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Common Molecular Mechanisms Regulating Fetal Bone Formation and Adult Fracture Repair

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INTRODUCTION

Skeletal formation involves synchronized integration of genetic programs governing the specification, proliferation, differentiation, and programmed death of cells, remodeling of the extracellular matrix, and vasculogenesis. These same cellular and extracellular events occur during adult bone repair, leading us and others to propose that the molecular machinery responsible for fetal skeletogenesis also plays a role in the process of skeletal repair *(1–5)*. The goal of this review is to highlight recent advances in understanding molecular and cellular mechanisms regulating fetal skeletal development and adult fracture repair. We are optimistic that these advances will ultimately facilitate the manipulation of molecular programs in order to prevent bone disease and treat traumatic injury.

BONE FORMATION DURING DEVELOPMENT

The skeleton can be divided into three parts based on anatomical location and embryonic origin. The axial skeleton arises from condensations of paraxial mesoderm that form adjacent to the embryonic notochord and that comprise the future vertebral column. The appendicular skeleton is derived from localized proliferation of lateral plate mesoderm in the trunk and, along with the axial skeleton, forms bone through endochondral ossification. The skeleton of the head has a far more complex developmental history, being derived from paraxial mesoderm as well as the cranial neural crest. Cranial skeletal tissues form bone through both endochondral and intramembranous ossification. Despite these differences in embryonic origin, cartilages and bones in the head are histologically indistinguishable from those tissues found elsewhere in the body. For the sake of simplicity in this review, we will focus the remaining discussion on development of the appendicular skeleton. However, two issues should be kept in mind. First, mechanisms initiating and controlling skeletal development in the head may be qualitatively different from those regulating appendicular or axial skeletogenesis. Second, these differences may be reflected in the mechanisms by which these tissues undergo repair and/or regeneration.

Appendicular skeletal development begins shortly after the onset of limb bud outgrowth, at a time when the limb primordia consist only of mesenchymal cells sheathed in an ectodermal jacket. Histologically, the mesenchymal cells in these early limb buds may appear identical to one another, but a "molecular map" of the limb field belies this fact. *Sonic hedgehog* (*Shh*), which encodes a secreted protein involved in patterning and growth in a number of systems *(6)*, is expressed in a localized region of the posterior limb mesenchyme *(7)*. Shh directly or indirectly regulates the expression of a wide variety of growth and transcription factors, including members of the Bone Morphogenic Protein (BMP) and fibroblast growth factor (FGF) families *(8)*. At this early stage of appendicular skeletal development, all of the mesenchymal cells in the limb are competent to adopt a chondrogenic fate *(9,10)*.

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Only with time does this chondrogenic potential become restricted to a group of cells that later forms the skeleton. The precise mechanisms by which this restriction in competence is achieved are not well understood, but recent evidence from null mutations in *Sox9*, a transcription factor related to the sex-determining gene *Sry*, suggest that regulation of *Sox9* is central to this process *(11,12)*.

Transcription factors, including many homeobox genes, are important regulators of early skeletal patterning and growth. Some homeobox genes in the *HoxA* and *HoxD* complexes are required for proliferation of skeletal progenitor cells, and specific combinations of *Hox* gene products determine the lengths of the upper arm, the lower arm, and the digits. A reduction in the dosage of some *Hox* genes results in truncations or the complete absence of skeletal elements *(13,14)*. Overexpression of *Hox* genes in chick limb buds can also cause shortening of long bones, by affecting the rates of cell division in the proliferative zone of growing cartilage *(15)*. *Hox* genes affect the expression of both BMPs and FGFs, which may account for some of their effects in mesenchymal cell proliferation, although all of the targets of *Hox* gene regulation have not been identified. Another transcription factor that affects the initial specification of skeletogenic mesenchyme is *Meis2 (16)*. *Meis2* is expressed in the proximal region of the developing limb bud, up to the presumptive radiohumeral joint.

BMPs and their antagonists also play important, but poorly understood, roles in defining the population of cells that give rise to skeletogenic tissues. In addition to their roles in patterning the early limb bud *(17)*, BMPs and anti-BMP molecules such as chordin, noggin, gremlin, and follistatin influence the competence of cells to become chondrogenic *(18)*. Cells expressing BMP-2, BMP-4, and BMP-7, for example, are located in mutually exclusive domains to those cells expressing *gremlin*. These findings indicate that BMPs and their antagonists function in specifying boundaries between cell populations.

