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The Convergence of Fracture Repair and Stem Cells: Interplay of Genes, Aging, Environmental Factors and Disease

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Abstract

The complexity of fracture repair makes it an ideal process for studying the interplay between the molecular, cellular, tissue, and organ level events involved in tissue regeneration. Additionally, as fracture repair recapitulates many of the processes that occur during embryonic development, investigations of fracture repair provide insights regarding skeletal embryogenesis. Specifically, inflammation, signaling, gene expression, cellular proliferation and differentiation, osteogenesis, chondrogenesis, angiogenesis, and remodeling represent the complex array of interdependent biological events that occur during fracture repair. Here we review studies of bone regeneration in genetically modified mouse models, during aging, following environmental exposure, and in the setting of disease that provide insights regarding the role of multipotent cells and their regulation during fracture repair. Complementary animal models and ongoing scientific discoveries define an increasing number of molecular and cellular targets to reduce the morbidity and complications associated with fracture repair. Last, some new and exciting areas of stem cell research such as the contribution of mitochondria function, limb regeneration signaling, and microRNA (miRNA) posttranscriptional regulation are all likely to further contribute to our understanding of fracture repair as an active branch of regenerative medicine.

Keywords

ANIMAL MODELS; CELLS OF BONE; INJURY/FRACTURE HEALING; ORTHOPAEDICS; AGING; CELL/TISSUE SIGNALING; PARACRINE PATHWAYS; STEM AND PROGENITOR CELLS; GENETICALLY ALTERED MICE

Introduction

Approximately 16 million bone fractures occur in the United States each year. Although most heal normally, approximately 10% of fractures are complicated by delayed healing or

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nonunion. Nonunions result in prolonged pain and additional surgical procedures, impair the quality of life and work productivity, reduce income, and inflate healthcare costs.^(1,2) Although treatment of nonunion depends on patient factors, anatomic location, and quality of the surrounding soft tissues, surgery remains the definitive treatment.^(2–5) Thus, there is a pressing need to develop therapeutic strategies to accelerate normal physiological fracture repair. Because of their general importance in tissue regeneration, stem cells are now a focus of research aimed at treating fracture repair.

Fracture repair is complex, involving release of cytokines, chemokines, and growth factors; induction of signaling pathways with activation of thousands of genes; and progenitor cell proliferation and differentiation, all of which occurs in a highly organized spatial and temporal process that eventually leads to tissue regeneration.⁽⁶⁾ The ultimate goal of the reparative process is to restore the biochemical, biomechanical, and morphological properties of the bone, because this is essential for locomotion, normal function, and survival of vertebrate organisms.⁽⁷⁾ The hematoma that develops immediately following fracture creates a microenvironment rich in hormones, growth factors, and cytokines that initiates the reparative process. Signals associated with the injury stimulate progenitor cell proliferation and lead to expansion of a critical mass of progenitor cells necessary for tissue regeneration. Although the periosteal cells lining the surface of the bone are the primary source of the progenitor cells, bone marrow stromal cells (BMSCs) and other progenitor cells located in surrounding skeletal muscle contribute to the process.^(8,9) The recruitment, proliferation, migration, and accumulation of periosteal progenitor and bone marrow stromal cells precede and are concurrent with differentiation into osteoblasts and chondrocytes. Chondrocytes and osteoblasts secrete collagen matrices that calcify and bridge the fracture site and are also responsible for the secondary signals that maintain the regenerative process and induce angiogenesis and remodeling (Fig. 1A–G). Although preclinical models have shown small numbers of progenitor cells derived from the systemic circulation in early fracture callus, these cells have a limited, if any capacity to differentiate into the bone and cartilage cells necessary for regeneration.⁽¹⁰⁾ In humans, progenitor cells are rare in the systemic circulation, if they are present at all, and there is no evidence to date demonstrating a role for them in fracture healing.^(11,12)

Fracture repair also involves both intramembranous ossification, whereby progenitor cells differentiate directly into osteoblasts, and endochondral ossification, the formation of a cartilage template that calcifies and is secondarily remodeled and replaced by bone.^(13,14) Intramembranous ossification occurs along the periosteal surface of the bone adjacent to the fracture site whereas endochondral ossification is predominant at the center of the fracture site.⁽¹⁴⁾ Chondrocytes tend to differentiate and form a cartilaginous soft callus, primarily in the more hypoxic environment lacking in blood vessels.^(15,16) The low oxygen tension in the fracture callus activates hypoxia-related molecular pathways that induce angiogenesis-related genes, stimulate angiogenesis, and ultimately restore the circulatory system.^(16–20) As the cartilage calcifies it is invaded by blood vessels that are a source of the chondroclasts that remove the calcified cartilage as well as the osteoprogenitor cells that form bone.^(21,22) Evidence suggests that these secondary osteoprogenitor cells are derived from pericytes present in the invading blood vessels.⁽²¹⁾ Upon completion of remodeling, bone returns to its

original architecture and function. Enhancing angiogenesis and/or increasing oxygen tension in the callus has been shown to enhance fracture repair and reduce cartilage callus, but direct effects of oxygen tension on the fate of progenitor cells in the direction to the osteoblast or chondrocyte lineage remains speculative.^(16,23–25)

Herein we review the role of progenitor cells in fracture repair and the manner in which the regenerative potential of this key cell population is modulated by aging, environmental factors, disease, and genetics. We are at the cusp of a more detailed understanding of the multiple factors that influence fracture repair and are poised to develop novel therapies to manipulate this process and improve bone regeneration.

Stem Cell Populations in Fracture Repair

Stem cells have self-renewal capacity, persist throughout life, and are responsible for tissue homeostasis where they replace damaged cells. Stem cells also play an essential role in tissue regeneration in response to injury, disease, and aging.^(26,27) Progenitor cells are organized hierarchically. Multipotent stem cells are metabolically active cells that proliferate and have unlimited renewal capacity. These cells are capable of differentiating into multiple cells and tissues, but progressively become more lineage-restricted and with differentiation they lose the capacity for self-renewal. Immature tissue lineage cells are typically located in tissues where they can rapidly respond to injury or disease. Although immature tissue lineage cells are necessary for the regenerative process, there is currently limited understanding of the specific markers and characteristics that distinguish these cell populations. Figure 2 provides a broad conceptual framework of the cell populations involved in tissue regeneration. This is an area where ongoing discovery is essential because the manner in which progenitor cells are modulated by specific signals, genes, aging, environmental factors, and disease is a key to optimizing the regenerative process.

Although the periosteum is the primary source of progenitor cells, BMSCs and muscle progenitor cells contribute to the bone repair process. In contrast, the role of circulating progenitor cells in fracture repair is not clearly established. Progenitors in the circulation are rare and, when identified in fracture callus, are present in low numbers. In this section we review information supporting a secondary role of bone marrow, muscle, and circulating cells during the fracture repair process.

