

Cellular Biology of Fracture Healing

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ABSTRACT: The biology of bone healing is a rapidly developing science. Advances in transgenic and gene-targeted mice have enabled tissue and cell-specific investigations of skeletal regeneration. As an example, only recently has it been recognized that chondrocytes convert to osteoblasts during healing bone, and only several years prior, seminal publications reported definitively that the primary tissues contributing bone forming cells during regeneration were the periosteum and endosteum. While genetically modified animals offer incredible insights into the temporal and spatial importance of various gene products, the complexity and rapidity of healing—coupled with the heterogeneity of animal models—renders studies of regenerative biology challenging. Herein, cells that play a key role in bone healing will be reviewed and extracellular mediators regulating their behavior discussed. We will focus on recent studies that explore novel roles of inflammation in bone healing, and the origins and fates of various cells in the fracture environment. © 2018 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res* 37:35–50, 2019.

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Injuries to the appendicular skeleton heal through two distinct processes: Direct (primary) or indirect (secondary) healing. Primary healing involves a direct transition of mesenchymal cells to bone-forming osteoblasts (intramembranous ossification). Secondary healing progresses through a cartilage intermediate before bone is formed by osteoblasts (endochondral ossification). The cellular and molecular factors that coordinate fracture callus formation and resolution are complex and highly orchestrated. This review will primarily discuss secondary healing, since the vast majority of fractures that occur clinically heal in this manner.

The process of bone healing has a variety of cellular components required for the progression of healing (Fig. 1). Inflammatory cells (i.e., T-cells, B-cells, mast cells, macrophages, eosinophils, and neutrophils) are the initial cellular component of the fracture environment, followed by mesenchymal progenitor cells, endothelial cells, chondrocytes, osteoblasts, and finally osteoclasts. The process of fracture healing can be easily considered in discrete temporal segments; however, it is important to recognize that there is significant overlap of the temporal segments of healing, and associated cell-types coexist. This is an important concept to consider, because cell-to-cell signaling, in a heterotypic manner (across cell-types) is undoubtedly important. For example, both chondrocytes and osteoblasts can promote blood vessel in-growth through their production of vascular endothelial growth factor

(VEGF).¹ Conversely, endothelial cells promote bone formation through production of bone morphogenetic protein (BMP) and new data suggest that vasculature guides the formation of a cartilaginous template and stimulates conversion of hypertrophic chondrocytes to osteoblasts.²

For this review we will consider the cells of fracture in a well-described and useful temporal sequence, familiar to many in the field. Where appropriate, we will discuss signaling factors regulating or produced by those cells, and in some cases consider signal transduction cascades and molecular programs that guide cellular physiology. While this review is not expected to be a comprehensive treatise on all known signaling factors, nor all known transcriptional regulators, we hope to present a detailed examination of the cells involved in bone regeneration.

INFLAMMATORY PHASE—INFLAMMATORY CELLS Acute Inflammation

The acute pro-inflammatory response is essential for initiating fracture healing (Fig. 1B and C and 2). After fracture, bone architecture and vascular supply are disrupted (Fig. 1A). This results in a loss of mechanical stability, a decrease in tissue oxygenation and nutrient supply, and the release of bioactive factors at the site of injury.^{3,4} The inflammatory cells themselves, along with the cytokines and extracellular matrix they produce, appear essential in facilitating normal healing, as mice deficient in innate and adaptive immunity have significantly impaired endochondral bone repair.⁵

Within the first minutes of fracture, a fibrin-rich blood clot forms to achieve hemostasis (Fig. 1B). The role of this fibrin-rich clot during fracture healing has been examined in mice lacking the key enzyme for fibrin degradation, plasminogen. While fibrin is not required for bone healing, repair does not properly

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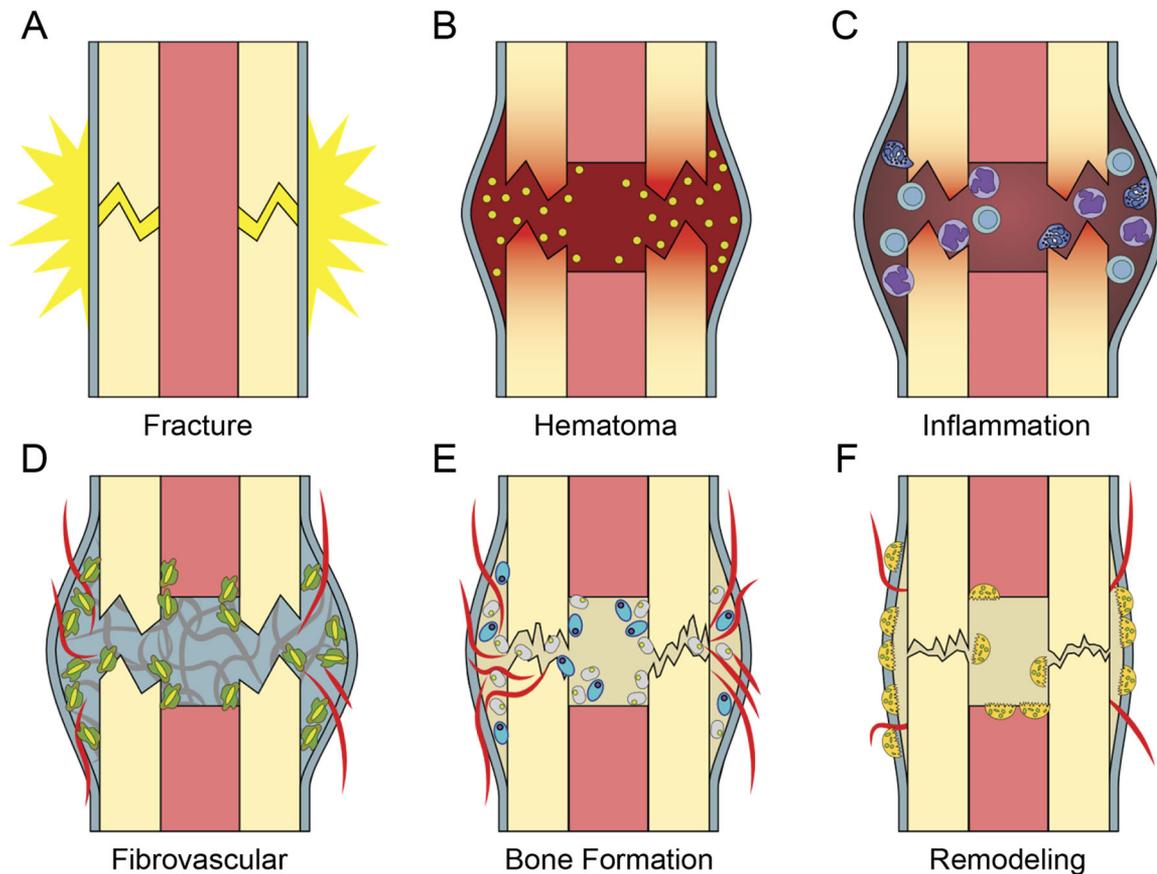