Condensation of the Mesenchyme

Upon this molecular map of the limb bud, populations of loosely associated, undifferentiated mesenchyme begin to aggregate and form condensations (**Fig. 1**). This aggregation marks the initiation of skeletal development and is an essential first step that positions cells adjacent to one another, thus facilitating cell–cell signaling *(19)*. Limb mesenchyme consists solely of chondrogenic condensations, and the SRY-related transcription factor Sox9 is one of the earliest markers of these cells *(20)*. *Sox9* is essential for differentiation of limb mesenchyme into chondrocytes. In chimeric mice, *Sox9–/–* cells are excluded from all cartilaginous condensations, and instead contribute to the adjacent noncartilaginous mesenchyme *(12)*. *Sox9* can bind to sequences in the enhancer regions of collagen type II α1 (Col2α1α1) *(21,22)*, collagen type IX α1 (Col9α1) *(23)*, and collagen type XI α2 (Col11α2) *(24)*, suggesting that *Sox9* activation upregulates the expression of genes encoding cartilaginous collagens, which in turn induces and/or maintains a cartilaginous phenotype in these cells. In addition, widespread ectopic *Sox9* expression in the chick limb, achieved with an RCAS virus encoding *Sox9,* resulted in both ectopic Col2α1α1 expression and ectopic cartilage nodules *(25)*.

A number of other genes are important for the process of mesenchymal cell condensation. *Noggin* is first expressed in condensations of the cartilaginous limb skeleton, and persists into the late stages of chondrogenesis *(26)*. Noggin binds with high affinity to BMPs, and blocks their ability to bind to cell-surface receptors *(27)*. In this way Noggin acts as an endogenous BMP antagonist, apparently limiting the range of BMP action and establishing the boundary between the condensing mesenchyme of the skeleton and the surrounding connective tissue. Mice carrying deletions in the *Noggin* gene exhibit a grossly altered cartilage skeleton with enlarged, misshapen skeletal elements and numerous joint fusions *(26)*. These phenotypic alterations lend support to the hypothesis that *Noggin* participates in defining the boundary of skeletal condensations. Consistent with this hypothesis is the observation that overexpression of BMPs, which may perturb the Noggin/BMP expression domains, can affect the size and shape of appendicular skeletal elements *(28)*.

Proteins in the transforming growth factor-β (TGF-β) superfamily also participate in mesenchymal cell condensation. For example, TGF-β1, acting through a cell-surface receptor, stimulates fibronectin