BMSCs in clinical applications

Friedenstein and colleagues^(28,29) were the first to demonstrate the osteogenic potential of BMSCs in a study in which a diffusion chamber filled with bone marrow was placed into the peritoneum, and in another where bone marrow fragments were placed under the renal capsule.^(25,28,29) These early experiments showed that ectopic transplantation of bone marrow cells results in osteogenesis.^(28,29) It was in the 1980s that preclinical studies established that bone marrow cell aspirates delivered directly into fracture sites influenced bone repair. For example, Takagi and Urist⁽³⁰⁾ treated a large rat femoral gap defect with a combination of bone morphogenetic proteins (BMPs) and bone marrow aspirate and achieved high rates of union. The authors concluded that bone marrow contains both preexisting osteogenic precursor cells and mesenchymal cells that differentiate into

osteoblasts in the presence of BMPs. Paley and colleagues⁽³¹⁾ injected bone marrow into a 2-cm critical-sized rabbit radii defect and showed that callus volume, breaking load, tensile strength, and crosssectional area of callus at the bone defect were all significantly increased compared to saline-injected controls. Grundel and colleagues⁽³²⁾ evaluated a porous biphasic hydroxyapatitecalcium phosphate ceramic filled with autogenous bone marrow for the repair of a canine 2.5-cm segmental diaphyseal ulna defect and showed accelerated healing. Werntz and colleagues⁽³³⁾ used a rat femoral segmental defect model to test and evaluate the ratio of the marrow volume to the defect, implantation of live or dead marrow, and remodeling of established nonunions by implantation of live marrow. The implanted live bone marrow induced formation of woven bone at 3 weeks with progression to early lamellar bone by 6 weeks.⁽³³⁾ Last, the potential for transplanted cultured bone marrow derived stem cells to stimulate criticalsized bone segmental defects was initially demonstrated by a series of studies in the late 1990s.^(34,35)

Poorly controlled early retrospective clinical studies in humans reported that iliac crest bone marrow aspirates injected into the fracture site stimulated healing of nonunion. A study reported that 20 patients receiving autologous bone marrow aspirate injection into tibia nonunion sites had a 90% progression to radiographic healing.⁽³⁶⁾ In another study with a similar approach, 20 patients with long-bone nonunion achieved 85% radiographic healing.⁽³⁷⁾ A third study reported an 82% union rate in 11 patients with long-bone fracture nonunion.⁽³⁸⁾ Based on these early clinical reports bone marrow aspirates continue to be used for the treatment of fracture nonunion despite no definitive evidence in humans showing efficacy based on well-designed controlled clinical trials. Consequently, there is no evidence to define the concentration and exact number and type of stem cells (hematopoietic or stromal) required for successful repair or even how factors such as gender, age, site, and volume of aspiration should be considered.⁽³⁹⁾ For the most part, early preclinical studies and those following used bone marrow– derived progenitors that were isolated, cultured, and transplanted in well-controlled models in which the impact on bone healing could be quantified.^(34,35) Although preclinical studies demonstrate that a unique population of bone marrow cells can promote fracture healing, there remains no evidence basis for clinical use in humans. This presents a major gap in our understanding of fracture repair due in part to: ⁽¹⁾ the large cost of clinical studies; ⁽²⁾ the fact that fractures in humans are heterogeneous, making comparison groups difficult; and ⁽³⁾ the absence of sensitive surrogate measures of fracture healing in humans as opposed to terminal studies in animals where tissues are harvested and quantitatively evaluated.

Circulating progenitor cells

Endogenous circulating progenitor cells—Observations suggest that circulating progenitor cells may contribute to the repair process in preclinical models, but their role remains uncertain and controversial in humans. In 2001, Kuznetsov and colleagues⁽⁴⁰⁾ isolated adherent fibroblast-like cell clones from the blood of mice, rabbits, guinea pigs, and humans. The osteogenic potential of these cells was demonstrated following transplantation into the subcutis of immune-compromised mice.⁽⁴⁰⁾ These circulating connective tissue precursors were subsequently shown to be extremely rare in humans.⁽¹¹⁾ More recently, Hoogduijn and colleagues⁽¹²⁾ were unable to detect circulating mesenchymal stem cells

(MSCs) in the blood of healthy subjects, or in any of 10 patients with end-stage renal or end-stage liver disease. Similarly, no circulating MSCs were observed in eight heart transplant patients tested. However, circulating MSCs were detected in the blood of a single patient with multiple fractures. Although this suggests that disruption of bone marrow as a result of skeletal injury may allow egress of MSCs into the circulation, the evidence is limited to a single patient observation.⁽¹²⁾ Thus the potential role for circulating BMSCs in either solid organ or skeletal tissue regeneration is controversial.

Evidence more clearly supports a role for circulating BMSCs in animal models involving skeletal repair. A study using a parabiosis mouse model (transgenic green fluorescent protein [GFP] and wild-type mice) combined with a transverse fibular fracture in the wild-type mouse showed that circulating GFP+ cells are recruited to fracture callus.⁽¹⁰⁾ Histomorphometry of the fracture site revealed a steady increase of GFP+ cells over 3 weeks as compared to nonfracture bones.⁽¹⁰⁾ However, the maximal number of circulating progenitor cells recruited to the callus by 3 weeks was only 5.6%, suggesting that although these cell populations contribute, their role in fracture repair is limited.⁽¹⁰⁾ Whereas alkaline phosphatase (AP) expression in GFP+ cells in 2-week fracture callus was interpreted as evidence of a direct role in bone formation, AP expression is not a definitive marker of the osteoblast differentiation.⁽¹⁰⁾ Moreover, the origin of the GFP+ cells in the parabiosis model is uncertain. Although the GFP+ cells could have originated in bone marrow, it is possible that they were released from other tissues. Despite the establishment of circulating progenitor cell recruitment to fracture sites, the tissue of origin and the fate of the cells remain uncertain.

AMD3100 is an antagonist of the chemokine receptor 4 (CXCR4) that mobilizes stem cell populations from the marrow into peripheral blood.^(41,42) AMD3100 was used in combination with various growth factors (insulin-like growth factor 1 [IGF1], stem cell factor [SCF], platelet-derived growth factor [PDGF], or vascular endothelial growth factor [VEGF]) in a mouse segmental defect model. Combined treatment with IGF1 and AMD3100 had superior bone healing compared to other treatments as measured by DXA and micro-computed tomography (μ CT) scanning.⁽⁴¹⁾ The more robust healing was attributed to increased mobilization of hematopoietic stem cells and endothelial progenitor cells, resulting in enhanced angiogenesis. However, because there is no promoter specific enough for tracing BMSCs, the source of these cells as bone marrow-derived is speculative.⁽⁴¹⁾ In a second study, the delivery of AMD3100 by subcutaneous injection into mice with femur fractures resulted in a higher number of circulating stromal cells, hematopoietic stem cells, and endothelial progenitor cells in treated mice.⁽⁴²⁾ AMD3100 mice had a larger fracture callus (~40% more total volume) at 21 days and enhanced remodeling at 84 days following fracture compared to vehicle-treated mice.⁽⁴²⁾ These studies show that AMD3100 induces mobilization of various populations of stem and progenitor cells. Although this is associated with increased bone regeneration, a direct versus indirect role in bone repair is not defined by these studies. Any definitive role for circulating progenitor cells in human skeletal repair remains unknown.