Figure 1. Fracture healing is temporally-defined process. (A) At injury there is disruption of periosteum and bone (B) A clot forms immediately providing a provisional matrix. Platelet degranulation releases chemokines to recruit inflammation. (C) Inflammatory phase leads to a period of (D) Mesenchymal expansion and migration from the periosteum and endosteum and angiogenesis, (E) Bone is formed via both endochondral (blue large oval cells) and intramembranous ossification (smaller grey cells), (F) Osteoclasts (multinucleated cells) resorb primary bone and the process of remodeling restores bone shape and structure.

progress without fibrinolysis. Specifically, the absence of plasminogen results in ectopic ossification and poor healing.⁶

Cytokines released by the clot (particularly during platelet degranulation) recruit inflammatory cells including lymphocytes, macrophages, eosinophils, and neutrophils.^{3,4,7,8} As one example, C-C Motif Chemokine Ligand 2 (also known as Monocyte Chemoattractant Protein-1) (CCL2 or MCP1) and its receptor Chemokine Receptor type 2 (CCR2) stimulate monocyte chemotaxis in the inflammatory response.⁹ CCL2 is expressed from days 1–3 in the fracture site.¹⁰ When subject to fracture, *Ccl2*-null and *Ccr2*-null mice both exhibit delayed fracture healing and decreased callus volume as a result of diminished mesenchymal cell infiltration and impaired vascularization.^{10,11}

Inflammatory cells are deposited throughout the clot during hemorrhage and migrate to the injury site from local sources. While, the contribution of inflammatory cells derived from circulation versus those that are locally derived is not fully-understood, tissue resident macrophages, called ostealmacs, are necessary for fracture healing. One role of inflammatory cells, particularly neutrophils and macrophages, is debridement of injured and devitalized tissue.

Inflammatory cells also produce cytokines that positively and negatively influence healing (Table S1).^{12–14} Some of these cytokines are detected at the fracture site within the first 24 h post-injury and are important for the expansion of the inflammatory response by acting on cells in the bone marrow, periosteum, and hematoma.^{15,16}

Macrophages secrete the pro-inflammatory molecule Interleukin 1 (IL1). IL1 in-turn regulates expression of cyclooxygenases (Cox1 and Cox2), which are the enzymes that synthesize prostaglandins in injured tissues.¹⁷ Non-steroidal anti-inflammatory drugs, which inhibit cyclooxygenase activity, cause delays in fracture healing.^{14,18–22} These delays have been attributed to inhibition of Cox2 activity during fracture healing.^{14,15,23,24} Interestingly, marrow stromal cells derived from *Cox2*-null mice have diminished ability to form bone nodules in vitro, and this deficiency can be alleviated by the addition of prostaglandins to the culture media.¹⁵ However, eliminating signaling through IL1-beta does not appear to affect fracture repair.²⁵

Other pro-inflammatory factors are also essential for fracture healing. For example, mice lacking the gene that encodes the Tumor Necrosis Factor alpha (TNF-alpha)

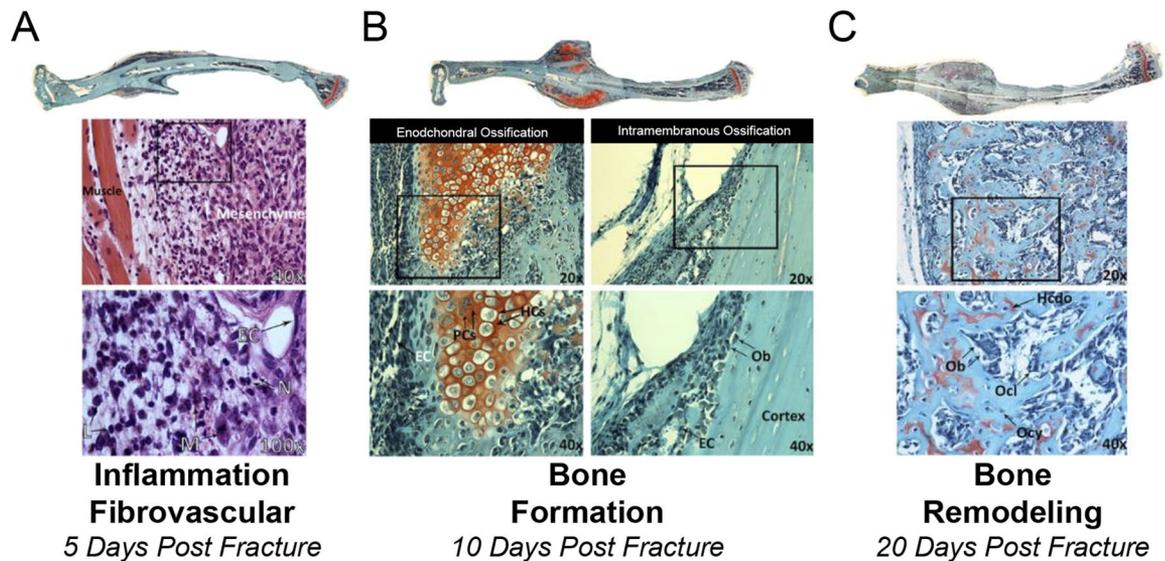


Figure 2. Multiple cell-types present during the process of bone regeneration. Tibiae were harvested 5 (A), 10 (B), and 20 (C) days post closed fracture and fixation with an intramedullary pin. Longitudinal histological sections were stained with H&E (A) or safranin-o (B and C) imaged at 2.5X and images stitched together and higher magnification images at 20X, 40X, and 100X obtained. (A) 5 day post-fracture undifferentiated mesenchymal cells are present in the callus and areas of inflammation remain (boxed area in 40X image is magnified in 100X) EC, endothelial cell; N, neutrophil; L, lymphocyte; M, macrophage. (B) 10 days post-fracture there is both endochondral ossification (red staining, safranin-o stains cartilage) and intramembranous bone formation occurring. Boxed areas in 20X images are magnified in 40X images. EC, endothelial cell; PC, proliferation chondrocytes; HC, hypertrophic chondrocytes; OB, osteoblast. (C) 20 days post-fracture. An extensive network of primary bone has formed and endochondral ossification is complete. Boxed areas in 20X images are magnified in 40X images. Ob, Osteoblast; Ocl, Osteoclast; Ocy, osteocyte; Hcdo, hypertrophic chondrocyte derived osteoblast.

receptor have a substantial delay in the onset of chondrocyte differentiation,²⁶ and a delay in endochondral ossification.¹² Interleukin 6 (IL6) has also been implicated in bone healing. Genetic ablation of IL6 in mice disrupts healing due to delayed callus mineralization, maturation, and conversion to bone.²⁷ Early fracture healing in IL6-null mice is marked by decreases in osteocytes and callus strength.²⁸ In addition to providing inflammatory cytokines, inflammatory cells also produce growth factors such as Fibroblast Growth Factors (FGF), Platelet-Derived Growth Factor (PDGF) and Transforming Growth Factor beta (TGF-beta), which initiate the repair process by facilitating proliferation and differentiation of the stem cells that give rise to the fracture callus.^{4,29,30}