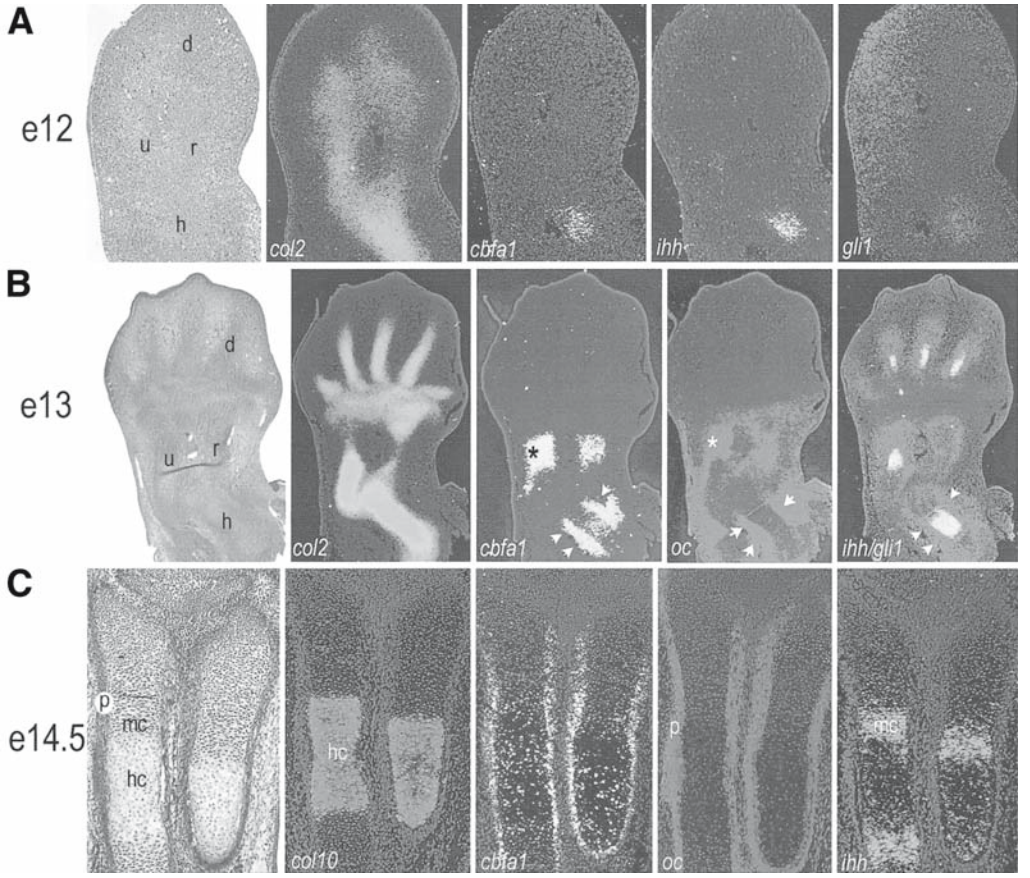


Fig. 1. Gene expression during mesenchymal cell condensation and cartilage development. **(A)** The aggregation of mesenchymal cells begins at approximately embryonic d 12 (e12) in the mouse forelimb. Even at this early time point, the expression of Col2 in an adjacent section indicates that these cells are committed to a chondrogenic lineage *(60)*. In a near-adjacent section, Cbfa1 transcripts are detected in cells of the presumptive humerus (h). These same cells express Ihh and Gli1. In addition, Gli1 transcripts are also detected in the posterior mesenchyme. **(B)** By e13, Safranin O/fast green staining indicates that mesenchymal cell condensations are beginning to generate a cartilaginous matrix (faint red staining) in the humerus (h), radius (r), and ulna (u); this matrix is absent from the digits. Maturation proceeds in a proximal-to-distal direction in the limb. Therefore, mature chondrocytes are located in the humerus, whereas more immature cells are located in the digit region (d). Col2 is expressed in chondrocytes throughout the humerus, radius, and ulna, and in the presumptive digits. In an adjacent section, Cbfa1 is expressed in chondrocytes of the humerus, radius, and ulna (asterisk). In addition, Cbfa1 is expressed in the perichondrium (white arrows). Osteocalcin transcripts are detected throughout the mesenchyme of the limb. Note that at this stage of development, Osteocalcin expression overlaps with Cbfa1 in the perichondrium of the humerus (arrows) and in the chondrocytes of the radius and ulna (asterisk). Ihh and Gli1 are expressed in reciprocal patterns: Ihh transcripts are restricted to chondrocytes in the humerus, radius (out of the plane of section), ulna, and digits, whereas Gli1 is expressed in the perichondrium of these elements. **(C)** By e14.5, mature (mc) and hypertrophic chondrocytes (hc) are arranged longitudinally in the radius and ulna, which is surrounded by a thickened perichondrium (p). No bone is visible at this stage of development. Col10 is detected in hypertrophic chondrocytes. In an adjacent section, Cbfa1 is expressed in the perichondrium and, to a lesser extent, in hypertrophic chondrocytes. Osteocalcin is expressed in the perichondrium, coincident with Cbfa1 expression in this tissue. At this stage, Ihh is restricted to mature and early hypertrophic chondrocytes, where it overlaps slightly with Cbfa1. (From ref. *2*, with permission.) (Color illustration in insert following p. 212.)