Infused circulating progenitor cells—Based on an apparent role for endogenous circulating progenitor cells in bone regeneration, the administration of progenitor cells into

the systemic circulation has been explored as a potential method to enhance bone repair. Many studies have used labeled cell populations. Following systemic intravenous injection, both adipose-derived and bone marrow-derived progenitor cells were identified within the callus tissue of mouse tibia fractures, transverse femoral fractures, and mouse tibia segmental defects, respectively.^(43–45) In all three studies, enhanced bone formation was associated with the localization of the labeled infused circulating stem cells at the fracture callus site. The systemically administered cells localized to the bone injury site as early as 3 days following injury and remained present for up to 14 days.^(43,45) In addition, the systemic injection of endothelial progenitor cells accelerated bone regeneration in a rat femoral segmental defect (5-mm) model. After 10 weeks all rats receiving systemic administration of endothelial progenitor cells had complete union, whereas none of the rats in the control group achieved union.⁽⁴⁶⁾ Although all of these studies showed enhanced healing, there was no direct evidence that the injected cells were responsible for generating bone. Thus the mechanism is not known and the findings are mostly descriptive.

In one study in which human adipose-derived stem cells (hADSCs) were placed directly into a calvarial defect in nude mice, bone formation was induced and the hADSCs expressed the osteoblast markers ALP, Col1a1, and Runx2. However, the human cells only persisted for up to 2 weeks and then were replaced by host cells, leading the authors to conclude that the key function of the hADSCs was to influence cells in the local environment to undergo osteoblast differentiation so that the final healed and remodeled tissue was of host origin.⁽⁴⁷⁾ A mounting concern is that despite limited mechanistic insights obtained in preclinical studies, the findings have provided a rationale for the systemic administration of progenitor cells in humans with bone injuries. This has resulted in an unregulated and highly competitive industry that is based on unsupported claims of success. This industry attracts a vulnerable population of patients that in many cases are desperate for successful bone regeneration and which could easily be exploited and harmed.

Muscle progenitor cells

Skeletal muscle precursor cells reside within the muscle interstitium and along the muscle fibers.^(48–50) The “satellite cell” is the mesodermal progenitor cell in muscle that is capable of self-renewal (Fig. 2). Satellite cells are Pax7 + /MyoD–.^(49,51,52) The major purpose of satellite cells is to respond to injury and to maintain muscle mass with aging.^(51–54) Muscle injury results in the proliferation and expansion of the satellite cell population with subsequent differentiation into a pool of myogenic progenitor cells that express MyoD and coalesce to form muscle fascicles involved in contraction.^(53,54) The Notch, TGF- β , and BMP signaling pathways are involved in the maintenance of the satellite cell population in muscle and their differentiation along the myogenic pathway.^(51,52,55,56)

Although not inherently chondrogenic or osteogenic, the myogenic progenitor population is capable of forming bone tissues.^(9,48,57) Muscle is a site of ectopic bone formation following injury and BMPs were initially described for their ability to induce ectopic bone formation following injection into muscle.^(48,58) Animal models suggest that under optimal conditions for bone repair muscle progenitor cells have a minimal role as a source of cells for regeneration.^(57,59) However, a recent study suggested that in cases where there is loss of the

periosteal progenitor cell population that myogenic progenitor cells can serve as a secondary source of cells necessary for regeneration. This was demonstrated in a model using mice expressing the human AP gene under the control of the MyoD promoter (MyoD-Cre + ; Z/AP+ reporter mice).⁽⁵⁹⁾ Following injury with limited disruption of the adjacent soft tissues, the AP+ expressing myogenic progenitor population was absent from callus tissues and did not participate in the repair process. However, in mice where the periosteum was disrupted, the callus contained an abundant population of AP+ cells undergoing osteoblast differentiation that were presumably derived from the myogenic progenitor cells.⁽⁵⁹⁾ At this point only a few preclinical studies suggest a role of myogenic progenitors in bone repair, and the potential of muscle-derived progenitor cells as either a primary or secondary source of cells in bone repair has not been established in humans.

Periosteal progenitor cells

Increasing evidence shows that progenitor cells in the periosteal tissue lining the surface of the bone serve as a major and primary source of progenitor cells for fracture repair.^(13,60–62) Experimental ablation of these cells leads to nonunions in animal models of fracture healing.^(63,64) Cell fate studies that trace the periosteum progenitor cells during bone repair confirm that these cells proliferate and differentiate into chondro-osseous tissues in the initial fracture callus.^(13,62) Periosteal progenitor cells have been isolated and demonstrated to express markers of progenitor cell populations.⁽⁶⁵⁾ These cells undergo differentiation into various mesodermal lineages, including bone, cartilage, and adipose tissues in vitro.⁽⁶⁵⁾ Following transplantation via a collagen scaffold into nude mice, labeled periosteum-derived progenitor cells form bone tissue.⁽⁶⁵⁾

Periosteal cells also respond to both autocrine and paracrine factors secreted by the surrounding cells and tissues.^(60,61,65–67) The reduced regenerative potential of bone with aging likely involves changes in levels of the signaling molecules in the local environment, as well as innate changes in the periosteal progenitor cells.⁽⁶⁰⁾ The initial response of the progenitor cell leads to the formation of the callus, which then drives the subsequent processes of recruitment of cells from surrounding tissues for angiogenesis and remodeling.⁽⁶²⁾ Although periosteal cells are a major driver of regeneration, this cell population resides within a small tissue compartment that has minimal accessibility. Thus, periosteal progenitor harvest and cell transplantation is not an area of easy clinical translation. However, many tissue engineering approaches are now designed to create a periosteum-like membrane containing cells with properties that mimic periosteal progenitor cells.^(68,69) In an elegant series of experiments, Colnot⁽¹³⁾ demonstrated that the periosteal progenitor cells lining the outside surface of the bone have unique characteristics compared to cells lining the endosteal surface of the bone. Using a tibia unicortical bone-periosteum transplantation model, the periosteum could be placed into a bone defect with the periosteum facing the outside of the bone, as normally found, or it could be placed so that the periosteum faced the inside of the bone.⁽¹³⁾ The experiments established that periosteum-derived progenitors have unique characteristics compared to endosteal progenitor cells. The periosteal progenitor cells underwent both osteogenic and chondrogenic differentiation when placed either facing the outside or inside of the bone.⁽¹³⁾ However, when facing outside, periosteum formed relatively more cartilage in comparison to when it faced the inside of the

bone. In contrast, the endosteum-derived progenitor cells only underwent osteogenic differentiation and bone formation in either location.⁽¹³⁾ These and other experiments in animal models show that the periosteum is a robust source of pluripotent progenitor cells that respond to skeletal injury and drive bone regeneration. Children, who have a thick periosteum with abundant progenitor cells, rarely develop fracture nonunion.

Genetically Altered Mice: Linking Genes to Stem Cell Behavior

Genetically altered mice have contributed enormously to our understanding of the role of progenitor cells during fracture repair. A summary of a sample of the studies linking genetic modification of mice with progenitor cells and fracture repair are described.