The multifactorial role of the acute pro-inflammatory response together contributes to its significance in healing and inhibition of inflammation is associated with delays in fracture repair.^{14,18–22,31} For example, depletion of macrophages during the early phases of fracture repair has been shown to reduce both callus size and chondrogenesis resulting in impaired fracture union.^{32,33} Defects in fracture healing can also be seen in mice lacking macrophage migration inhibitory factor (MIF).³⁴ While endochondral bone regeneration is the primary mechanism of fracture repair, depletion of macrophages also impacts osteogenesis during intramembranous healing.³⁵ Similarly, the absence of T and B lymphocytes and neutrophils also alters fracture healing.^{36–38}

The role of complement factors in healing has been investigated by Ignatius and colleagues. Their work has demonstrated that mice deficient in C5, but not C3, show reduced bone healing. Interestingly, C5a activation

is independent of C3.³⁹ Furthermore, recent work demonstrates that both C5a receptors, C5aR1 and C5aR2, are required for bone healing, particularly in cartilage-to-bone transition.⁴⁰ Interestingly, this is in contrast to C5aR antagonists in polytrauma fracture healing, which has been shown to improve bone healing.⁴¹ As many cell types involved in bone healing express complement receptors, including, inflammatory cells, osteoclasts, and osteoblasts, fully understanding the role of complement in bone healing will require temporal and cell-type specific allelic disruption.

Resolving Inflammation

While the inflammatory phase of fracture healing begins during the earliest stages of repair, current evidence indicates that the inflammatory cells are also present throughout later phases and appear to undergo changes as healing proceeds (reviewed in ref.⁴²). Analysis of fracture healing in mice lacking the TNF-alpha receptor reveals delays not only during acute inflammation, but in later stages of healing as well.^{12,26} Likewise, IL6 expression appears bimodal during fracture healing suggesting a temporally specific role for inflammatory cytokines during bone repair.⁴³

Changes in macrophage phenotype may explain this dual role in fracture healing. Macrophages can polarize along a continuum of pro- to anti-inflammatory states (Fig. 3). In the first few days post injury, pro-inflammatory macrophages are produced by “classical activation” that is typified by the innate immune response to bacterial pathogens and tissue injury

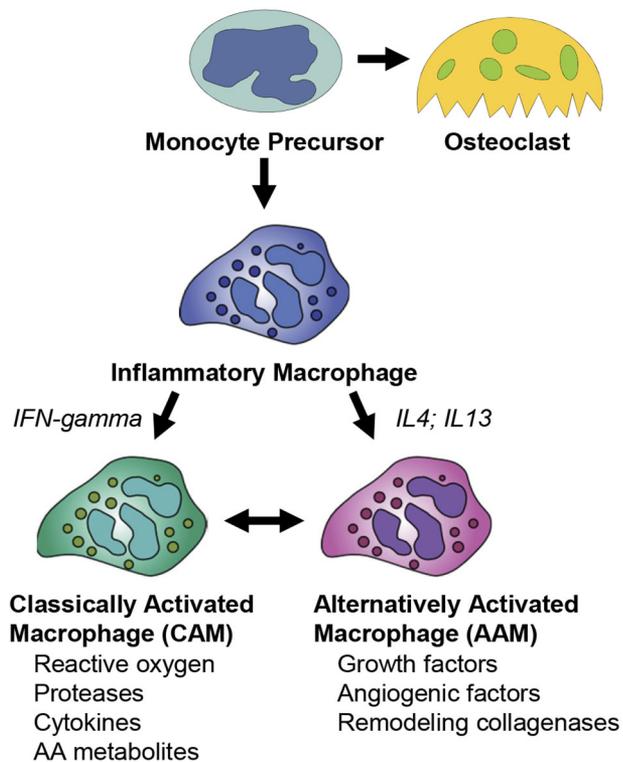


Figure 3. Macrophage precursors develop into both classically activated and alternatively activated macrophages. Monocyte precursors give rise to both the osteoclast lineage and to inflammatory macrophages. Various factors, such as IFN-gamma, IL4, and IL13 control transitions between classically activated macrophages (CAM) and Alternatively activated macrophages (AAM).

through toll-like receptors (TLR). Classically activated macrophages (CAMs) are primed by exposure to interferon-gamma (IFN-gamma). Thereafter, pathogen-associated molecular pattern binding to TLR-family receptors on CAMs up-regulates the pro-inflammatory cytokines TNF-alpha, IL1 and IL6 through the NFκB pathway. Recent work from the Goodman laboratory has shown that CAMs indirectly promote osteogenesis by regulating MSC, albeit these studies have not been supported by in vivo studies.⁴⁴

Once macrophages have debrided the wound and are no longer classically activated, they can assume an anti-inflammatory state. Anti-inflammatory macrophages, also known as alternatively activated macrophages (AAMs), are generated through IL4 and IL13 signaling. In contrast to CAMs, alternative polarization of macrophages results in cellular activity that promotes collagen deposition and the return to tissue homeostasis. Production of TGF-beta, IL10 and arginase, as well as other secreted anti-inflammatory proteins, is associated with the repair of tissues following infectious and traumatic insults.^{45,46}

Chronic Inflammation

Chronic, non-resolving inflammation is detrimental to fracture healing. Experimental evidence indicates fracture healing and osseointegration are disrupted in conditions where there is chronic, non-resolving inflam-

mation, such as diabetes.⁴⁷⁻⁴⁹ Elevated TNF-alpha signaling may underlie some chronic inflammatory conditions,⁴⁹ and evidence suggests blocking this pathway may have a therapeutic benefit in fracture healing.⁵⁰

Aging is also associated with a non-resolving inflammatory state and impaired bone healing.^{16,51-54} In elderly animals, there are alterations to both progenitor and inflammatory cells.⁵⁵ In particular, macrophages exhibit significant age-related alterations in function that change the inflammatory environment of aged animals and impact healing (reviewed in ref.⁵⁶). Cutaneous wound healing is delayed in aged mice; however, these delays can be overcome by grafting macrophages derived from younger animals.⁵⁷ Similarly, rejuvenation of the inflammatory system in aged animals significantly accelerates fracture repair.^{53,58,59} Thus, the functional capacity of juvenile macrophages appears to be more beneficial for healing than that of elderly macrophages. Compared to young mice, the innate and adaptive immunity cells of aged mice are more highly enriched during fracture healing.⁶⁰ Bone regeneration is inhibited by increased CD8+ T cells which produce interferon-gamma and TNF-alpha and increased expression of CXCL8, CXCL9, and CXCL5 cytokines. The specific contribution of CD8+ T cells on fracture healing was demonstrated in a murine osteotomy model.⁶¹ Depletion of CD8+ T cells improved callus formation and bone mineral density. Conversely, increasing the CD8+ T cell population using adaptive transfer resulted in delayed callus formation and decreased bone mineral density. This work supports CD8+ T cell number as a potential prognostic marker for bone healing. Contrary to CD8+ T cells, IL-17A producing gamma delta T cells are essential for fracture repair. Loss of IL-17A disrupts proliferation and differentiation of MSCs resulting in delayed callus formation and lower bone mineral density.⁶²

The cellular mechanisms causing dysregulation of immune cell function in aged animals is still being investigated. However, age-related changes to the macrophages appear to alter production of inflammatory cytokines. For example, decreases in Cox2 expression, an enzyme required for prostaglandin production, were observed during fracture healing in aged mice, and age-related delays were mitigated by activation of the prostaglandin receptor.⁵⁴ Ultimately, understanding the altered functional characteristics of the macrophages may be essential for addressing the decreased healing in the elderly⁶³; however, it is also important to consider that there are other non-inflammatory cell-autonomous explanations for altered healing with aging and metabolic conditions, such as reductions in progenitor cell number and function.⁶⁴

FIBROVASCULAR PHASE—ENDOTHELIAL AND MESENCHYMAL PROGENITORS CELLS

Following inflammation, the angio-mesenchymal phase of repair begins (Fig. 1D and 2A). This phase

has been termed the “fibrovascular phase” and is defined by vascular remodeling (angiogenesis and neovascularization) and recruitment of mesenchymal progenitor cells, sometimes referred to as mesenchymal stem cells (MSCs), that will ultimately differentiate into chondrocytes and osteoblasts to regenerate the fractured bone.