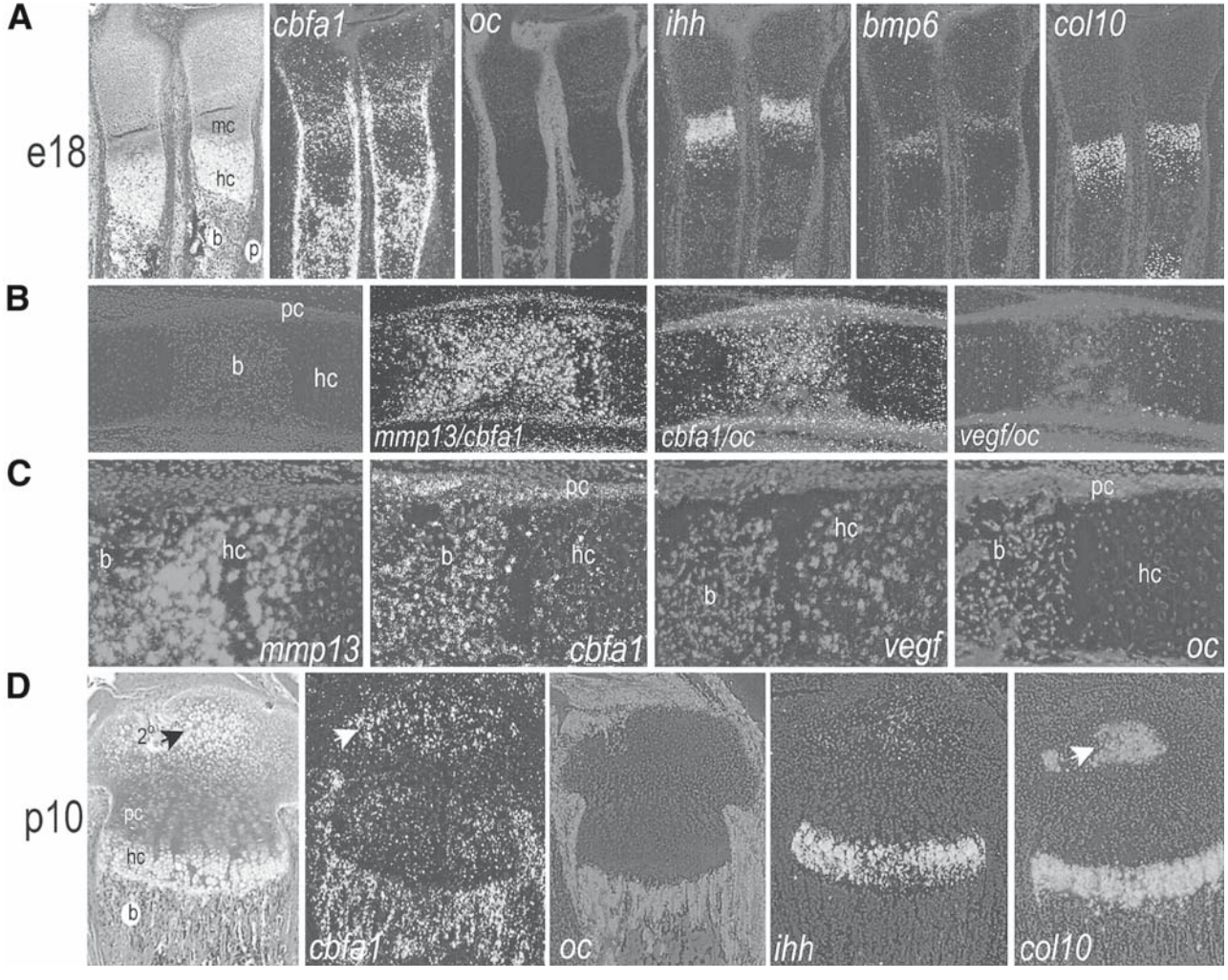
expression, which in turn regulates the cell adhesion molecule N-CAM *(17,29)*. This alteration in cell–ECM contact is a prerequisite for condensation. Another member of the TGF-β superfamily, growth and differentiation factor-5 (GDF-5), affects condensation size by increasing cell adhesion, which is a critical determinant of condensation *(30)*. Later in development, GDF-5 stimulates the proliferation of chondrocytes. However, mice carrying deletions in GDF-5 exhibit only subtle alterations in skeletal development, specifically a loss or abnormal development of some joints *(31)*.

Chondrogenesis

During condensation, mesenchymal cells begin to alter their phenotype from small, fibroblast-like cells to rounded, enlarged cells (**Fig. 2**). At the same time, there is a shift from the production of a mesenchymal matrix, characterized by collagen types I and III, to the production of a cartilaginous matrix, typified by the expression of collagen types II, IX, and XI. The transition from an undifferentiated mesenchymal cell to a differentiated, mature chondrocyte is incremental. Apparently, cells must continue to express Sox9 and Col2α1α1 before becoming irrevocably committed to a chondrogenic lineage. In the head, for example, mesenchymal cells that contribute to the cranial vault express Col2α1α1, yet these cells do not progress to form a mature cartilage *(19)*. After their initiation into chondrogenesis, however, cells must downregulate *Sox9* in order to mature *(11,32,33)*.