Genetically modified mice with altered inflammatory mediators

In 2001, Gerstenfeld and colleagues⁽⁷⁰⁾ used a mouse model with tumor necrosis factor α (TNF- α) receptors (p55^{-/-} and p75^{-/-}) ablation and demonstrated impaired healing of transverse, middiaphyseal tibia fractures. Fracture callus in TNF- α receptor-deficient mice had delayed: ⁽¹⁾ osteoprogenitor cell differentiation and complete absence of intramembranous bone formation along the periosteal surface; ⁽²⁾ cartilage differentiation and resorption; and ⁽³⁾ expression of osteogenic-related (ie, *collagen type I* and *osteocalcin*) and chondrogenic-related (ie, *collagen type II* and X) genes.⁽⁷⁰⁾ The authors concluded that a primary role for TNF- α in fracture repair is to facilitate the recruitment of osteoprogenitor stem cells, simulate apoptosis of hypertrophic chondrocytes, and enhance recruitment of osteoclasts to the calcified cartilage callus.⁽⁷⁰⁾

A recent study used cyclooxygenase-2 (COX-2^{-/-} mice and a 4-mm murine live femoral autograft model. Transplantation of bone grafts from a COX-2^{-/-} donor into a COX-2^{-/-} host led to 96% reduction of bone formation compared with similar transplantation in wild-type mice.⁽⁷¹⁾ Limited donor cell-initiated periosteal bone formation was observed in these mice lacking COX-2.⁽⁷¹⁾ A critical role for COX-2 in periosteal stem cell proliferation and differentiation was shown directly in tibia fractures in COX-2^{-/-} mice that were administered BrdU to label proliferating cells in vivo during the repair process. Absence of COX-2 resulted in a 50-fold decrease in the proliferation in cells along the periosteal surface of the bone at 3 days, with a decreased rate of proliferation remaining through 10 days following fracture.⁽⁷²⁾ Predictably, COX-2 gene deletion resulted in reduced fracture callus volume.⁽⁷²⁾ The major metabolite of COX-2 involved in fracture repair and bone formation is prostaglandin E2 (PGE2), which binds to four different G-protein-coupled receptors, EP1, EP2, EP3, and EP4.⁽⁷³⁾ In particular, activation of the EP2 and EP4 receptors, which both stimulate protein kinase A (PKA) signaling, enhances bone formation.^(72,74) Interestingly, a recent publication showed that EP1 gene deletion results in fracture calluses that are larger and have increased cartilage formation, faster completion of endochondral ossification, and enhanced mineralization and remodeling compared to wild-type mice.⁽⁷⁵⁾ Further, EP1^{-/-} mesenchymal progenitor cells isolated from bone marrow and placed in cell culture had enhanced osteoblast differentiation and increased bone nodule formation and mineralization.⁽⁷⁵⁾ Altogether, the studies suggest that COX-2-PGE2 signaling acts on EP2 and EP4 receptors to stimulate periosteal progenitor cell proliferation and differentiation

following fracture, but is balanced by EP1 receptor signaling which maintains progenitor cells in an immature state.

Genetically modified mice with altered growth factor signaling

Genetically modified mouse models are useful tools to gain understanding of the role of specific signals in tissue regeneration. Although these approaches for the most part are not focused specifically on tissue progenitor cells, they still provide some insight into the signaling mechanisms involved in regulating progenitor cell proliferation and differentiation during fracture repair. BMP-2 signaling was shown to be essential for the proliferation and accumulation of a progenitor cell population in a femur fractures in a paired-related homeobox 1 (Prx1)-Cre; BMP-2(f/f) mouse model.⁽⁶⁷⁾ Whereas control mice have fracture healing with bridging callus formation between 10 and 20 days, the heterozygous BMP2 (+/-) mice, which have half the normal BMP-2 expression, have reduced fracture callus size. More remarkably, Prx1-Cre;BMP-2(f/f) mice, which lack BMP-2 expression in femur fracture callus, completely failed to form a fracture callus.⁽⁶⁷⁾ There was no activation of cell proliferation in the periosteum at the fracture site and there was no accumulation of a progenitor cell population necessary to drive the regenerative response.⁽⁶⁷⁾ BMP-2 in particular is essential for this process because neither BMP-4 or BMP-7 expression are required for fracture healing.^(76,77) Based on clinical studies, BMP-2 has been U.S. Food and Drug Administration (FDA)-approved to enhance the healing of open tibia fractures, suggesting that activation of progenitor cell populations is an important target to enhance fracture healing in humans.⁽⁷⁸⁾

Wnt/ β -catenin is necessary for bone regeneration in animal models.^(6,79,80) Inhibitor of β -catenin and TCF4 transgenic mice (ICAT-Tg mice), which have reduced β -catenin signaling, have impaired fracture healing. The intramembranous bone formation necessary for mandible regeneration is impaired in low-density lipoprotein receptor-related protein knockout mice (LRP5^{-/-}).^(81,82) In another study, LRP5^{-/-} mice showed impaired repair fracture repair when compared to Lrp5^{+/+} mice, as indicated by reduced callus area, bone mineral content, and bone mineral density, and biomechanical properties.⁽⁸³⁾ In contrast, genetic models with increased β -catenin signaling have more robust healing. Sclerostin ^{-/-} mice heal bone defects more rapidly, as do Axin2^{-/-} mice and glycogen synthase kinase (GSK)-3 β ^{+/-} mice.⁽⁸⁴⁻⁸⁶⁾ GSK-3 β inhibitors increase β -catenin signaling and enhance bone formation/regeneration.⁽⁸⁷⁾ Sclerostin increases with aging and elevated levels are seen in postmenopausal women; this could be one factor reducing bone repair with aging.⁽⁸⁸⁾ Although β -catenin signaling is critical for fracture repair, its actions are complex; differential effects have been observed at different stages of the repair process.⁽⁸⁹⁾ With regard specifically to progenitor cell populations, mice with secreted frizzled related protein 1 gene deletion (sFRP1), a Wnt antagonist, had a shift in the differentiation of the progenitor population predominantly to osteoblasts with reduced cartilage formation.⁽⁹⁰⁾ This promoted an increase in intramembranous bone formation with bone bridging after 14 days and caused earlier bone remodeling by 28 days.⁽⁹⁰⁾ The accelerated fracture repair was associated with enhanced biomechanical strength.⁽⁹⁰⁾ Thus, enhanced osteoblast differentiation of the stem cell progenitor cell pool with increased intramembranous ossification relative to endochondral bone formation improves fracture repair.

An elegant study employed the transplantation of primary BMSCs expressing IGF-I into wild-type and into insulin receptor-deficient mice. Insulin receptor substrate 1 (*Irs1*^{-/-}) mice have a failure of fracture repair.⁽⁹¹⁾ The local delivery of the IGF-I-expressing BMSCs into tibia fractures of wild-type mice increased intramembranous bone formation and improved the biomechanical properties of fracture repair. Transplanted IGF-I-expressing BMSC cells induced osteoblast differentiation in both the transplanted cells and in the local host cells through mechanisms involving activation of the phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathway.⁽⁹¹⁾ Similarly, transplantation of IGF-I-expressing MSCs into tibia fractures of *Irs1*^{-/-} mice induced abundant bone and cartilage formation and stimulated fracture repair. However, when control MSCs lacking increased IGF-I expression were transplanted, the fracture healing in *Irs1*^{-/-} mice was not restored.⁽⁹¹⁾ Fracture callus in *Irs1*^{-/-} mice had accumulation of fibroblastic tissue and an absence of cartilage or bone formation. Thus, the autocrine action of IGF-I on the transplanted BMSCs was sufficient to stimulate new bone formation in *Irs1*^{-/-} mice and to partially rescued fracture healing.⁽⁹¹⁾

Another example of an important signal regulating mesenchymal progenitors during fracture healing was recently shown in A2B adenosine receptor (*A2BAR*)^{-/-} mice.⁽⁹²⁾ A2BAR is a G-protein-coupled receptor that binds adenosine and signals via cAMP, which in turn, has been demonstrated to regulate the differentiation of progenitor cells into osteoblasts.⁽⁹³⁾ In a closed transverse femur fracture model, fracture calluses of *A2BAR*^{-/-} mice had a decreased ratio of bone volume to total callus volume at 14 and 21 days following fracture.⁽⁹²⁾ Histomorphometry showed increased cartilage area, indicating delayed endochondral ossification.⁽⁹²⁾ Analysis of gene expression showed a decrease in the osteoblast markers, Runt-related transcription factor 2 (*Runx2*) and Osterix at postfracture days 3 and 7, but increases in the chondrogenic marker *Col2a1* (Collagen II) compared with the wild-type mice.⁽⁹²⁾ The findings show that A2BAR is involved in various phases of the fracture repair and is necessary for normal healing.