Revascularization

During the initial fracture trauma, the periosteal, cortical, and medullary vascular supply are disrupted leading to acute cellular necrosis and acidosis. The lack of vascularization causes local hypoxia, in which oxygen tension is lowered to 0.1–2%^{65–67} from 5%. Revascularization is required for perfusion of the callus with oxygen, nutrients, inflammatory and progenitor cells to facilitate repair, and the egress of waste products. In most cases, vascular supply is reestablished rapidly through the development of a new vascular network.⁶⁸

Formation of the network occurs by two distinct processes: Angiogenesis and vasculogenesis. Angiogenesis is the process by which new blood vessels are formed by sprouting from existing vasculature. Vasculogenesis is de novo formation of blood vessels from *in situ* endothelial progenitor cells (EPCs) within the callus. Endothelial cells in forming callus vasculature can develop from a variety of sources, including, existing vessels of the periosteum and the intramedullary vasculature⁶⁹; circulating EPCs⁷⁰ that are increased during fracture repair⁷¹; or the bone marrow.⁷² Circulating EPCs are not only increased in rodent models, but are significantly increased in human patients at day three post-fracture.⁷³

Vascular endothelial growth factor (VEGF) is a well-characterized driver of angiogenesis and vasculogenesis.⁷⁴ VEGF is produced by a variety of cells in the fracture callus, including inflammatory cells and mesenchyme, but also osteoblasts and hypertrophic chondrocytes. VEGF binds the VEGF family of receptors VEGFR1 (FLT1) and VEGFR2 (FLK1) activating signaling cascades that lead to increased proliferation and sprouting of endothelial cells, and recruitment of EPCs to the fracture. In a model of distraction osteogenesis, blockade of VEGF activity via antibodies to VEGFR1 and VEGFR2 results in decreased vessel volume and reduction of callus formation.⁷⁵ Neutralization of VEGF by the soluble VEGF receptor (IgG-Flt) recapitulated this delay of callus vascularization.⁷⁶

VEGF is a classical downstream target gene of hypoxia inducible factor 1- α (HIF1- α), which is stabilized in hypoxic⁷⁷ and other conditions including when lactate levels are increased, as they are after fracture.⁶⁷ Induction of HIF1- α and VEGF protein production peak at day 10 post-fracture in mice, during the period of endochondral ossification.^{2,78} Mice with increased expression of HIF1- α develop hyper-vascularized long bones with enhanced healing.⁷⁹

On the other hand, *HIF1- α* -null mice,⁸⁰ and mice with HIF1- α disrupted in osteoblasts,¹ have delayed callus formation in fracture healing.

One interesting aspect of VEGF signaling is that during endochondral ossification, VEGF protein binds to the cartilage matrix until liberated by matrix metalloproteases (MMPs). MMPs are a family of extracellular proteases that degrade and remodel the extracellular matrix during development and repair. MMPs-2, -9, and -13 are robustly expressed during fracture repair and their absence results in impaired healing.^{13,81–84} While the *Mmp2*-null mutation delays only bone remodeling, the *Mmp9*- and *Mmp13*-null mutations affect bone formation by altering cartilage remodeling and vascularization. Importantly, administration of rVEGF to *Mmp9*-null mice during fracture healing rescues the null phenotype, indicating that VEGF release from the matrix by MMP9 is required for angiogenesis. MMP9 regulates VEGF availability.

The extracellular matrix (ECM) can also influence the angiogenic response to fracture healing. For example, thrombospondins (TSP) are a family of non-fibrillar matricellular proteins with a potent anti-angiogenic function.⁸⁵ *Tsp2*-null mice exhibit increased angiogenesis in the fracture callus,^{86,87} resulting in enhanced ischemic bone healing and alterations in callus composition in non-ischemic conditions. As such, targeting this pathway is an attractive therapeutic target for enhancing vascularity in bone regeneration. Osteopontin is also a modulator of fracture vascularization. Mice deficient in osteopontin show delayed angiogenesis and smaller calluses.⁸⁸ Cell-type specific deletion of TSP2 and osteopontin using Cre-LoxP has not been described but would serve to better elucidate the mechanistic bases for these observations.

As the angiogenic response is a required event in fracture healing, deficiencies in angiogenesis result in delayed or insufficient fracture repair. Clinically, the non- or delayed-union rate increases from a basal level of 10–20% in the normal fracture population, to 46% when there is concomitant damage to the vasculature.⁸⁹ Multiple preclinical models have been used to investigate the underlying mechanisms for this defect in healing. In experimental models of ischemia, fracture healing is significantly altered due to massive apoptosis of the periosteum.⁹⁰ Similarly, de-vascularization of the periosteum proximal to the fracture site results in delayed healing and inhibits new bone formation.⁹¹ Some of the negative effects of ischemic bone fractures can be mitigated by environmental hyperoxia. In an experimental model of an ischemic tibia fracture, mice in hyperoxic conditions (50% environmental oxygen) demonstrated an increase in callus volume and cartilage content.⁶⁷ The mice also were less likely to progress to non-union.

Co-morbidities such as aging, diabetes and smoking are also associated with delayed fracture healing, likely due to underlying vascular defects. Elderly and middle aged mice exhibit a decreased callus volume

formation coupled with inhibited angiogenesis, and reduced expression of VEGF and MMP9 relative to juvenile fractures.⁹² In an obesity-induced model of type II diabetes mellitus, neovascularization of the fracture callus is inhibited resulting in decreased formation of woven bone.⁹³ In distraction osteogenesis, cigarette smoking inhibits neovascularization and delayed tibial lengthening.⁹⁴ Taken together, identifying clinically relevant conditions that affect angiogenesis are required to improve outcomes in fracture healing.

Mesenchymal Progenitor Cells

The other primary cellular component of the fibrovascular callus, is the mesenchymal progenitor cell (MSC). MSCs are multipotent cells that give rise to osteoblasts, chondrocytes, fibroblasts, myocytes, and adipocytes.⁹⁵ While these cells are referred to as “stem cells,” it is notable that in most cases, criteria of stemness are not well-established for these cells. Even within the MSC population, sub-populations have been identified that differ in lineage potential and function. Nestin, an intermediate filament fiber, has been used to differentiate between populations of MSCs that are mesodermal- or neural crest-derived. Nestin-negative MSCs primarily contribute to skeletogenesis in the fetus whereas nestin-positive cells assume this role later in life.⁹⁶ In bone fracture repair, quiescent MSCs reprise their developmental role as osteochondral progenitor cells (Figs. 1, 2, and 4).

