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The Role of Chondrocytes in Fracture Healing

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Commentary

Inflammation plays important roles in the early steps of fracture healing including, the recruitment, expansion, growth and differentiation of mesenchymal stem cells (MSCs), the coordinated interplay of many other cell types, growth factors, and extracellular matrix (ECM) components, as well as the production of cartilage and bone matrix in a temporally controlled manner [1–4]. There are three stages of fracture healing: the reactive stage, the reparative stage, and the remodelling stage. After a bone fracture, the reparative process initiates from a hematoma and inflammatory reaction at the fracture site. In the inflammatory phase, the shortage of peripheral vasculature causes an anoxic environment that leads to the formation of a cartilaginous template, which initiates the differentiation process that restores endochondral (EC) ossification. The proliferation and differentiation of bone marrow stem cells (BMSCs) into chondrocytes and osteoblasts is a critical component of this phase of fracture healing [1,4-6]. In the reparative phase, chondrocytes facilitate ECM deposition at the fracture site, which forms a transient soft callus [2,7]. During the initial stage of remodelling, the callus of the femoral head is transformed into vascularized and mineralized tissue, allowing the initial stages of osteoclast resorption to commence [8]. Later, during bone remodelling, skeletal elements heal into the appropriate shapes [9]. The importance of invading vascular EC during bone formation has been established, and defective bone vasculature was reported in osteoporosis and rickets patients [10]. Therefore, vascular endothelial growth factor (VEGF) expression in bone repair and neovascularization is driven with the support of nutrition, oxygen transport and tissue oxygenation, which are required for the differentiation of osteoblasts [11]. Pharmacological inhibition of angiogenesis has also been shown to impair fracture healing and reduce or delay mineralization of the callus, which further suggests the need for a cascade of angiogenesis in the repair process [12]. Finally, the mechanical requirements of the tissue are achieved after the transformation of osteoclasts [13,14].

EC ossification is characterized by the condensation of mesenchymal cells that produce a cartilage primordium surrounded by a perichondrium, which consists of pre-chondroblasts, osteoblasts, and fibroblasts [15,16]. Previous studies have shown that cartilaginous callus formation plays an important role in the development of bone tissues [17]. The early periosteal formation of membranous new bone, followed by EC ossification results in a linear increase of callus bone during the healing process. EC ossification occurs naturally in most fracture healing processes; therefore, it can be used to improve almost any orthopedic bone regeneration, especially in anoxic conditions caused by critical size defects in the femoral head that are conducive to initial cartilage and not direct intramembranous (IM) ossification. However, EC fracture healing involves a well ordered sequence of cellular events that are similar to those that occur during embryologic bone development and postnatal skeletal growth. Calcification of the temporary bridge of cartilaginous tissue is a pivotal mechanism in secondary bone healing. The possible mechanisms could involve chondrocyte apoptosis and replacement with osteoblasts or the acquisition of an osteogenic phenotype by the chondrocytes. New blood vessels then grow into the cartilaginous callus and vascular osteogenic tissues, namely the chondroid matrix, gradually substitute the avascular cartilaginous callus, leading to the formation of true bone tissues. Together, EC ossification is an essential process for the reparative phase of fracture healing [18,19], which starts with the differentiation of BMSCs into chondrocytes and is followed by chondrocyte proliferation, differentiation, maturation, and apoptosis, as well as vasculature invasion [20].

This paper describes how chondrocytes are incorporated into the process of fracture healing through ECM secretion, the formation of cartilage callus or EC ossification, and the regulation of bone regeneration involved in the healing process. Here, we only focus on bone healing processes that involve cartilage ossification after the formation of new bone. The wealth of recent data on this topic necessitates an update of the existing models of EC bone repair with an emphasis on the potential opportunities for enhancing bone repair and new treatment approaches for the repair of cartilage. Bone revascularization is a normal biological function consisting of a series of carefully coordinated events that require interactions between healing tissue and blood vessels. In this review, we will also discuss how these interactions can be utilized in clinical application.

Role of Chondrocyte in Fracture Healing

EC ossification

EC bone formation occurs during the normal processes of embryonic development, postnatal growth, and fracture healing [1]. As the process continues, capillaries invade the tissue, chondrocytes undergo apoptosis, and there is proteolytic degradation and resorption of the mineralized cartilage matrix concurrent with the deposition of new bone by osteoblasts [9,21,22].