Shortly after the induction of Col2α1α1, the secreted factor Indian hedgehog (Ihh) is expressed in mesenchymal cells in the central region of the condensation *(34–37)*. Ihh binds to a cell-surface receptor complex encoded by *Patched (Ptc)* and *Smoothened (6)*. Ihh expression persists throughout fetal chondrogenesis and postnatal growth, and then disappears around the time of puberty *(35)*. Mice carrying deletions in *Ihh* develop condensations, yet they have a delay in chondrocyte maturation *(38)*. Ihh appears to regulate the rate of chondrocyte maturation through a feedback loop involving parathyroid hormone-related protein (PTHRP) and its receptors *(37)*. Ihh appears to regulate angiogenesis as well

Fig. 2. (*Opposite page*) Gene expression during cartilage maturation, vascular invasion, and ossification. **(A)** By e18, bone formation has begun in the forelimbs. Mature (mc) and hypertrophic chondrocytes (hc) border the primary ossification center (b), which is evident within the center of the distal ulna. The periosteum (p) has formed a bony collar and the perichondrium is visible as a thickened epithelium adjacent to the mature chondrocytes. Cbfa1 is expressed in the perichondrium and periosteum, in mature and hypertrophic chondrocytes, and in bone (b). On an adjacent section, Osteocalcin is expressed in both the perichondrium and periosteum, and in the primary ossification center. The Ihh, BMP-6 and Col-10 expression domains overlap with Cbfa1. **(B)** Nuclear Hoechst stain illustrates the cellular outline of the primary ossification center at e18.25. Note that the periosteum (p) has formed around the periphery of the skeletal element, and bone (b) is forming in the central region, surrounded on either side by hypertrophic chondrocytes (hc). Cbfa1 (yellow) and MMP-13 (aqua) signals are superimposed to show the extent of overlap between the two transcripts in hypertrophic chondrocytes. Note the absence of MMP-13 in the periosteum, where intramembranous ossification is occurring. Cbfa-1 (yellow signal) and Osteocalcin (red) are co-expressed in areas of new bone formation, including the periosteum and in the primary ossification center. VEGF is expressed strongly in hypertrophic chondrocytes and weakly in bone, where it overlaps with Osteocalcin. **(C)** Higher magnification shows that MMP-13 transcripts are limited to the hypertrophic and terminally differentiated chondrocytes, similar to VEGF. Cbfa1 is detected in chondrocytes, bone, and periosteum, coincident with Osteocalcin. **(D)** In the tibial growth plate of a 10-d-old mouse, there is an orderly progression of chondrocytes from a proliferative (pc) to a hypertrophic state (hc). New bone formation is evident distal to the hypertrophic zone (b). In addition, the secondary ossification center (2°) is evident; the arrow indicates the location of hypertrophic chondrocytes in this center. At this stage, Cbfa1 is expressed in mature and hypertrophic chondrocytes in both the growth plate and secondary ossification center. Cbfa1 is expressed in regions of new bone formation. Osteocalcin transcripts are detected throughout the trabecular bone of the growth plate. Ihh is restricted to mature and early hypertrophic chondrocytes of the growth plate, with very low levels detected in the secondary ossification center. The Col10 expression domain overlaps with that of Cbfa1 in the secondary ossification center (arrow) and, to a lesser extent, in hypertrophic chondrocytes of the growth plate. (From ref. *2* with permission.) (Color illustration in insert following p. 212.)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(39), which may account, in part, for the delay in ossification seen in the *Ihh*-null mutant. Curiously, the ability of mesenchymal cells to undergo condensation and initial chondrogenesis is apparently unaffected in the *Ihh*-null mutant *(38)*. Either *Ihh* does not participate in the programs of condensation and initial chondrogenesis, or it does not play an essential role. However, some clues about Ihh function come from the expression of PTC, which is found in the perichondrial mesenchyme surrounding the skeletal condensations. Ihh and PTC expression patterns are complementary, strongly suggesting that even at this early stage of skeletogenesis, cell communication has been established between the future chondrocytes and cells of the perichondrium.

