Published in final edited form as:

Stem Cell Rev. 2016 October ; 12(5): 524–529. doi:10.1007/s12015-016-9665-5.

Stem Cells in Bone Regeneration

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Abstract

Bone has the capacity to regenerate and repair itself. However, this capacity may be impaired or lost depending on the size of the defect or the presence of certain disease states. In this review, we discuss the key principles underlying bone healing, efforts to characterize bone stem and progenitor cell populations, and the current status of translational and clinical studies in cell-based bone tissue engineering. Though barriers to clinical implementation still exist, the application of stem and progenitor cell populations to bone engineering strategies has the potential to profoundly impact regenerative medicine.

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Disclosures: The authors indicate no potential conflicts of interest

Author Contributions:

GGW, ERZ, TL, RCR, JSF, MSH, and ASL wrote the manuscript. MTL and DCW provided technical expertise and edited the manuscript. All authors read and approved the final manuscript.

Keywords

stem cell; bone; regeneration; tissue; engineering

Introduction

The remarkable capacity of the skeleton for spontaneous bone regeneration has long been observed and appreciated due to the direct relationship between its structure and function (1, 2). Aside from classical roles in load-bearing, movement, and soft tissue/organ protection, the skeleton acts as a supportive niche for hematopoiesis, and is an active regulator of endocrine homeostasis (3–7). The process of spontaneous skeletal regeneration is of particular interest, because mechanisms must be executed rapidly to re-establish this range of diverse vital functions (8). However, in the setting of skeletal injury beyond a critical size, spontaneous healing does not occur, leading to nonunion and the formation of scar tissue. Skeletal defects resulting from congenital deformities, complex trauma, degenerative disease, and tumor resection often require large amounts of bone for reconstruction. Clinically available sources of bone for skeletal reconstruction are limited by several factors including accessibility and amount of available autologous grafts, infectious risks of cadaveric materials, and durability of synthetic substitutes. While autogenous bone grafts remain the gold standard for reconstructing large skeletal defects, alloplastic materials still remain part of the clinical armamentarium. However, the limited supply of autogenous bone grafts and the risk for infection associated with alloplastic materials have fueled the search for an alternative approach to repair large skeletal defects.

The processes of skeletal growth, regeneration, and repair are each controlled by stem and progenitor cells that are genetically and phenotypically distinct (9–11). In this review, we present recent findings on stem and progenitor cells in bone regeneration, focusing on the material composition of bone, skeletal development, mechanisms of fracture healing, the role of adult stem and progenitor skeletal cells in bone regeneration, and translational cell-based studies, as well as early clinical findings to-date. A better understanding of the regenerative potential of adult skeletal stem and progenitor cells will facilitate the translation of novel therapies to enhance skeletal repair.

Bone Composition

The hierarchical structure of bone, extending from the macro-scale to the subnano-scale, is irregular and anisotropic. At the macroscopic level, compact outer cortical bone surrounds porous inner trabecular bone. Microscopically, cortical bone is composed of repeating osteon units containing collagen fibers and calcium phosphate crystals, whereas cancellous bone is an interconnecting framework of trabeculae with a surrounding marrow space. A single osteon unit consists of concentric layers of collagen fibers, called lamellae, running perpendicular to a central canal containing blood vessels and nerves (12).

Bone is mineralized dense connective tissue that consists mainly of a mineral component and an organic matrix. The organic matrix is composed primarily of type I collagen with less type III collagen. Type I collagen is a 300 nm long triple-helical structure that arranges into

fibrils that form collagen fibers. Hydroxyapatite $\text{Ca}_3(\text{PO}_4)_2 \cdot (\text{OH})_2$ nanocrystals are embedded between individual collagen molecules and serve to substantially increase the rigidity of bone (13). Together with non-collagenous proteins such as fibronectin, osteocalcin, osteopontin, and osteonectin, collagen forms a scaffold for mineral deposition, and proteoglycans (PGs) such as decorin and biglycan regulate collagen fibril assembly and diameter. Type 1 collagen is evenly distributed throughout the skeleton, while non-collagenous proteins and proteoglycans are found in an irregular distribution pattern.