Genetically modified mice with altered integrins

Direct evidence showing a role for the $\alpha 1\beta 1$ integrin collagen receptor on the function of bone progenitor cells in bone regeneration was obtained in a study conducted using $\alpha 1\beta 1$ integrin (collagen receptor)-deficient mice.⁽⁹⁴⁾ Mice deficient in $\alpha 1$ integrin have no bone defects during growth and development. However, closed diaphyseal tibia fracture model showed that the fracture calluses in $\alpha 1$ integrin knockout mice were smaller, contained less cartilage, and had decreased numbers of proliferating bone marrow-derived progenitors in comparison to the wild-type mice.⁽⁹⁴⁾ Moreover, isolated BMSCs from $\alpha 1$ integrin knockout mice showed a ~50% decreased proliferation rate relative to the wild-type cells, consistent with the in vivo observation.⁽⁹⁴⁾ Molecular analyses revealed decreases in the mRNA levels of a number of cartilage-related matrix genes (*aggrecan*, *decorin*, and *biglycan* as well as *collagen I, II*, and *IX*) as early as postfracture day 9.⁽⁹⁴⁾ These findings defined a role in for integrin-signaling during progenitor cell proliferation and formation of fracture callus. Given the importance of integrins in serving as the cell anchors on collagens, it is no surprise that these detrimental results were obtained with the $\alpha 1$ knockout mice.

Aging: Exerting Its Inhibitory Effects

There are two aspects of aging which potentially affect tissue regeneration: ⁽¹⁾ intrinsic (genetic and epigenetic) changes in stem cell populations and ⁽²⁾ changes in the microenvironment (hormone and growth factors, etc.) that alter the biological activity of progenitor cells. Adult progenitor cells are present in essentially all tissues where they regulate tissue homeostasis and regeneration. Depletion and/or senescence of progenitor cells are associated with age-related tissue degeneration as well as the reduced potential for regeneration following injury.^(95,96) Defining the mechanisms involved is important because reversal of this process can delay the effects of aging and enhance regeneration.

Telomeres are repetitive nucleotide sequences at the end of each of the chromosomes that enable DNA replication without loss of genetic information. With each cell division, telomeres shorten, and the capacity for ongoing proliferation is reduced.⁽⁹⁷⁾ Telomere maintenance is essential for unlimited self-renewal and is a characteristic of stem cells. Multipotent tissue resident progenitor cells, although not having capacity for unlimited renewal, have the enzymatic machinery necessary to maintain telomere length through multiple rounds of cell division. The various proteins in the telomerase enzyme complex and in the shelterin telomere protection complex are involved in controlling telomere length.^(98–103)

Telomerase is present rarely and in select populations in adult tissues. Its presence has been related to the limited self-renewal capability required for tissue regeneration.⁽⁹⁷⁾ Several studies show that cell populations critical for regeneration in various tissues express increased levels of telomerase, including both the protein telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC), a noncoding RNA that is part of the telomerase enzyme complex.^(96,97,104,105) Shortened telomeres promote senescence by inducing expression of the tumor suppressor p53 and the cyclin-dependent kinase (CDK) inhibitors p16 and p21.^(95,97,106,107) These genes and others that cause cell-cycle arrest and senescence markedly impair progenitor cell self-renewal function.^(108–111) Cumulative oxidative stress and DNA damage with aging is another factor that promotes cell senescence and apoptosis.^(95,105,106,112–114) The importance of maintaining robust stem cell populations is demonstrated in mice with telomerase gene deletion.^(95,97) These mice develop senescent stem cell populations and have reduced capacity for tissue maintenance and regeneration.⁽⁹⁷⁾ In bone, telomerase gene deletion results in accelerated aging and reduced bone mass.⁽⁹⁵⁾ However, the role of telomerase in the reduced potential for bone regeneration with aging has not been defined. Because the cell senescence pathway is the major physiologic mechanism preventing the development of malignant cells,^(107,110) therapeutic approaches using either delivered or endogenous stem cell populations need to consider the potential consequence of unrestricted cell growth and cancer.^(107,109,110) This represents one of the major challenges of enhancing tissue regeneration with aging.

Uniformly, animal models mimic humans by having reduced capacity for bone repair with aging.^(24,74,115–117) Several studies suggest a cell-autonomous effect by demonstrating that stem cells in the local environment have reduced expression of critical regenerative factors in aged mice compared to young mice. Progenitor cells in the periosteum of healing

fractures in aged mice have reduced expression of COX-2 compared to similar progenitor cell populations in young mice.⁽⁷⁴⁾ The reduced callus formation and fracture healing observed in aged mice can be enhanced with delivery of an EP4 receptor agonist to the fracture site.⁽⁷⁴⁾ Another study showed that, as compared with 4-week-old mice, middle-aged (6 months) and old mice (18 months) exhibit a progressive decrease in the expressions of hypoxia-inducible factor-1 α (HIF1 α) and matrix metalloproteinase 9 (MMP9) early in fracture repair and have reduced vascularization of fracture callus and decreased bone regeneration.⁽²⁴⁾ A murine calvaria critical-defect model was used to examine the relative regenerative potential of BMSCs from young mice compared to aged mice. Bone formation was markedly impaired when BMSCs from aged mice were placed into the defect. The BMSCs derived from aged mice had reduced β -catenin signaling and the bone healing potential was restored in progenitor cells derived from older mice following Wnt3a treatment.⁽¹¹⁸⁾ A recent study examined the early periosteal progenitor cell response in rigidly fixed murine proximal tibia osteotomies.⁽⁶⁰⁾ A more robust periosteal response was observed in the young mice compared with aged mice. In addition, the rate of proliferation, expression of cyclin D1, and the amount of matrix formed were significantly elevated in young mice compared to aged mice. Interestingly, whereas the progenitor cell populations in both young and aged mice had an anabolic response to PTH1–34, the effect was larger in the young mice.⁽⁶⁰⁾ These various studies suggest that aging results in intrinsic differences in the population of regenerative (progenitor) cells that limit the capacity for new tissue formation.