MSCs derived from the periosteum and chondrocyte differentiation

Initially, disrupted vasculature and bone marrow during the inflammatory phase facilitates a coagulation cascade along with an influx of progenitor cells, including BMSCs, into the fracture space forming a hematoma. However, without permanent vasculature the fracture space becomes hypoxic, and it remains unclear whether enough stem cells survive the initial inflammatory phase to play an

active role in subsequent tissue regeneration [1,23–26]. Signalling molecules from the inflammatory phase and ECM subsequently recruit progenitor cells from both the exposed periosteum and bone marrow that migrate into the fracture space, initiating both bone developmental pathways: IM and EC ossification [1,23-26]. The primary differences in these pathways reside in precursor requirements. Bone marrow stromal cells differentiate into chondrocytes or osteoblasts through a process called IM ossification and form a film that is directly deposited onto bone. The EC ossification pathway involves cartilage cell proliferation, hypertrophy, mineralization, and new bone is made through a process of small deposits of cartilage and ossified matrix ECs [1,23-26].

Bone marrow is known to contribute to bone repair after injury because its removal delays fracture repair, but the underlying mechanism of this phenomenon is unclear [27]. Taguchi et al. concluded that bone marrow-derived MSCs are osteoblast progenitor cells in the callus, especially near the fracture site using chimeric mice generated from bone marrow transplantation (BMT) from green fluorescent protein (GFP) transgenic mice into wild-type recipients [21]. However, the results of a similar experiment showed that bone marrow-derived MSCs may contribute to fracture repair along with factors from another source that increase MSC differentiation [28]. Finally, lineage analysis from a recent study clearly showed that bone marrow MSCs directly act on osteoblasts and bone cells, rather than chondrocytes [29]. Another group subsequently confirmed that the bone cells involved in fracture repair were derived from the periosteum [30]. After injury, periosteal cells proliferate extensively and are known to contribute to chondrocyte development [27]. Similarly, destruction of the periosteum, rather than the bone marrow, inhibits the production of cartilage and EC ossification, further supporting the model that includes local origin from the periosteal cells. The periosteum is also the main source of cartilage cells in the gap defect model of callus. This confirms that the soft tissue does not suppress bone marrow-stimulated differentiation of the unique chondrocytes required for normal fracture healing [27,30-32].

Chondrocyte differentiation

The process of chondrogenic differentiation includes six phases: mesenchymal cells (chondroprogenitors), condensed mesenchymal cells, chondrocytes, proliferating chondrocytes, pre-hypertrophic chondrocytes and hypertrophic chondrocytes.

EC ossification, therefore, can further be divided into three generalized steps: (1) chondrogenesis, (2) cartilage hypertrophy, and (3) ossification. However, regardless of the pathway, neo-vascularization and angiogenesis are necessary before ossification can proceed.

In the inflammatory phase, there is a stage of both mesenchymal and angiogenesis activation. Vascular and bone marrow MSCs are recruited to the injury site and proliferate. The second stage is only required for bone regeneration.

Signalling the migration, proliferation, and differentiation of stem cells into the injured site is complex and has not been fully elucidated. However, many factors are known to influence these processes, including the basic fibroblast growth factor (bFGF), bone morphogenetic protein (BMP), Wnt/β-catenin and Notch signalling pathways, as well as other physiological stimuli, such as hypoxia and mechanical loading [32,33].

Members of the transforming growth factor beta (TGF-β) superfamily, such as growth and differentiation factors (GDFs) and BMPs, regulate bone shape [27]. BMP family members are important in the formation of mesenchymal condensations [26,34]. BMP signalling induces the expression of the transcription factor sex determining region Y-box 9 (SOX9), which is required for commitment of undifferentiated mesenchymal cells in the condensations to chondrocytes [35-37].

By harnessing growth factors to stimulate endogenous BMSCs, either alone or in combination by using exogenous BMSCs, therapeutics could be developed to promote BMSC proliferation and differentiation to heal fractures and treat conditions of low bone mass.