Another transcription factor that plays an essential role in chondrogenesis is Runx2 (previously termed Cbfa1, Aml3, Pebp2αA, or Osf2). Runx2 is expressed in chondrogenic condensations of the limb and osteogenic condensations *(40,41)*. Mice with null mutations in *Runx2* form mesenchymal cell aggregations in the limb, but later have an arrest in chondrocyte maturation and osteoblast differentiation *(41–43)*. Runx2 directly regulates *Osteopontin* and *Osteocalcin*, two genes associated with hypertrophic and terminally differentiated chondrocytes and osteoblasts *(40,44)*. One function of *Osteopontin* is to mediate the attachment of cells, such as osteoclasts, to the extracellular matrix *(45–47)*. In addition, Runx2 directly induces at least one matrix metalloproteinase, collagenase-3 *(48)*, which is also expressed by hypertropic chondrocytes. The loss of *Runx2* may therefore result in a misregulation of genes associated with the degradation of the hypertrophic cartilage matrix. Accordingly, *Runx2–/–* cartilage fails to undergo vascular invasion *(43)*. This mouse mutant demonstrates an important connection between the differentiation of chondrocytes, remodeling of the extracellular matrix, angiogenesis, and bone formation.

Angiogenesis and Osteogenesis

Vascular invasion is essential for the formation of bone during both intramembranous and endochondral ossification. In intramembranous ossification, the endothelial cells are incorporated into growing mesenchymal cell condensations and provide a blood supply for subsequent ossification. In endochondral ossification, chondrocytes undergo hypertrophy, terminal differentiation, and apoptosis. The hypertrophic cartilage matrix simultaneously is degraded by matrix metalloproteinases, such as MMP9, and invaded by blood vessels. The molecular regulation of new blood vessel formation during endochondral ossification is beginning to be understood, and a number of key angiogenenic regulators have been identified. These molecules include members of the FGF, insulin-like growth factor (IGF), TGF-β, and vascular endothelial growth factor (VEGF) families *(49,50)*. VEGF is of particular importance to the vascularization of the cartilaginous skeleton. Several forms of VEGF bind to tyrosine-kinase receptors, Flt-1 and Flk-1, and the coreceptors Neuropilin-1 and Neuropilin-2 *(51,52)*. *Flt-1* and *Flk-1* are expressed in endothelial cells. VEGF is essential for embryonic development, and even the loss of a single *VEGF* allele in restricted embryonic domains causes embryonic death *(53)*. VEGF induces endothelial cell proliferation, stimulates cell migration, and inhibits programmed cell death. Whether apoptosis of hypertrophic chondrocytes is the stimulus for vascular invasion, or conversely, whether blood vessel recruitment is the trigger for cell death, is not clear. However, the coordination of the two steps is essential for osteogenesis.

VEGF-mediated angiogenesis is critical for coupling the resorption of cartilage with the deposition of bone *(50)*. One possible mechanism by which this is achieved is that VEGF is produced by hypertrophic chondrocytes but is only active in or adjacent to those cells that also express *MMP9*. *MMP9* may function to release VEGF from the extracellular matrix and initiate a series of signaling cascades that induce endothelial cell proliferation and invasion, the result of which would be the introduction of osteoprogenitor cells from marrow or endothelial pericytes. As opposed to this indirect mechanism, VEGF may have direct effects on Flt-1-expressing osteoblasts *(50)*. VEGF-mediated angiogenesis is an essential step in the replacement of cartilage by bone during development. As is becoming clear from recent experiments in our laboratory, the same events are important in both proper skeletal development and healing (Colnot et al., unpublished observations).

BONE FORMATION DURING FRACTURE REPAIR

Histologically, bone formed during skeletogenesis has much in common with bone formed during fracture repair *(1,2,35)* (**Fig. 3**). In response to injury, mesenchymal cells from surrounding tissues invade the wound site, where they proliferate, condense, and differentiate into cartilage or bone, much like that seen during development. The similarities and differences between fetal skeletal development and adult fracture repair will be outlined in the following sections.