The majority of MSCs recruited to the fracture site are derived locally from the periosteum and bone marrow. The original experimental evidence for this came from Colnot who used lineage analysis to demonstrate that locally derived progenitors from the periosteum, endosteum, and bone marrow are the major cellular contributors to the fracture callus.⁹⁷ Subsequently, using the mesenchymal marker α -Smooth Muscle Actin-9 (α -SMA9), Cre-recombination based fate mapping shows α -SMA9⁺ cells invade the fracture site from the periosteum 2-days post fracture, and by 6 days post fracture this α -SMA9⁺ population has robustly expanded to provide a large source of osteocyte progenitors.^{98,99} Gremlin-CreERT2 and LepR-Cre cells have also been shown to give rise to osteoblasts and chondrocytes in the fracture callus.^{100,101} Table S2 provides a summary of recent investigations in bone healing that employed Cre systems and includes additional Cre mice relevant to fracture biology that have yet to be studied. Together, these various Cre mice will be useful for better defining the spatial and temporal regulation of cells contributing to bone healing.

Recruitment of MSCs in the fracture repair program is under molecular regulation by cytokines released at the fracture site, particularly CXCL12, also known as stromal cell-derived factor 1 (SDF1). SDF1 is released by the injured periosteum and drives mobilization and homing of MSCs through CXCR4.¹⁰²

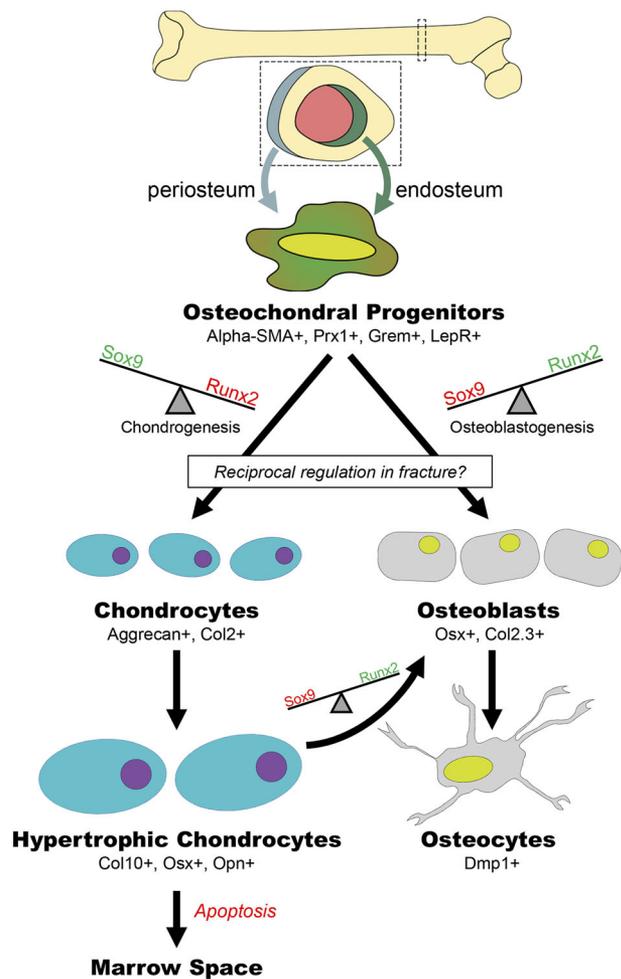


Figure 4. Mesenchymal precursors develop into both osteoblasts and chondrocytes. Osteochondral progenitors are activated at the time of bone injury and a balance in transcriptional activation results in the cells becoming either osteoblasts or chondrocytes. Hypertrophic chondrocytes can differentiate to become osteoblasts.

Partial disruption of SDF1/CXCR4 in mouse allografts lead to decreased MSC chemotaxis and bone formation. In a live bone graft model of repair, both antibody sequestering of SDF1 and pharmacologic inhibition of its receptor CXCR4 resulted in inhibited MSC chemotaxis and decreased formation of bone in the callus.¹⁰² SDF1 is under transcriptional regulation by HIF1-alpha in response to ischemia,¹⁰³ demonstrating a role for the hypoxic condition of the fracture environment in directing MSC recruitment, as well as vascularization. In a tibial fracture model in mice, SDF1 increased callus formation as well as induced expression of VEGF and Runx2 in the soft tissue callus, indicative of increased angiogenesis and osteogenesis.¹⁰⁴ Recently, implantation of bone marrow derived MSCs (BM-MSCs) overexpressing SDF1 in a bone defect model resulted in improved new bone formation relative to BM-MSC implantation alone.¹⁰⁵ It is notable that the SDF1/CXCR4 homing pathway is also required for EPC recruitment in tibial fracture healing.

Cxcr4-null mice exhibit decreased callus formation as a result of inhibited EPC recruitment, decreased blood flow in the fracture site, and decreased VEGF and CD31 (an endothelial cell marker) expression in the callus one week post fracture.¹⁰⁶ Exogenous SDF-1 was not sufficient to rescue this phenotype, indicating an exclusive requirement of CXCR4 in SDF-1 stimulated EPC recruitment.

Notch signaling is another potentially important factor in both regulating MSC number and activation. Mice with Notch signaling disrupted through *Mx1*-Cre mediated overexpression of the dominant negative mastermind (*DnMAML*) show alterations in callus size.³⁸ Mice with complete disruption of canonical Notch signaling by *Prx1*-Cre mediated disruption of the Notch transcription factor CSL have non-unions. Notch signaling appears to be required for the proliferation and/or migration of mesenchymal progenitor cells.¹⁰⁷

While the data using reporter mice support the concept that MSC in fracture callus are derived from periosteal and endosteal activation, there is still some potential that MSC could be derived from the circulation. Using CMV-Cre-R26R-LacZ-MSCs transplanted into a fractured mouse, it was shown that MSCs in circulation localize in the endosteum, but not periosteum, of the fracture site as early as 3 days post injury.¹⁰⁸ To study if circulatory MSCs could contribute to fracture healing, BMP-2-Lac-Z-MSCs were transplanted which demonstrated BMP-2 expression in the endosteum. However, it is still unclear if MSCs in circulation contribute significantly to bone remodeling by differentiating into chondrocytes and osteoblasts or if they promote healing indirectly in a paracrine fashion through the release of growth factors and cytokines.

BONE FORMATION—OSTEOBLASTS AND CHONDROCYTES

Following the fibrovascular phase of healing, many of the MSC that formed the fibrovascular callus undergo differentiation to either osteoblasts or chondrocytes to initiate the bone formation phase of healing⁹⁸ (Fig. 1E, 2, 4).

Differentiation of MSCs into bi-potential osteochondral progenitor cells is initially regulated by Sox9.¹⁰⁹ Sox9 is required for chondrogenesis and genetic disruption studies demonstrate that absence of this transcription factor leads to the complete elimination of the cartilaginous anlagen in the developing skeleton.¹¹⁰ Conversely, in osteoblasts, downregulation of Sox9 in bi-potential cells releases repression of Runx2.¹¹¹ Runx2 deletion results in a complete loss of osteoblasts in mouse embryos.^{112–114} However, disruption of Runx2 in chondrocytes is embryonic lethal and inhibits endochondral ossification.¹¹⁵ While Runx2 has been traditionally called a “master regulator” of osteoblastogenesis, it may play a larger upstream role as a regulator of bi-potential osteochondral progenitor cells.