Bone Development

Cartilage, bone, and bone marrow stroma, like individual bones, arise at specific time points during development and from multiple embryonic lineages. Cranial neural crest gives rise to facial bones, while the remainder of the skeleton is derived from paraxial and lateral plate mesoderm. A bona fide skeletal stem cell in mice (mSSC) gives rise to bone, cartilage, and nonhematopoietic stroma of long bones during development (9, 14). Furthermore, the development of the skeleton and its cellular hierarchy proceeds in a manner that is similar to – and tightly coordinated with – the hematopoietic system.

Skeletogenesis occurs through either intramembranous or endochondral ossification. Endochondral bone formation occurs through a cartilage intermediate that is replaced by bone and marrow. Mesenchymal condensations contain cells which transition to cartilage centrally and mature further into hypertrophic cartilage, later to be replaced by bone and marrow. The outer envelope of mesenchymal cells later give rise to primitive perichondrium of loose connective tissue and articular soft tissues. The perichondrium then gives rise to both chondrocytes and osteogenic cells. As the ossification centers and growth plates are later established, peripheral and hypertrophic chondrocytes contribute to osteogenic cell types (15). In membranous bones, chondrogenesis is typically aborted after transient expression of type II collagen as mesenchymal condensations undergo direct ossification. In some membranous bones, such as parietal bones of the skull, cartilage appears prior to degradation by matrix metalloproteinases and chondrocyte apoptosis, while bone forms at the periphery in concert with central cartilage regression (16).

Fracture Healing and Bone Remodeling

Fracture healing is a complicated physiological process that involves a variety of different cell types and cellular responses. Fracture healing is categorized into two different groupings: direct or primary cortical fracture healing and indirect or secondary fracture healing. Primary fracture healing occurs when there is anatomic reduction of the fractured fragments by rigid internal fixation and decreased interfragmentary strain. This is characterized by the fact that there is no callus formation, which is a result of there being no periosteal response. Instead, the cortex actively attempts to restore mechanical continuity by reestablishing new Haversian systems (17). In contrast, secondary fracture healing is characterized by hematoma formation, micromotion, and relies on a dynamic process of construction and deconstruction of the wounded area. These dynamic processes are the focus of this review (18).

Secondary fracture healing is further broken down into subsets based on two types of bone formation - intramembranous and endochondral (19). Secondary fracture healing is also distinguished by the contributions of four different spatial areas, the cortex, the periosteum, the bone marrow, and the external soft tissue, for each type of bone formation. The five temporal stages of fracture healing include the initial stage of hematoma formation, followed by inflammation and angiogenesis, which leads to early cartilage formation, subsequent consolidation and calcification, and finally long term bone remodeling. It should be stated that these stages do not have clear cut boundaries, but rather overlap one another with certain angiogenic and osteogenic factors present throughout (20). By viewing the process both temporally and by type of bone formation, it is possible to focus on the orchestration of all the physical, biochemical, and cellular factors contributing to the healing of the fracture.

The fracture healing process starts with hematoma formation and subsequent inflammatory cell infiltration. The hematoma is formed from blood entering the area which begins to be reabsorbed within hours. Inflammatory cells (macrophages, granulocytes, lymphocytes, and monocytes) then enter the hematoma and prevent infection, release growth factors, secrete cytokines, and advance the clotting process (17). Degranulating platelets are also present in the clot that may release TGF-beta and PDGF. This series of events sets the stage for subsequent steps by initializing angiogenesis, chemotaxis, and mesenchymal stem cell differentiation (18). BMPs are also released from the area by the bone matrices and are expressed primarily by recruited stem cells. BMPs help facilitate the ossification process and have a role in differentiating MSCs (21).

At this point, high levels of proangiogenic factors such as FGF, VEGF, and angiopoietin 1 and 2 can be found at the fracture site. It is believed that angiopoietin is the major player in the beginning stages while VEGF is prominent primarily during endochondral bone formation (17). Endochondral bone formation begins with the differentiation of MSCs into chondroblasts that cause a soft callus to form around the area. Once mechanical stability has been established, the cartilage starts to mineralize and eventually, as vasculature invades the area, chondroclasts remove the chondroblasts and woven bone formation occurs (19). The major contributing areas to this process are the underlying cortical bone, the periosteum close to the fracture site, and the bone marrow within the fracture. Initially, cells from the bone marrow differentiate into cells with an osteoblast phenotype. Then, osteoblasts from the cortex start to form woven bone in the area creating a hard callus (18).

