell-seeded versus the cell-free constructs (Mestak et al. 2013; Tasso et al. 2009; Jin et al. 2009). While the outcomes of these studies generally support the notion that the presence of progenitor (or even differentiated cells) will benefit wound repair and tissue regeneration, the clinical implementation of this concept may still be limited by numerous problems surrounding the use of cells, such as cell sourcing (what kind of cells to use, at what stage of differentiation, how to obtain enough of them, etc.) and potential immunogenicity and teratogenicity in the case of stem cells (whether embryonic or iPS). Moreover, from a translational standpoint, handling, storing, transporting cell-based tissue engineered constructs, is complex, to say the least, and may thwart the commercial success of technically/scientifically/clinically promising regenerative biomaterials, e.g. recently happened with some "living" skin substitutes.

3.5 Conclusions

The induction of de novo tissue formation around the scaffold suggests that our scaffolds *per se* are permissive and promote proper host integration. Given their mechanical properties, these scaffolds hold potential promise for treating non-load bearing bone injuries. While tissue integration and immunosuppression are of upmost concern, the end goal is to engineer a scaffold that is osteoconductive and will lead to fully function bone tissue. Our results suggest that the presence of hydroxyapatite greatly enhances the osteogenic capacity of these scaffolds and leads to mineralized tissue formation by month 3. Osteoconduction can be improved with the presence of MSCs. Quantitatively there was up to a 5 fold increase in defect closure versus scaffolds without hydroxyapatite and MSCs. Further, MSC seeded hydroxyapatite-containing scaffolds only showed ~10 % more wound healing than hydroxyapatite-containing scaffolds without cells, indicating that the mineralized scaffolds by themselves were fully capable of inducing enhanced wound healing without the need for a cellular component. This makes these scaffolds clinically relevant with the added benefit of off-the-shelf availability and no time (and additional expenses) required for cell culture and scaffold preparation prior to implant. Combined with the findings of endochondral tissue formation on the composite scaffolds after 3 months of implantation, we can conclude that this de novo generated tissue is in the early stages of endochondral ossification and that mineralized ECM is beginning to replace cartilage tissue. Interestingly, the normal development process of cranial bone is intramembranous ossification.

Further studies into the mechanisms involved in tissue formation on these genipin-cross-linked mineralized chitosan scaffolds are warranted and may yield a new and improved manner to initiate endochondral bone healing in cranial bones.

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