Runx2 transcriptionally regulates osteoblastogenesis in part through the transcription factor Sp7 (Osterix).^{114,116} Knock-out of Osterix is also associated with lack of osteoblasts, however, Osterix expression is absent following deletion of *Runx2*, suggesting Osterix is downstream.^{114,116} Sox9 also actively represses osteogenic potential by suppressing *Runx2*,¹¹¹ thereby these opposing programs appear to act as a molecular switch between cartilage and bone fate in osteochondral progenitor cells.¹¹⁷

Factors regulating the decision of progenitor cells towards the chondrogenic or osteogenic fate are multifactorial, integrated and still being defined. Extrinsic factors, mechanical factors and oxygen tension are undoubtedly important variables regulating fate decision.^{118,119} These microenvironmental cell-extrinsic factors then lead to very specific cell-intrinsic regulation of chondrogenesis and osteoblastogenesis.

Increased motion has been shown to induce the formation of more chondrocytes and in-turn increases endochondral ossification,^{118,120,121} while stabilization results in the generation of more osteoblasts and direct bone repair via intramembranous formation.¹²⁰ Specifically, strains smaller than 5% and hydrostatic pressures less than 0.15 MPa promote intramembranous formation.¹²¹ Morgan et al.¹²² have assessed strain distribution during healing in a loaded osteotomy model and then associated strain patterns with the type of bone formation determined histologically. Higher octahedral shear strain and maximum principal strain increased cartilage and decreased woven bone, while volumetric strain was less reliably associated with intramembranous bone versus a cartilage phenotype.

Another putative environmental signal that may regulate the fate decision of MSC is oxygen tension. The relationship between oxygen tension and MSC differentiation in vitro has been extensively investigated, and the preponderance of evidence suggests that hypoxia promotes a chondrogenic phenotype, whereas higher levels of oxygen promote osteoblast differentiation. In vivo, this relationship between oxygen and MSC fate decision has been computationally modeled and experimentally validated through directed callus oxygenation.^{123,124} However, other work has demonstrated that reducing inspired oxygen levels leads to problems with healing, but does not appear to alter the mode of fracture healing.⁶⁷

Secreted growth factors also have a direct effect on MSC differentiation. BMPs are the classic osteogenic molecule associated with bone formation. In vitro BMPs directly stimulate MSC osteoblast differentiation and canonical bone programs characterized by the activity of the Runx2 and Sp7 (Osterix) transcription factors which are direct, downstream targets of BMP signaling.¹²⁵ In vivo, after trauma, periosteal cells express BMP2 and BMP4 and over time they proliferate in response to BMP5 and BMP6.^{126,127} Notably, BMPs are also important in dictating chondrocyte

differentiation, so that mice with conditional disruption of *Bmp* (in particular *Bmp2*) show non-unions.^{128–130} Signaling via BMP2 is also absent during intermediate stages of intramembranous repair, which is critical for preventing cartilage formation.¹³¹ At day 10 post-injury, BMPs (2, 4–8), extracellular BMP antagonists (BMP3 and noggin), BMP receptors (1A, 1B, and II), and effectors (p-Smads 1, 5, and 8) are not detected in osteoblasts, osteoclasts, or the periosteum within a fracture site's new bone.¹³² Addition of recombinant human BMP2 (rhBMP2) to stabilized fractures results in formation of new cartilage primarily at the periosteal surface, which ultimately leads to a callus with increased cartilage and total volume, but no increase in intramembranous bone formation.¹³¹ The dual role of BMP signaling in regulating both osteoblast and chondrocyte differentiation of multipotent mesenchymal progenitors is not fully understood in the context of fracture repair. Presumably, co-acting factors, in association with as yet undefined epigenetic changes, influence the balance of key osteoblast transcription factors, such as Runx2 and Osterix, relative to key chondrogenic transcription factors, such as Sox9.

Another secreted growth factor family that could play a role in regulating MSC fate determination in bone healing is the Wnt family (reviewed in ref.¹³³). In non-fracture environments, inhibiting beta-catenin activity in the osteoblast lineages leads to decreased bone mass and increased chondrogenesis,^{134–136} while ablation of Wnt inhibitors, DKK¹³⁷ or sclerostin,¹³⁸ increases bone formation and bone mass. While the developmental role of canonical Wnt has been demonstrated, less is known about its role during fracture healing.¹³³ In areas of intramembranous ossification in murine femur fractures, Dishevelled and beta-catenin have been localized to osteoblasts lining regions of newly formed woven bone and in those destined to be trapped in new bone.¹³⁹ Fracture studies in *Wnt* deficient mice suggest impaired healing compared to wild type littermates,^{140,141} likely as a result of disturbed osteoblast function since cartilage formation and osteoclasts numbers degrading the mineralized matrix are unaltered in its absence.¹⁴² Conversely, mice deficient in Sclerostin (an inhibitor of the canonical Wnt/beta-atenin pathway) heal single cortical, fully stabilized mid-diaphyseal femur fractures more robustly than wild type mice.¹⁴³ This is in large part due to increased osteoblast numbers and bone surfaces 7–14 days post-injury. Importantly, a therapeutic benefit to fracture healing has been shown when canonical Wnt signaling was stimulated by adding a monoclonal antibody to the Wnt inhibitor DKK.^{144,145}

Intramembranous Ossification—Osteoblasts

Direct differentiation of mesenchymal progenitors to osteoblasts is the exclusive mechanism of bone repair in fully stabilized defects (intramembranous ossification), but also occurs along the periosteal and endosteal surfaces of the bone in less stabilized fractures.

(Fig. 1E, 2B and 4) Periosteal progenitor cells appear to have a bi-potent osteo-chondral potential, with differentiation linked to the mechanical microenvironment, as detailed previously. Osteogenic differentiation of the periosteal MSC gives rise to intramembranous bone locally along the bone surfaces adjacent to the fracture; while these same periosteal progenitor cells migrate into the fracture gap to undergo chondrogenesis. In contrast, endosteal stem cells exhibit uni-potent osteogenic potential. Intramembranous bone formation from these endosteal stem cells is thus responsible for rapidly bridging across the marrow cavity.⁹⁷

Endochondral Bone Formation—Chondrocytes

Temporally, chondrogenic differentiation of fracture callus progenitor cells is closely aligned with resolution of the pro-inflammatory response and occurs on the fibrin scaffold that was generated as part of the hematoma. Spatially chondrogenesis occurs primarily in the fracture gap, with periosteal stem cells being the primary source of the chondrocytes¹⁴⁶ (Fig. 1E, 2B and 4).