However, the molecular events that regulate the differentiation of mesenchymal cells into chondrocytes are still largely unknown. Condensations of mesenchymal cells reportedly express the transcription factor SOX9, which is a key regulator of chondrogenesis and give rise to cartilage primordia, which consists of round immature chondrocytes that continue to express SOX9 [36,38]. It is also evident that canonical Wnt signalling promotes progenitor cell differentiation towards the osteoblast and chondrocyte lineage in developing skeletal elements. Wnt pathway components (Wnt4, Fzd2, Lrp5 and β-catenin) were up-regulated at the fracture site within 3–5 days after injury in mice [39]. Collectively, the studies described previously provide compelling evidence in the cartilage and bone formation phases of fracture repair. The data show that pluripotent BMSCs differentiate into osteochondral progenitor cells and then further differentiate into chondrocytes and osteoblasts [40]. Once cells begin to show phenotypic features of either chondrocyte or osteoblast precursors, they exhibit β-catenin-mediated and TCF-dependent transcriptional activity, demonstrating the role of β-catenin signalling in the initial phase of BMSC differentiation [41]. SOX9, which is also used as a marker for chondrogenic differentiation [42,43], was downregulated by Dickkopf-1 (DKK1), a secreted glycoprotein and potent Wnt antagonist, in the early stage fracture healing [44]. It has also been reported that DKK1 prevents the early stages of bone repair by blocking Wnt/β-catenin signalling and the differentiation of MSCs into chondrocytes or osteoblasts [45,46].

Chondrogenic and osteogenic programs in the progenitor cell pool oppose each other at a molecular level. For example, activation of Wnt/β-catenin during osteogenesis specifically prevents differentiation toward the chondrogenic lineage by suppressing SOX9 [47,48].

The SULF1/2 enzymes are critical for modulating cell signalling pathways that require heparan sulfate proteoglycan (HSPG) as a co-factor for ligand–receptor interactions (Dhoot et al., Morimoto-Tomita et al., Rosen and Lemjabbar-Alaoui).

This leads to the inhibition of 6-O sulphate requiring ligands, such as FGFs, hepatocyte growth factor (HGF) and VEGF but also to the promotion of other signalling pathways such as those involving glial cell-derived neurotrophic factor (GDNF) and Wnts. Many BMPs and their receptors are also expressed during bone formation, although BMPs and FGFs have opposite effects on chondrocyte differentiation [49,50].

Stabilization of hypoxia-inducible factor 1α (HIF-1α) under the hypoxic environment, directly regulates SOX9 expression in the condensed mesenchymal cells to promote survival and chondrogenesis [22,51,52].

Relationship between ECM secretion by chondrocytes and bone fracture healing

Cartilage ossification is the process of indirect bone formation through a cartilage intermediary, and results in part from callus-induced differentiation of bone progenitor cells to chondrocytes. SOX9 regulates the synthesis of type II collagen (Col2) and protein polysaccharides, typical markers of cartilage [43,53-55]. The progenitor cells that form the fracture callus are locally recruited from bone and periosteum [29]. In a fracture that is not fixed, these cells are subjected to a specific regional pattern of bone and cartilage differentiation. In the fracture space, progenitor cells form bone by the ossification of the cartilage.

In this pathway, SOX9 expression promotes condensation and commitment of the osteochondral progenitors toward the chondrogenic lineage. After specification, subsequent SOX9 activity is necessary to maintain cell morphology and the chondrogenic phenotype through maturation to hypertrophy. In a process that resembles the well-described process of EC ossification in the growth plate [49]. Chondrocyte proliferation in the cartilage of the fracture callus, where the bone phenotype is obtained, also experiences hypertrophic maturation. However, in the fracture callus growth plate where there is a lack of tissue, SOX9 expression during the early stage of bone callus condensation is easily detected. Subsequently, SOX9 regulates the production of cartilage ECM by directly binding to enhancer elements that control the expression of type II collagen and aggrecan [43,53,54,56]. Hypoxia enhances ECM synthesis through the HIF-1α/SOX9 signalling pathway [22,51,52].

Similar to the growth plate cartilage, differentiation of cartilage cells in the fracture callus is controlled and these cells mature gradually. Hypertrophic maturation of the chondrocytes is marked by increased expression of Type X collagen (Col10) in the area where cartilage transitions to bone.