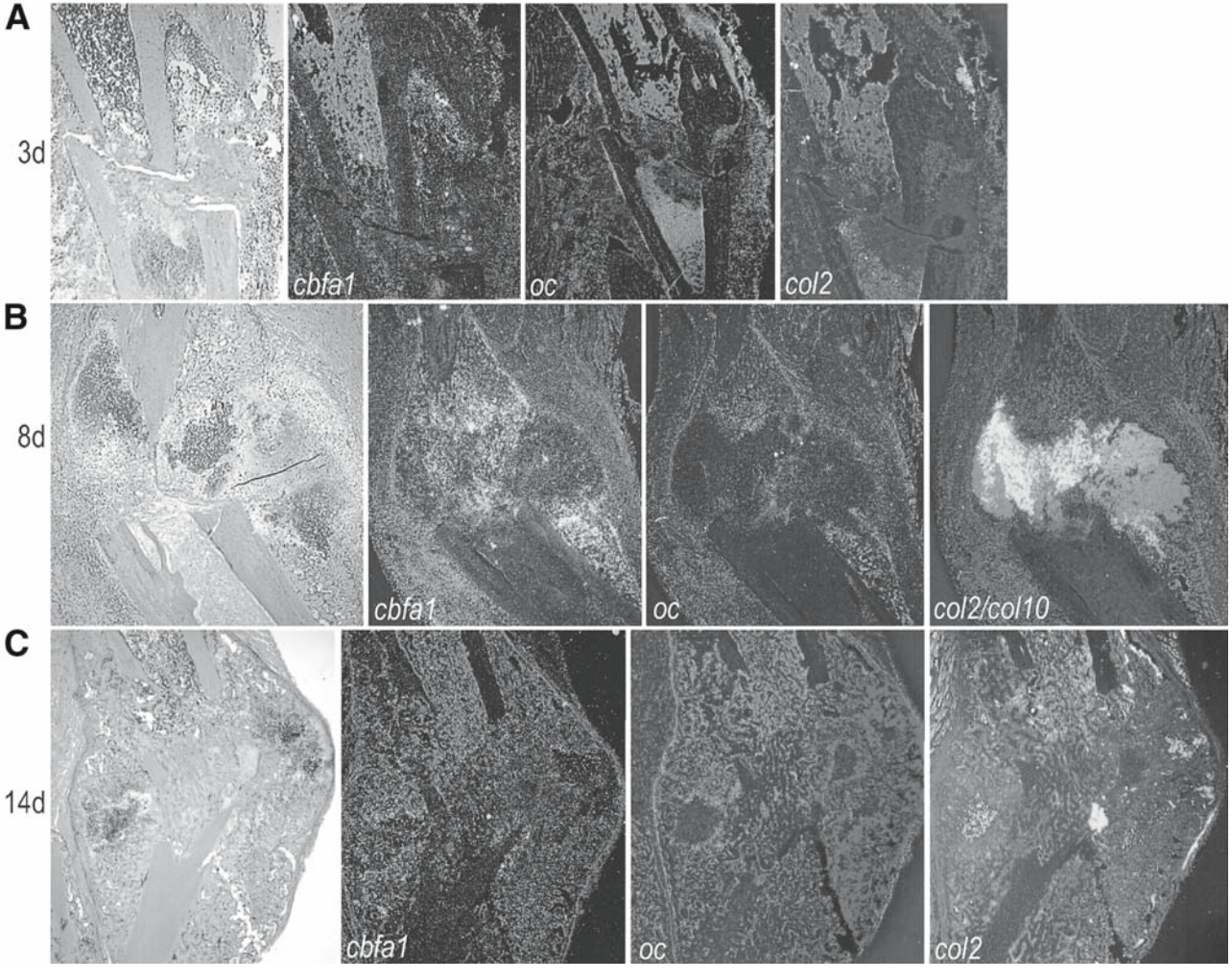
Mesenchymal Cell Aggregation Following Injury

Immediately after tissue injury, vascular and inflammatory processes trigger a cascade of signaling events that coordinate the invasion of macrophages and other inflammatory cells to the site of injury. Unlike skeletal development, the inflammatory process is a component of adult fracture repair, although its precise contribution remains unclear. One important element of the inflammatory response is the local increase in the number of macrophages, which release molecular signals regulating differentiation *(54)*. Precisely which cells respond to these cues is not entirely clear, but one possibility is mesenchymal stem cells. These cells may be capable of differentiating into chondrocytes or osteoblasts, as suggested by analogy with mesenchymal cells present during the initial stages of skeletal development. Our own analyses of cells populating a fracture site during this inflammatory stage of healing support this hypothesis. Mesenchymal cells populating the site of injury in a closed murine fracture express genes such as *Runx2*, *Sox9*, and *Col2α1*, similar to mesenchymal cells in the developing limb (Miclau et al., unpublished observations).