Other studies suggest that the differing potential for tissue regeneration is related to a unique profile of systemic factors in young compared to aged mice.⁽¹¹⁹⁾ Inflammation increases with aging.⁽¹²⁰⁾ In a distraction osteogenesis model, aged mice had increased circulating serum levels of IL-6 and TNF α and a 60% reduction in bone formation compared to young mice.⁽¹²¹⁾ The reduced bone formation in aged mice could be reproduced in young mice treated with recombinant TNF α , and it could be reversed in old mice that had TNF α signaling inhibited by the administration of a soluble TNFR1.⁽¹²¹⁾ The induction of senescence by inflammation in local progenitor cell populations was evident from the observation that mice with a p21 gene deletion did not have impaired bone healing when treated with recombinant TNF α .⁽¹²¹⁾ A role for differences in systemic inflammatory signals is further suggested by another study in which bone marrow in 4-week-old and 1-year-old mice was ablated with radiation and then reconstituted with marrow from mice of different ages.⁽¹²²⁾ This approach created aged chimeric mice in which tissue progenitor cells, osteoblasts, and chondrocytes were host-derived, but inflammatory cells in the callus were derived from younger mice. The chimeric aged mice developed larger fracture calluses and had accelerated remodeling compared to the control aged mice, indicating that the inflammatory cells from young mice supported a more robust bone-injury regenerative response. However, in young chimeric mice, the transplantation of inflammatory cells from aged mice did not alter or inhibit the accelerated healing observed in these mice.⁽¹²²⁾ Thus, the anabolic activity of host stem cells in the young mice was not impaired by the transplantation of an older inflammatory cell population and suggests that although systemic inflammation and other factors influence local stem cells in a non-cell-autonomous manner,

intrinsic differences in stem cell populations are an important determinant of the reduced repair that occurs with aging.

Environmental Factors: Compounding the Inhibitory Effects

Cigarettes, nicotine, and smoke

Cigarette smoke, alcohol abuse, poor nutrition, and heavy metal exposure are implicated in reduced healing. Animal models have been useful for understanding the molecular, cellular, and tissue responses to various exposures. A limitation of these models is that humans are often subjected to long-term exposures that cannot be reproduced in animals. Nonetheless, these models have provided important insights regarding pathogenesis as it relates to fracture repair.

It is well established now that cigarette smoke contains more than 4000 different compounds. A larger number of studies have been completed with nicotine, because administration of nicotine in animals is technically easier than the delivery of cigarette smoke exposure. Although nicotine exposure is only one among the many chemicals in cigarette smoke and thus has limitations as a model for cigarette exposure, it is highly relevant as a drug used for smoking cessation.⁽¹²³⁾ Furthermore, electronic cigarettes (e-cigarettes), which are rapidly growing in popularity and public acceptance, are electronic nicotine delivery systems lacking the other components of cigarette smoke.⁽¹²⁴⁾ Each cigarette delivers a dose of approximately 2 to 3 mg of nicotine.⁽¹²⁵⁾

Although a common chemical exposure, the effect of nicotine on bone regeneration is uncertain. Some in vitro studies have shown that low doses of nicotine can enhance osteoblast differentiation through direct effects on nicotinic receptors,^(126–128) whereas other studies suggest that nicotine enhances proliferation but delays differentiation of osteoblast precursors.^(129,130) For the most part, preclinical studies suggest that nicotine does not alter stem cell recruitment or expansion in areas of skeletal injury, but reduces the subsequent differentiation of chondrocytes and osteoblasts.⁽¹³¹⁾ However, studies have shown both positive and negative effects on fracture repair. Nicotine decreased BMP expression and impaired bone formation in a rabbit distraction osteogenesis model.^(131,132) Tibia osteotomy repair was decreased in rabbits treated with a transdermal nicotine patch.⁽¹³³⁾ However, in some cases, nicotine has been shown to enhance tissue repair. For example, nicotine accelerated skin healing by stimulating epithelial progenitor cell proliferation, migration, and vasculogenesis.⁽¹³⁴⁾ In stabilized rat femur fractures, nicotine increased torsional strength and stiffness compared to vehicle treatment after 3 weeks of healing.⁽¹³⁵⁾ Thus, although nicotine regulates tissue regeneration, the manner in which this common environmental toxin regulates progenitor cell populations and modulates bone repair requires further study.

In contrast, preclinical studies with cigarette smoke exposure clearly define an inhibitory effect on bone repair. Mice exposed to cigarettes in smoking chambers formed a normal early fracture callus, but had delayed formation of cartilage and bone and bone remodeling.⁽¹³⁶⁾ Kung and colleagues⁽¹³⁷⁾ used smoke chambers to show that fracture calluses in mice exposed to cigarette smoke had activation of the aryl hydrocarbon receptor

(AHR). The AHR receptor is stimulated by the polycyclic aromatic hydrocarbons compounds, including dioxin, which are present in cigarette smoke.⁽¹³⁸⁾ The AHR receptor does not yet have a naturally identified ligand, but it signals through the ARNT pathway, and thus shares a cytoplasmic binding partner and transcriptional coactivator with the HIF signaling pathways.⁽¹³⁸⁾ Both cigarette smoke extract (CSE) and the AHR agonist benzo(a)pyrene (BAP) inhibited chondrogenesis in E11.5 mouse embryonic limb bud micromass cultures.⁽¹³⁷⁾ Because of the complexity of cigarette smoke and the number of toxins present, a combination of molecular events likely influence regeneration and will be challenging to fully understand. An interface with unique genetic traits is suggested by the finding that wound healing is differentially affected by cigarette smoke in mice from distinct strains.⁽¹³⁹⁾ Last, cigarette smoke, particularly with long-term exposure, results in accelerated aging as it acts to shorten telomeres.^(140,141) As described above, telomere shortening leads to stem cell senescence, a major factor associated with the age-related reduced capacity for tissue homeostasis and regeneration.^(112,114)

Heavy metals

Heavy metal exposures are an environmental hazard present in the modern and developing world. Major metal toxicants include lead and cadmium. Cadmium is a component of cigarette smoke, which remains the major risk factor for cadmium exposure. Cadmium is also an occupational exposure hazard, air pollutant, and environmental contaminant that enters the food system in significant levels^(142,143) and leads to DNA damage and telomere shortening.⁽¹⁴⁰⁾ Consistent with its negative effects on telomeres, increased cadmium exposure results in reduced bone density and increased risk for osteoporotic fractures.^(142,144) Unfortunately, limited work has been done regarding its effects on progenitor cells and fracture repair in animal models.

Although lead exposure has been more comprehensively studied, work has similarly focused more on the cell differentiation effects rather than on the regulation of progenitor cells. In large human population studies, lead is associated with osteopenia and a reduced rate of childhood growth.^(145,146) Rats exposed daily to normal water or water containing 50 parts per million (ppm) of lead for 18 months develop low-level chronic lead exposure with serum concentrations similar to those observed in human populations, and have osteopenia with reduced trabecular and cortical bone, alterations in mineralization of bone, reduced osteoblasts, and increased bone marrow fat.⁽¹⁴⁷⁾ Gene expression from harvested bones showed that lead increased expression of the adipocyte-specific genes *PPAR gamma*, *adipocyte protein 2 (aP2)*, and decreased expression of the osteoblast specific genes, *osteocalcin* and *Runx2*. In vivo and in vitro experiments established that lead increases sclerostin levels and reduces β -catenin signaling (a key stimulator of osteoblastogenesis in stem cell populations) in the bone microenvironment.⁽¹⁴⁷⁾

Reduced osteoblast formation was also observed during fracture repair in lead-exposed mice.⁽¹⁴⁸⁾ Lead exposure increased the relative formation of cartilage tissue and reduced the formation of bone tissue in the fracture callus.⁽¹⁴⁸⁾ The maturation and disappearance of cartilage was impaired, resulting in delayed healing in mice exposed to a low lead

concentration, and formation of a fibrous nonunion in mice exposed to a high concentration of lead.⁽¹⁴⁸⁾

Disease: Additional Inhibitory Factors

Obesity and diabetes

Obesity and diabetes is a worldwide epidemic. The incidence of obesity in the U.S. population is now over 30% and approximately one-third of obese individuals develop type II diabetes. Diabetes is associated with reduced fracture repair in humans. Humans with type II diabetes have reduced numbers of circulating peripheral blood mononuclear cells that express osteocalcin, and have elevated levels of serum sclerostin and reduced levels of serum β -catenin.^(149–151) Factors involved in reduced bone repair in diabetes have been studied in several rodent models.