The fracture healing process ends with the bone remodeling stage. Bone remodeling is defined by a joining of the hard callus of intramembranous ossification and the soft callus of endochondral ossification (20). It is essential for bone remodeling to occur in order for the bone to become weight bearing once again. Bone remodeling involves osteoclasts that orchestrate the resorption of mineralized bone in hard and calcified soft callus. While osteoclasts are resorbing bone, osteoblasts are depositing new, lamellar bone that is able to bear weight and eventually becomes a restored cortical structure (21).

Adult Stem and Progenitors Cells in Bone

Bone regeneration is a dynamic process that balances the breaking down of old bone, the generation of new bone, and the infiltration of these areas with blood vessels (17). Two broad categories of cell populations available as sources of bone regeneration include osteoblasts and multipotent cells. Osteoblasts are cells committed along a bone lineage, possessing a limited number of divisions, and readily form mineralized matrix. Multipotent stem cells have the ability for prolonged division while maintaining the capacity to differentiate along multiple lineages with proper biological cues. Resident stem cells can be found in many adult tissues, and are active in endogenous mechanisms of repair and regeneration.

Postnatal skeletal stem and progenitor cells differentiate into a multitude of specialized cells that participate directly in bone regeneration. Their origins and roles differ depending on the type of regeneration occurring at the area: bone remodeling, intramembranous ossification, or endochondral ossification. However, the mechanisms governing recruitment and contribution of skeletal stem and progenitor cells to long-term bone maintenance and acute injury repair have remained largely unknown.

Cellular sources of bone have been identified in bone marrow, periosteum, skeletal muscle, fat, and umbilical cord blood (22). Though the limits of their long-term self renewal capacity and differentiation potential have been less defined, research in this area has demonstrated the ability of these postnatal progenitor cells to differentiate along multiple lineages *in vitro*. The most commonly studied source of postnatal multiprogenitor cells is the bone marrow, comprised of hematopoietic and mesenchymal stromal cells. The hematopoietic component provides progenitors that give rise to all mature blood cell phenotypes *in vivo*. Bone marrow mesenchymal stromal cells (BMSCs) are easily isolated from hematopoietic components owing to their adherent quality to polystyrene culture dishes. Substantial work has demonstrated the ability of BMSCs to be guided along multiple mesenchymal lineages *in vitro*, including bone, cartilage, muscle, ligament, tendon, and stroma. However, only recently have efforts begun to identify and isolate bone, cartilage, and stromal progenitors for rigorous functional characterization.

Our laboratory has identified a stem cell for skeletal tissues and a system of more restricted, downstream progenitors, and defined their respective surface phenotypes in the mouse skeleton (9). These skeletogenic progenitors are diverse, with distinct cell-surface marker profiles and skeletal tissue fates, similar to the diverse hematopoietic progenitor cells that generate various differentiated blood cells. Indeed, skeletogenesis proceeds through a developmental hierarchy of lineage-restricted progenitors as occurs in hematopoiesis. The mouse skeletal stem cell (mSSC) [CD45⁻Ter119⁻Tie2⁻AlphaV⁺Thy⁻6C3⁻CD105⁻CD200⁺] resides at the top of the hierarchy with the capacity to both self-renew and differentiate to cartilage, bone, and marrow stroma. The mSSC subpopulation generates all of the other (seven) subpopulations through a sequence of stages beginning with generation of two multipotent progenitor cell types: first the pre-bone cartilage and stromal progenitor (pre-BCSP) [CD45⁻Ter119⁻Tie2⁻AlphaV⁺Thy⁻6C3⁻CD105⁻CD200⁺] and the bone, cartilage, and stromal progenitor (BCSP) [CD45⁻Ter119⁻Tie2⁻AlphaV⁺Thy⁻6C3⁻CD105⁺CD200⁺].

The different cellular subpopulations possess intrinsic skeletogenic potential that is heavily guided by bone morphogenetic protein (BMP) signaling from within the skeletal niche. Moreover, the *in vivo* directed differentiation of mSSCs to cartilage and bone fates can be accomplished through BMP and VEGF pathway manipulation.