Following initial fate specification of the MSC to a chondrocyte, SOX9 expression plays an essential role in maintaining the cartilaginous phenotype and hypertrophic maturation.^{147–149} SOX9, along with transcriptional co-factors SOX5 and SOX6, regulate the expression of collagen II^{150–152} and aggrecan.¹⁵³ These are the canonical extracellular matrix proteins of cartilage, and together make up ~90% of the dry weight of the tissue, imparting cartilage with its characteristic biophysical properties. This dense cartilage callus bridges the fracture gap and helps stabilize the defect. At this stage the cartilage tissue becomes avascular, repressing angiogenesis and vascular invasion.¹⁵⁴

Conversion of the cartilage callus to bone occurs following a highly regulated maturation of chondrocytes from a proliferative through a hypertrophic state (Fig. 5).² Hypertrophic maturation is distinguished morphologically by a dramatic increase in cell volume. Hypertrophic chondrocytes in the growth plate increase in size ~20-fold by taking on both volume and dry mass.¹⁵⁵ At a molecular level, the hypertrophic chondrocyte is distinguished by the expression of collagen type X. While the exact function of collagen X is not clear, it is uniquely expressed by hypertrophic chondrocytes and matrix deposition is believed critical in priming the matrix for mineralization.

Chondrocyte hypertrophy represents a pivotal state during endochondral ossification. Hypertrophic chondrocytes are highly angiogenic and facilitate a second phase of vascular invasion into the cartilage callus by synthesizing VEGF,^{156–158} PDGF (platelet derived growth factor),¹⁵⁹ and PIGF (placental growth factor).¹⁶⁰ Adjacent to the invading vasculature, hypertrophic chondrocytes lose Sox9 expression, which subsequently relieves repression of osteogenic promoters Runx2 and beta-catenin.^{2,147} Subsequently,

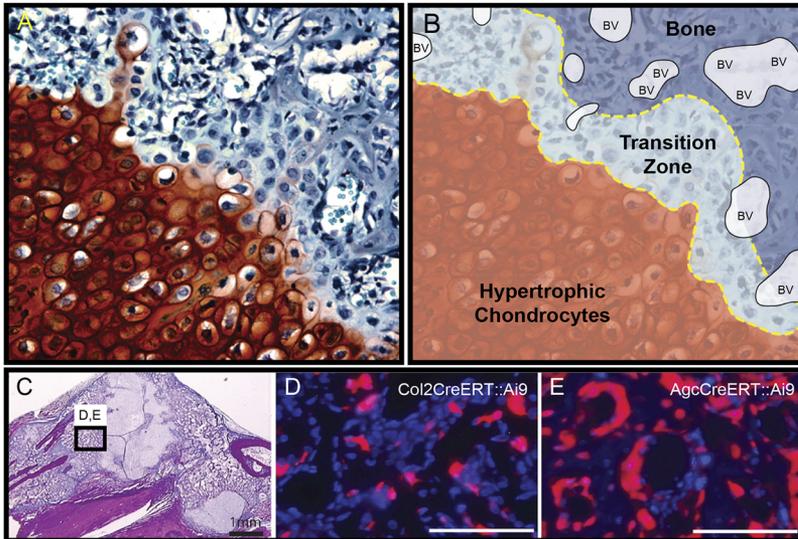


Figure 5. Hypertrophic chondrocytes develop into osteoblasts and osteocytes. Tibiae were harvested post fracture and stained with (A) Safranin-O to define the chondrogenic front as outlined in panel (B). (B) shows zones of hypertrophic chondrocytes, transition zone, Bone, and blood vessels (BV). (C) is a low magnification H&E image showing the localization of panels D and E in areas of bone. Cells of bone can be traced to the chondrocyte lineage using the (D) Col2CreERT2::Ai9 or the (E) AgcCreERT::Ai9 mouse with a tamoxifen pulse.

hypertrophic chondrocytes begin to express canonical markers of bone, including, alkaline phosphatase, osterix, osteopontin, and osteocalcin.¹⁶¹ Together, activation of osteogenic programs and angiogenesis result in calcification of the cartilage matrix.¹⁶² From a functional perspective this calcification provides additional rigidity to the fracture.

The molecular trigger for calcification is not completely clear, but BMPs likely play a key role in this process. BMP are expressed by both hypertrophic chondrocytes¹³² and vascular endothelial cells,¹⁶³ suggesting that there are both cell-autonomous and paracrine effects of BMP signaling that may drive calcification. Invasion of the vasculature also provides hypertrophic chondrocytes with other systemic factors such as extracellular calcium, parathyroid hormone, vitamin D, and insulin-like growth factor that play a role in controlling mineral homeostasis during fracture repair. Whether it is BMP expression alone from the vascular endothelial cells that drives calcification of the cartilage, or whether additional secreted factors¹⁶⁴ may also contribute to this process remains unclear.

Following calcification of the cartilage, bone formation occurs. In this vascularized transition zone between cartilage and bone, histological staining reveals hypertrophic chondrocytes entrapped in a bone matrix adjacent to the vasculature.² As cartilaginous matrix is lost and bone matrix is laid down the large round hypertrophic morphology of chondrocytes is gradually converted into morphology characteristic of the osteocytes with cellular extensions existing in canaliculi. How this shape change is facilitated remains an outstanding question, but reductive cell division of the hypertrophic chondrocytes may be one mechanism enabling this morphogenesis.² Similarly, the mechanism by which the cartilage matrix is degraded remains debated and will be discussed later.

The ultimate fate of the hypertrophic chondrocyte at the time of bone formation has recently been redefined both in the growth plate and fracture callus

(Fig. 2 and 5). The traditional model held that hypertrophic chondrocytes were a terminally differentiated, post-mitotic cell, fated for apoptosis. According to this model, new bone was formed by osteoprogenitors or pre-osteoblasts that invade acellular cartilage matrix along with the vasculature.¹⁶⁵ This dogmatic view of cell death in the hypertrophic chondrocyte overshadowed early work suggesting that chondrocytes could directly give rise to bone during endochondral ossification.^{166–169} However, more recently a number of genetic lineage tracing studies using chondrocyte-specific, temporally-regulatable promoters (Table S2) have clearly demonstrated that chondrocytes live and differentiate to become osteocytes both in the growth plate during development and during fracture repair^{164,170,171,2} (Fig. 5).

The mechanism by which chondrocytes transform into osteocytes remains poorly defined, but a few possibilities have been proposed. The osteocyte could just be the terminal fate of the chondrocyte, representing the natural phenotypic progression of these cells during maturation; or the chondrocyte could de-differentiate to a progenitor-like state prior to activating the osteoblast programs, and then becoming an osteoblast.^{2,164,172} Another proposed mechanism is that the hypertrophic chondrocytes undergo an asymmetric cell division, at which point one of the daughter cells becomes an osteoblast/osteocyte and the other undergoes apoptosis.^{173–175} These suggested pathways are not mutually exclusive. For example, activation of the stem cell genes may not truly impart multipotency, but rather reactivate the cell cycle or enable the chromatin remodeling required for osteoblast gene expression. Significantly more work is required to understand the molecular details that regulate conversion of hypertrophic chondrocytes to osteoblasts, and to understand how chondrogenic matrix is converted to an osteogenic matrix.

It should be noted, that some amount of apoptosis of hypertrophic chondrocytes and osteoblasts/cytes is

required to create marrow space. Similarly, evidence suggests that in the growth plate at least some of the endochondral bone is formed by osteoblasts.¹⁶⁵ Consequently, it is important to recognize that this new model does not exclude the possibility that chondrocyte apoptosis and invading osteoblasts contribute to the newly formed bone at the fracture site. Determining the contribution of the various cell sources will require more study utilizing cell-type-specific temporally-regulated Cre-based studies.