A clear difference between fetal development and adult repair is the influence of the mechanical environment on the differentiation of mesenchymal stem cells into chondrogenic or osteogenic fates *(55,56)*. In an unstable mechanical environment, mesenchymal cells differentiate into cartilage, whereas a stable environment favors the differentiation of these cells into osteoblasts. How this is achieved remains largely unknown, but some biomechanical and molecular data suggest at least one possible mechanism. Fracture site instability may prevent formation of an intact vasculature. In such a scenario, the fracture site would develop a low oxygen tension, permit the formation of an avascular tissue such as cartilage, and inhibit the generation of a highly vascular tissue such as bone. In a sense, the cartilage stabilizes the fracture site for intact vascularity. As avascular chondrocytes differentiate and eventually hypertrophy, they express angiogenic factors such as VEGF that induce new blood vessel formation. In addition, conditions such as lower oxygen tension lead to the induction of hypoxia-inducible factor, which directly regulates the expression of VEGF *(57)*. This mechanism may account for the formation of cartilage in an avascular situation, but whether an unstable mechanical environment actually disrupts angiogenesis is still an untested hypothesis. Although stabilizing the fracture results in an intramembranous form of healing *(58)*, this scenario additionally does not explain how mesenchymal cells can sense their mechanical environment. These are areas that remain open to inquiry and will likely yield important clues about how bone healing can be stimulated in different mechanical environments such as a stabilized fracture or distraction osteogenesis *(59)*.

Chondrogenesis, Osteogenesis, and Angiogenesis

The maturation of a cartilage callus following a fracture closely parallels cartilage maturation during development. Similar growth and transcription factors expressed during development are also detected during the soft and hard callus phases of fracture repair *(2,5)*. For example, with the conversion of the cartilage callus to woven bone, *Runx2*, *BMP-6*, *Ihh*, and *Col2α1α1* are expressed in chondrocytes of the callus. Similarly, BMP-6, Gli-3, osteocalcin, and collagen type X are detected as the cartilage is replaced by bone. Therefore, the cellular and molecular programs for endochondral and intramembranous ossification during adult fracture healing may recapitulate those operating during development. Recent findings suggest that, as during development, angiogenesis is a key regulator of the conversion of cartilage to bone during fracture repair (Colnot et al., unpublished observations), and also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demonstrate that similar to the process of embryonic development, the breakdown of the extracellular matrix is important for the vascularization of the fracture callus.

CONCLUSIONS

During both fetal skeletal development and adult fracture repair, the creation of bone requires a precise coordination of genetic programs that mediate chondrogenesis, osteogenesis, angiogenesis, and bone remodeling. Substantial advances have been made in identifying some of the key molecules and mechanisms that regulate the processes of skeletal development and repair. Collectively, this work indicates that there are remarkable similarities between the cellular and molecular programs for bone formation that function in both embryos and adults. Whether during fetal skeletogenesis or adult healing, bone formation clearly involves a series of discrete phases that are highly coordinated to produce a complete, intact skeleton. Future studies focusing on the molecular and cellular regulation of skeletal morphogenesis and the development of new models of bone repair will undoubtedly provide the foundation for novel therapies to treat bone diseases and traumatic injuries.

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Fig. 3. (*Opposite page)* Gene expression during the early, intermediate, and late stages of nonstabilized fracture healing. **(A)** Three days after fracture, the periosteum near the fracture site appears thickened, with no cartilage detected by Safranin O/Fast Green staining. Cbfa1 transcripts are present in the thickened periosteal cells. Coincident with Cbfa1 expression, Osteocalcin is detected in the periosteum as well as in the periphery of the callus. Col2 is limited to a small region near the periosteum of the proximal fracture fragment. **(B)** By 8 d postfracture, abundant cartilage appears within the callus. Col-2 and ColX are detected throughout the callus. Osteocalcin is expressed on the periphery of the cartilaginous portion of the callus and within newly formed woven bone, overlapping with Cbfa1. **(C)** At 14 d postfracture, extensive new bone formation is evident, with small islands of residual cartilage persisting. Cbfa1 is detected at low levels in areas of ossification and within the cartilage islands, but is not detected in the periosteum. Osteocalcin is evident throughout areas of bone formation, but is excluded from the cartilage islands. Col2 shows a reciprocal expression pattern, being restricted to the small islands of cartilage in the callus. (Data from ref. *2*.) (Color illustration in insert following p. 212.)

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