Skeletal stem cells are recruited locally for fracture healing, without significant contribution from systemic circulation, and their ablation has been shown to disrupt bone regeneration and remodeling (10). The dramatic upregulation of mSSCs in the fracture callus, a milieu of growth factors released in response to injury, implies that extrinsic signals produced in the regenerative environment could locally activate these cells. Skeletal injury also results in the expansion of more restricted and downstream BCSPs. Injury-induced BCSP expansion precedes ossified callus formation shortly after injury, while irradiation suppresses BCSP expansion and delays callus formation, indicating a system of postnatal skeletal repair that depends on a functional progenitor response at the fracture site. In addition, injury-induced variations occur between BCSP phenotypes. Our group also identified a highly potent regenerative cell type termed the fracture-induced bone, cartilage, stromal progenitor (f-BCSP) in the fracture callus of adult mice, which is distinguished by the presence of the surface marker CD49 (11). CD49f+ f-BCSPs have enhanced regenerative potential and are active during acute periods of repair. The *in vivo* intrinsic bone-forming capacity and gene expression patterns of f-BCSPs resembles that of uninjured perinatal BCSPs more closely than that of adult BCSPs, suggesting that highly-regenerative skeletal progenitors maintain perinatal phenotypes in the postnatal adult skeleton. A better understanding of the requisite stimuli for bone repair offers translational promise because cell-specific activation could enhance osteogenesis amid dysfunctional bone health.

While we have shown previously that the mSSC and downstream BCSP facilitate the rapid repair of skeletal tissue, it is unknown whether aberrant stem and progenitor cell activity lead to impaired bone healing in the setting of pathologic conditions. Deviations in normal mSSC activity could be cell-intrinsic, or they could arise from alterations to the external regulatory niche environment. The importance of extrinsic signals in guiding skeletogenic fates at the cellular level has been highlighted by the *in vivo* directed differentiation of cells in skeletal and extraskeletal (adipose) tissue. Cells derived from fat do not exhibit an inherent skeletal potential that is observed in the mSSC lineage. However, extraskeletal mSSC formation is inducible with application exogenous BMPs and additional soluble factors which regulate the differentiation toward bone, cartilage, or stromal cells (9). The results in these settings suggest that cellular fate is controlled extrinsically. Future studies to define how intrinsic and extrinsic signals guide mSSCs to regulate skeletal shape and patterning at the single-cell level would likely provide key insights to facilitate the translation of mSSC-based strategies in the setting of impaired skeletal regeneration.

Isolation, characterization, and utilization of stem cells have provided regenerative medicine with many new promising treatment options to address problems of skeletal defects. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are attractive cellular therapies for their ability to maintain differentiation capability across all cell types and lack dedicated differentiation pathways. While pluripotent cells require strict culture conditions to prevent spontaneous differentiation and tumor formation *in vivo*, a growing

body of work has shown progress toward harnessing their differentiation potential for regenerative therapies. Further efforts to identify and enrich for adult skeletal stem cells in humans with continued advancements in pluripotent cell research may facilitate clinical trials and therapeutic options for reconstructive surgeons.

Translational Cell-based Studies

Current translational studies investigating the potential use of stem cells in bone tissue engineering require an optimal cell delivery method for effective *in vivo* osteogenesis. To that end, a large variety of strategies are being employed. Scaffolds to assist in bone regeneration can be broadly classified into biological, mineral, and polymer scaffolds. Biological scaffolds utilize biomaterials such as chitosan and collagen, though there is an increasing trend to combine these with mineral agents such as hydroxyapatite, in order to provide more precise replication of the natural bone ECM (23). Unfortunately, the use of natural materials limits the extent to which the scaffold's physical properties can be tailored. Thus, mineral scaffolds, such as glass and ceramic materials have shown much promise. For example, Sacak et al. demonstrated that bioactive glass was suitable for induction of *in vivo* osteogenic differentiation of adipose derived stromal cells (ASCs) in a critical size calvarial defect model. However, while the quality of the regenerate was similar to that obtained by bone grafting alone, it was not significantly different from that obtained using bioactive glass alone (24). More recently, Hendrikx et al. developed a homogenous gelatin-glass material that would allow for improved scaffold shaping, as it lacks the brittleness seen in bioactive glass alone (25).