Streptozotocin is toxic to the insulin-producing beta cells in the pancreas and thus produces a disease that mimics type I diabetes. Rodents treated with streptozotocin have impaired fracture repair characterized by reduced callus formation, accelerated loss of cartilage, and decreased healing.^(152–154) TNF α expression is increased in chondrocytes in a fracture callus in streptozotocin-treated mice. Inhibition of TNF signaling by systemic pegsunercept treatment restores fracture healing to normal levels.⁽¹⁵²⁾ In a rat model of streptozotocin-induced diabetes, the proliferation of chondroprogenitor cells is similarly reduced at postfracture days 4 and 7, with decreased cartilage formation, and delayed union.⁽¹⁵⁵⁾ The potential for endosteal bone formation is also diminished in the streptozotocin-treated diabetic rat model; bone ingrowth into titanium coated femoral implants is reduced compared to the level of osseointegration in control rats.⁽¹⁵⁶⁾ Last, BMSCs isolated from streptozotocin-treated diabetic rats have decreased colony forming units and reduced osteoblast differentiation compared to control BMSCs.⁽¹⁵⁷⁾

Genetic models of obesity-induced type II diabetes mice lacking the adipose secreted hormone leptin (ob/ob mice) or the leptin receptor (db/db mice), which is located in the hypothalamus, have been studied. Leptin receptor-deficient db/db mice have reduced fracture repair, delayed periosteal progenitor cell osteogenesis, premature apoptosis, and impaired vascular invasion of the cartilage callus.⁽¹⁵⁸⁾ Because leptin also regulates bone metabolism through the sympathetic nervous system, effects could alternatively be related to either systemic/local effects or central nervous system (CNS) effects alone or in combination. The Zucker diabetic fatty (ZDF) fa/fa rat is a spontaneous model of diabetes. These animals have reduced bone mass and mechanical strength in the vertebral bodies and in the femurs.⁽¹⁵⁹⁾ Bone repair was examined in a 3-mm stable femoral defect model and the diabetic rats had less than one-half as much bone formation within the defect compared to normal rats. Sclerostin antibody enhanced bone formation in both rat models, but with greater effects observed in the normal rats.⁽¹⁵⁹⁾ These experiments indicate that type II diabetes leads to reduced potential for bone repair and suggest diminished progenitor cell responsiveness to bone anabolic agents.⁽¹⁵⁹⁾

Other data suggests that insulin is directly anabolic for bone formation.^(160,161) BB Wistar rats develop spontaneous insulin-dependent diabetes. BB Wistar diabetic rats have reduced

progenitor cell proliferation by 2 and 4 days postfracture.⁽¹⁶¹⁾ By 6 weeks postfracture, diabetic rats have incomplete fracture repair with reduced bone formation and increased cartilage and fibrous tissues in the callus.⁽¹⁶¹⁾ The diabetic mice also have limited remodeling, and reduced biomechanical strength compared to normal mice. Fracture callus volume and biomechanical parameters of fracture repair are increased in control mice with normal glucose levels as well as in hyperglycemic mice that receive delivery of insulin directly at the fracture site through a local delivery pump.^(160,161) The delivery of local insulin to the callus normalized the rate of proliferation in spite of continued systemic hyperglycemia.⁽¹⁶¹⁾

Emerging Areas for Stem Cells and Fracture Repair: What the Future Holds

Mitochondria and regeneration

Stem cell populations present within various skeletal tissues are typically in a quiescent state, have limited mitochondrial activity, and meet energy demands through glycolysis.^(162,163) Activation of stem cell populations involves a shift to oxidative phosphorylation.^(162,166) Recent data suggests that there is a strong linkage between mitochondrial function, the expression and activity of cell cycle regulators, and cellular differentiation.^(163,165–167) Although glycolysis is not as efficient, it provides energy more rapidly than oxidative phosphorylation and it generates less reactive oxygen species that can lead to DNA damage and cellcycle arrest. Importantly, there also appears to be a gene regulatory relationship between mitochondria function and the fate of stem cell populations.⁽¹⁶²⁾ Inhibition of mitochondrial function results in glycolysis, enhanced self-renewal, and expression of genes associated with stem cells.⁽¹⁶²⁾ Similarly, the reprogramming of induced pluripotent stem (iPS) cells is associated with an opposite shift from oxidative phosphorylation to glycolysis.^(163,166) With age, there is a decline in mitochondrial metabolism, with a reduced capacity for oxidative phosphorylation and decreased regenerative potential.⁽¹⁶⁸⁾

Agents that stimulate mitochondrial activity and oxidative phosphorylation induce maturation and exit from the stem cell niche.⁽¹⁶²⁾ Stem cells have mitochondria with less differentiated features, including poorly formed cristae, reduced mitochondrial membrane potential, and an open mitochondrial permeability transition pore (mPTP) that causes leakage and inefficiency of electron transport. Pharmacologic or genetic factors that close the mPTP lead to mitochondrial maturation with oxidative metabolism and result in differentiation of progenitor cells.⁽¹⁶⁴⁾ The reprogramming of adult cell populations to iPS cells through the expression of essential stem cell–specific transcription factors results in the acquiring of structural and functional mitochondria characteristics consistent with the immature mitochondria observed in stem cell populations.^(162,163,166) Regulation of mitochondrial function is an emerging target in regenerative medicine.

Limb regeneration signals

Complete regeneration of complex structures composed of different tissue types remains an ultimate goal. Notch signaling and Wnt/ β -catenin signaling are key factors involved in limb regeneration in zebrafish and mammalian models. Zebrafish are capable of fin

regeneration.⁽¹⁶⁹⁾ Amputation of the fin results in the formation of a mass of undifferentiated mesenchyme possessing high levels of Notch signaling. Notch is required for the formation and expansion of this cell population and fin regeneration.⁽¹⁶⁹⁾ In the absence of Notch signaling, proliferation is impaired and fin regeneration is inhibited. Notch signaling has also been shown to be important in the regeneration of mammalian tissues, including cardiovascular development and repair, neural regeneration in the brain, and in bone, where it is necessary for expansion of stem cell populations required for development and regeneration.^(170–174)