CALLUS REMODELING AND OSTEOCLASTS

Remodeling of the bony callus is traditionally considered the last stage of fracture repair. Remodeling must occur to degrade the provisional bone that is first produced, referred to as woven bone, and replace it with mature lamellar bone. A key component of callus remodeling is bone degradation by osteoclasts¹⁷⁶ (Fig. 1F and 2). Osteoclasts are myeloid lineage multinucleated cells that form tight attachments to the bone surface via a specialized membrane structure called the sealing zone.¹⁷⁷ Vesicle trafficking delivers both soluble and membrane-bound lysosomal proteins to the sealing zone, and fusion of these transport vesicles with the intra-sealing zone plasma membrane creates the high-surface-area, manifold ruffled border that is the hallmark of a mature resorbing osteoclast.^{178–180} The acidic pH of Howship's lacuna facilitates dissolution of hydroxyapatite crystals comprising the mineral component of bone while proteases digest the underlying collagenous matrix. Osteoclast mediated degradation of the bone liberates bone-sequestered factors, such as TGF-beta as well as factors produced by the osteoclast itself, such as complement 3a, Wnt10b, BMP6, and SLIT3^{181,182} which are hypothesized to be critical in the subsequent stimulation of osteogenesis.^{183,184} Resorption is concluded with the apoptotic death of the osteoclast, an event that can be stimulated by the hormone calcitonin or 17-beta-estradiol-enhanced Fas ligand expression.¹⁸⁵

Osteoclasts originate from hematopoietic monocyte/macrophage lineage precursors. Proliferation and survival of osteoclast precursors is stimulated by interaction between monocyte/macrophage colony-stimulating factor (M-CSF) and its receptor c-fms, which is present on both macrophages and osteoclasts. Bone marrow macrophages differentiate into osteoclasts upon stimulation with Receptor Activator of Nuclear Factor kappaB Ligand (RANKL) which binds to its receptor, RANK.¹⁸⁶ Osteoclast differentiation occurs through multiple phases.^{176,187} Both M-CSF and RANKL are required throughout the differentiation process and also contribute to the survival of mature osteoclasts. M-CSF and RANKL are both necessary and sufficient for osteoclast formation and function, but multiple other cytokines and signaling pathways influence osteoclast differentiation, maturation, and survival.^{188–191}

Both RANK and RANKL knockout mice have demonstrated the critical role of osteoclasts in physiological bone remodeling, but the role of osteoclasts in fracture repair has been investigated only recently.^{192,193} The medaka fin ray fracture model has allowed for longitudinal observation of the cellular contribution to fracture repair and has revealed a role for osteoclasts in two stages.¹⁹⁴ Following the inflammatory phase of fracture, osteoclasts are recruited to smaller bone fragments which are partially resorbed. This partial resorption deburrs the edges of the fragments which are later incorporated into the callus, but whether osteoclast resorption of the fragments is necessary for their preservation in the growing fracture callus is unknown. Osteoclast activity is again induced near the conclusion of the healing process, wherein they remodel the hard callus and restore the bone to dimensions similar to those prior to injury. Inhibition of osteoclast protease activity using cathepsin K inhibitors during the bony callus remodeling phase results in calluses with greater mineral density, but also increases osteoclast surface and osteoblast numbers.¹⁹⁵ Pharmacological disruption of osteoclastogenesis by inhibiting transient receptor potential cation channel subfamily V member 1 (TRPV1) has been used as a treatment strategy for post-menopausal bone loss. However, fracture studies using TRPV1 knockout mice demonstrated an essential role of osteoclasts in soft-callus formation and remodeling.¹⁹⁶ The decreased osteoclast number in TRPV1 mice lead to enlarged malformed calluses and persistent fracture gaps. In addition, there was down regulation of RUNX2 and ALP in MSCs.

Remodeling during the process of endochondral bone formation is also necessary; however, the requirement for osteoclasts in this process are less-clear. Osteoclasts can be detected in histological sections of the endochondral callus and therefore are sometimes referred to as chondroclasts (though there is no evidence that these are a cell type distinct from osteoclasts). While osteoclasts are capable of resorbing cartilage, it is not clear that they have a functional role. Both human and animal studies have revealed that inhibiting osteoclast function does not significantly impact remodeling of the cartilaginous callus, but will delay bony callus remodeling.^{197–200}

If not mediated through direct interaction with osteoclasts, degradation of the cartilage callus may be accomplished indirectly through other cells expressing MMPs. As discussed earlier, MMPs are a family of extracellular matrix proteins with a demonstrated functional role in fracture repair. MMPs are expressed by many of the cells involved in bone healing, including osteoblasts and chondrocytes, and have differential specificity towards the collagens and proteoglycans found in the cartilage matrix. MMP13 has high specificity towards both collagen II and aggrecan and is made by both hypertrophic chondrocytes and osteoblasts. Similarly MMP9, with specificity towards

gelatin (or degraded collagen), is made by both vascular endothelial cells and macrophages.^{84,201} Interestingly, transplantation of wild type bone marrow into MMP9 mutants rescues the remodeling defect that is observed in the mutant animals,⁸³ but this same experiment does not rescue the remodeling defect in the MMP13 mutant.⁸¹ These outcomes suggest that MMP9 expressed by cells derived from the hematopoietic system and MMP13 derived from the chondrocytes work in concert to remove cartilage during endochondral ossification.

CONCLUSIONS

While we have discretely discussed the various phases of healing, the reality is that there is overlap of all phases of healing. This spatiotemporal heterogeneity of fracture healing has made studies of fracture cell biology challenging. However, the utilization of genetically modified mice has permitted a more rapid advancement in our understanding of the cells involved in fracture repair (Table S2). Cell-specific reporter mice have permitted us to prospectively identify the cells of the callus to provide spatial resolution. The development of tissue-specific promoters driving inducible Cre activity enables additional temporal resolution for lineage tracing. Indeed, it was only relatively recently that the periosteal and endosteal origin of mesenchymal cells was definitively determined based on promoter reporter lineage tracing. Similarly, recent temporally defined lineage-specific data from Bahney et al. has been able to demonstrate the transdifferentiation of chondrocytes to osteoblasts.² Traditional gene knockouts as well as tissue specific and temporally defined knockout models have permitted us to understand gene function in the context of defined cell types. This has been particularly true for probing the role of BMP. Over the next decade, this temporally-regulated, cell-type specific gene regulation will permit a more careful dissection of fracture cell biology. The next frontier will be to understand how multiple cell types and resultant signaling networks are integrated spatially over time to regulate healing. More advanced computational models of cellular behavior in complex environments will be useful for understanding these influences. From a translational perspective, advances in understanding the cell biology of fracture will then need to be extended to larger animal models, and to pathological fracture healing.

AUTHORS' CONTRIBUTIONS

Each author has contributed to the writing and editorial review of this review. KDH, RM, CSB, RLZ have produced the figures and the tables. All authors have read and approved the final submitted manuscript.

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SUPPORTING INFORMATION

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