In contrast to mineral scaffolds, polymer scaffolds encompass a range of biomechanical possibilities. The physical properties of hydrogels can be easily manipulated: thermoresponsive solutions that gelatinize at physiologic temperature can be premixed with either cells or growth factors prior to implantation (26). Alternatively, Han et al. have developed a hydrogel formulation consisting of microribbons with variable crosslinking ability, thus allowing for specification of pore size based on desired application. This has shown promise as a stem cell delivery vehicle for regeneration of mouse critical size calvarial defects (27).

Solid polymer scaffolds include poly-(l-lactic acid), poly-(l-lactic-co-glycolic acid), and poly(ε-caprolactone). The properties of these scaffolds are not as titratable as hydrogels, though they have demonstrated efficacy in bone repair. Our laboratory has previously shown that human ASCs seeded onto hydroxyapatite-coated PLGA scaffolds can heal critical sized calvarial defects in mice (28). As an alternative to the use of growth factors, Park et al. utilized PLGA microspheres seeded with bone marrow derived mesenchymal stem cells (BM-MSCs) in conjunction with high dose laser therapy (far red wavelengths) for the formation of ectopic bone in immunocompromised mice (29). They found significantly higher mineralization of PLGA microspheres by BM-MSCs that had been exposed to 60 seconds of laser light, in comparison to both unexposed and 90-second exposure times. Similarly, Nagasaki et al. found that a combination of nanohydroxyapatite scaffolds with exposure to low intensity pulsed ultrasound resulted in significantly improved ASC-mediated healing of calvarial defects (30).

Early Clinical Findings and Safety

While there are numerous combinations of scaffold materials, growth factors, and ancillary treatment modalities such as ultrasound/laser therapy which may be used clinically, incorporation of stem cell based strategies for bone regeneration remains challenging secondary to regulatory restrictions surrounding them. However, incorporation of cellular building blocks with demineralized bone matrix (DBM), an already approved growth-factor containing bone substitute, has shown promise in early clinical studies: Dufrane et al. utilized a combination of osteogenically differentiated ASCs and DBM to create three-dimensional autografts for treatment of bony nonunion in patients with pseudoarthritis or post-tumor resections (31). While effective, long-term consolidation was observed in approximately half of the patients receiving grafts, the clinical course of the other patients was complicated by either infection and/or disease-related events. Importantly, there were no complications directly attributable in their report to use of the ASC-DBM autograft. Similar studies have investigated the combination of bone marrow aspirate (containing both BM-MSCs and growth factors) and DBM, with and without the addition of platelet rich plasma (32) or allograft (33), and have found them to be effective and safe in the settings of distraction osteogenesis for bimalleolar fractures, and spine arthrodesis, respectively.

With such studies in mind, the efficacy and safety of stem cells is continuously being investigated in a variety of clinical trials. A Brazilian clinical trial has thus far found effective closure of alveolar clefts in pediatric patients utilizing a combination of autologous dental pulp stem cells and a commercially available bone substitute (Geistlich Bio-Oss®, Geistlich Pharma North America Inc., Princeton, NJ) (NCT01932164). Fracture healing is by far the most popular target for stem cell strategies in bone reconstruction, as there are currently seven open studies listed on ClinicalTrials.gov for investigation of stem cell-based therapies in this setting. The interventions in these studies vary from direct injection of MSCs at the fracture site, to implantation in conjunction with DBM or allograft, to combination of cells with platelet lysate. Though the diversity in the approaches being investigated in clinical trials is almost as great as that seen in the translational literature, it is these studies that will ultimately yield the most important data in terms of bringing stem cells treatments to clinical reality.

Conclusion

Despite progress made on the translational and clinical fronts, a number of fundamental questions persist. Translation of stem and progenitor cell populations to clinical practice requires a deep and fundamental mechanistic understanding of the tissue niches in which these populations reside, and further work must be performed on this front. Clinical translation is also limited by existing practices within tissue engineering surrounding use of extensive *in vitro* manipulation. Overcoming these limitations will be key to the future success of strategies seeking to harness the regenerative capacity of stem and progenitor cells in native bone tissue.

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