β -catenin signaling was recently identified as a major factor involved in the potential for mammalian limb regeneration, a complex process that occurs in humans and other mammals only following fingertip amputations (Fig. 3A–D). Takeo and colleagues⁽¹⁷⁵⁾ used conditional gene deletion experiments to show that induction of β -catenin signaling in proximal nail bed stem cells following digit amputation is required for complete regeneration of the bone, soft tissues, and nerve in mammals. β -catenin signaling in nail bed stem cells acts in a paracrine manner to induce the formation of a stem cell blastema that provides the cell population necessary for the regenerative process. *Runx2* was expressed in 90% of the proliferating mesenchyme progenitors that compose the blastema. Regenerating nerve fibers extending into the blastema express fibroblast growth factor 2 (FGF2), another key component of the limb regenerative process.⁽¹⁷⁵⁾

microRNA and regeneration

Recent evidence links expression of specific microRNAs (miRNAs) with control of commitment and differentiation of MSCs into specific lineages.⁽¹⁷⁶⁾ Individual miRNAs have been shown to serve as both positive and negative regulators of osteoblast differentiation from progenitor cells during skeleto-genesis.^(177–181) Dicer-null growth plates display reduced numbers of proliferating chondrocytes, associated with severe skeletal growth defects and premature death.⁽¹⁷⁹⁾ Thus, the absence of miRNAs (as a result of Dicer knockdown) suggests a critical role in normal chondrocyte maturation. Dicer was also shown to be required for morphogenesis but not for patterning of the vertebrate limb.⁽¹⁸⁰⁾ Targeted deletion of miR-140 resulted in a mild skeletal phenotype with a short stature,⁽¹⁷⁸⁾ whereas miR-182 was found to function as a FoxO1 inhibitor to antagonize osteoblast proliferation and differentiation, with subsequent negative effects on osteogenesis.⁽¹⁸¹⁾

The involvement of miRNAs in fracture repair has recently been demonstrated. A study in rats showed the differential expression of a number of miRNAs during the early stages of fracture repair following alcohol exposure.⁽¹⁸²⁾ Murata and colleagues⁽¹⁸³⁾ compared the plasma concentrations of miRNAs in four human patients with fractures and found that out of 134 miRNAs, six were dysregulated. They also showed that systemic administration of antimir-92a in mice increased callus volume and enhanced fracture repair via an increase in neovascularization.⁽¹⁸³⁾ More recently, a study reported on the presence of five free-circulating miRNAs and a number of other bone tissue miRNAs that are associated with human osteoporotic fractures.⁽¹⁸⁴⁾ Clearly, the number and mechanisms involved in miRNA regulation of fracture repair undoubtedly represents an emerging area of research.

Summary

Fracture repair is a complex process that requires the proliferation and expansion of a progenitor cell population for successful tissue regeneration. Although a reservoir of progenitor cells remains available for bone regeneration throughout a lifetime, this essential population of cells is subject to the accumulated effects of aging, changes in the microenvironment, environmental exposures, and disease. Moreover, the manner in which these factors alter progenitor cells and their potential for tissue regeneration is influenced by inherited and acquired genetic and epigenetic features. Complete understanding of the complex interface of environmental exposures, aging, disease, and genetics on the proliferation, accumulation, and differentiation of progenitor cell populations is key to unlocking bone regenerative therapies to improve skeletal diseases in the human population. Although the complexity of the factors controlling regeneration is daunting, the vast number and variety of potential points of regulation provides an exciting array of potential therapeutic targets that can lead to improvements in clinical care.

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Fig. 1.

The regenerative response to fracture. (A) Bone is a mineralized tissue that resists tension, compression, and torsional stresses. However, if the deforming energy surpasses a critical threshold structural failure of bone results in a fracture. (B) The initial response to the injury involves development of a hematoma and formation of a fibrin clot at the site of the fracture. Inflammatory cytokines, chemokines, and growth factors, such as TNF- α , CXCR4, SDF1, and PDGF, are released into the fracture hematoma. (C) Signals initiated by the injury activate stem cell progenitors located primarily in the periosteum and in the bone

marrow. This results in proliferation and expansion of the pool of progenitor cells necessary for repair. Key signals involved in these early events include BMP, COX-2/PGE2, Wnt/ β -catenin, and Notch signaling pathways. Marked changes in gene expressions occur in these cell populations and there is a shift in cell metabolism from glycolysis to oxidative phosphorylation. Evidence supporting a role for progenitor cells from the circulation has only been demonstrated in preclinical models, and a potential role in humans has not been established. (D) The expanded stem cell population undergoes differentiation with the expression of chondrocyte lineage genes (*Sox9*, *Col2a1*, *ColXa1*) and osteoblast lineage genes (*Runx2*, *Osterix*, *Col1a1*). Osteoblasts form bone directly through intramembranous ossification along the surface of the bone at adjacent ends of the fracture where injury is less severe. Cartilage is formed in the central, more hypoxic area of the fracture where the tissue injury is maximal. Undifferentiated mesenchyme persists longest in the most central region of the fracture and is flanked by cartilage and bone tissues at either end of the fracture. (E) The cartilage callus tissue undergoes calcification and is invaded by blood vessels that bring in osteoprogenitors and chondroprogenitors that initiate secondary bone formation and remodeling. Terminal differentiation, calcification, angiogenesis, and remodeling are associated with the expression of *Osteocalcin*, *VEGF*, *MMP13*, and *RANKL*. Pericytes in the vasculature provide a robust source of osteoprogenitors for secondary bone remodeling. (F) Fracture repair occurs with bridging of the fracture with calcified tissues, but is completed with remodeling of the fracture, which restores the bone to its normal size and shape. (G) Complete healing. SC = stem cell;

Fig. 2. Schematic of progenitor cell lineage. Multipotent stem cells have self-renewal capacity. A key feature of these cell populations is the expression of telomerase and the maintenance of telomere length. Multipotent cells are maintained in a quiescent state with inactive mitochondrial function until needed for tissue maintenance or regeneration. The multipotent cells have capacity to differentiate into different cell and tissue types. The various progenitor cell populations are present within specific tissues and begin to express lineage-specific transcription factors. For example, the mesodermal progenitor cell in muscle tissue expresses Pax7 and is referred to as a satellite cell. Upon tissue injury, progenitor cells proliferate and provide a pool of immature tissue lineage cells necessary for tissue regeneration. Immature tissue lineage cells have limited self-renewal but readily differentiate with the expression genes and transcription factors associated with mature cell and tissue function. The progression through the differentiation process is associated with loss of telomere length and reduced self-renewal capacity. The factors that stimulate progenitor cells to progress through this process, and the manner in which aging, disease, environmental factors, and genetics modulates these processes are focus areas in the study of tissue regeneration.

Fig. 3.

Regeneration of the fingertip in mammals. (A) The fingertip is a complex tissue that includes bone, tendon, nerve, and the nail matrix, which is a specialized epithelial tissue. Amputation of the fingertip with preservation of the proximal nail matrix is the only example of an appendage that undergoes complete regeneration in humans and other mammals. (B) Amputation of the fingertip results in activation of nail bed stem cells and induction of Wnt/ β -catenin signaling. Particularly important is the proximal nail matrix where the progenitor cell (SC) population is located. In the distal nail matrix, progenitor cells are a

transient cell population that is undergoing amplification and subsequent differentiation. Wnt/ β -catenin signaling in the nail bed leads to proliferation of mesenchymal stem cells and formation of a regenerative blastema distal to the bone and soft tissue injury. (C) The undifferentiated mesenchymal cells in the blastema have abundant expression the osteoprogenitor gene, *Runx2*. The blastema osteoprogenitor cell population expresses *Fgfr1*. The regenerative blastema is further enhanced by the invading regenerating nerve tissues that express *FGF-2*. (D) The interactions between the various tissues eventually results in complete regeneration of the fingertip. SC = progenitor cell; Fgfr = fibroblast growth factor receptor; FGF = fibroblast growth factor; MSC = mesenchymal stem cell.