Enlargement of the cartilage anlagen by chondrocyte proliferation, hypertrophy and ECM production

In the process of bone healing, most of the initial developmental procedures are conserved, as are the genetic mechanisms involved in the regulation of cell differentiation by a variety of cell types [57,58]. In development, cartilage production at the injured site is repaired by cartilage ossification. The fracture callus deposits ECM that includes Col2, which promotes the aggregation of cartilage cells, and their differentiation into hypertrophic chondrocytes is promoted by ECM deposits containing Col10. This is then partially mineralized, absorbed and replaced by a collagen matrix that is mainly composed of type I collagen (Col1) [34]. This complex developmental process requires strict control mechanisms that locally produce factors and their respective receptors, including ECM components and transcription factors through the coordinated action of hormones [59]. Strikingly, a similar phenotype was found in mice containing null mutations in the tumor suppressor gene PTEN, which regulates Akt activation upstream of the O-box transcription factors [60,61].

This process is affected by the invasion of osteoblasts and osteoclasts. These cells, whose development and function are closely linked, continue to transform the regenerated tissue into mature bone until the fracture is healed. Other aspects of bone repair are different from bone development; for example, bone repair may be affected by the mechanical environment. In unstable fracture healing through EC ossification, stable fracture healing occurs through the ossification of the deposited film. In this process, different recruitment mechanisms are used to bring mesenchymal precursors to the injured site that are only produced by compact (cortical) and sponge (loose) bone in the fracture site [7,62,63].

Skeletal elements are rich in ECM, and ECM remodelling is the core of bone development and repair [62,64]. Matrix remodelling is affected by many of the same proteases, and these enzymes determine the speed and effectiveness of the development and repair procedures [65]. The role of matrix metalloproteinases (MMPs) in skeletal development has been widely studied [66-71]. MMP13 promotes growth plate hypertrophic cartilage and bone formation in trabecular bone, the newly deposited tissue that requires absorption remodelling [68,69]. In addition, other work points to the need for MMPs in bone repair [62,63,72-74]. These reports suggest that MMP13 may also be involved in bone repair, but this hypothesis requires formal testing [63].

Previous studies have shown that the differentiation of chondrocytes is key to bone formation, bone remodelling and bone fracture healing [75,76]. During bone repair, ECM secreted by chondrocytes is deposited around the fracture, forming temporary cartilage callus, which is critical for the fracture healing process [75,77]. ECM remodelling is important during bone development and repair. Due to the increased blood supply and presence of mature of osteoclasts during the molding period, cartilage callus is gradually replaced by vascularized bone tissue, resulting in the formation of real bone tissue. EC vascular invasion in cartilage can induce the formation of a primary ossification center in cartilage, indicating formation of real bone tissue [10]. Bone formation occurs in the vicinity of angiogenesis, and these new blood vessels deliver nutrients, oxygen and mitogens secreted by osteoblasts for the bone progenitor cells [78,79]. Unsurprisingly, angiogenesis inhibitors can damage the bone fracture healing process [12].

Ideally, fracture healing depends on adequate blood vessel formation and, therefore, the formation of new blood vessels is necessary to meet the needs of different stages of fracture healing. In the process of fracture healing, the transition from the callus to new bone is the key stage in the repair process [2]. This stage includes four coordinative processes: the apoptosis of cartilage cells; the degradation and removal of cartilage matrix; angiogenesis in repair areas; and the recruitment and differentiation of osteoblasts to form bone matrix [80,81]. Failure of any of these processes can lead to delayed or blocked fracture healing.

EC fracture healing involves a well ordered sequence of cellular events that is similar to those occurring during embryological bone development and postnatal skeletal growth. The possible mechanisms responsible for the timely removal of chondrocytes, which are not normal cellular component of cortical bone, could involve chondrocyte apoptosis and replacement with osteoblasts or acquisition of an osteogenic phenotype by the chondrocytes [82-85].

Signalling Pathways involved in Bone Fracture Healing

Bone fracture healing is tightly controlled by the expression of crucial transcription factors including, Col2a1, ACAN, Col10a1, and MMP13 [86-88] in differentiating ATDC5 cells. Moreover, the pharmacological agent Tranilast has been shown to upregulate expression of essential signalling molecules involved in EC ossification such as Parathyroid hormone-related protein (PTHRP), Indian Hedgehog (Ihh), and AXIN2 [20,89-91]. Increased matrix proteoglycan synthesis, which induces alkaline phosphatase activity,

accelerates mineralization. Tranilast is a potential agent that accelerates fracture repair by promoting the regulatory steps of EC ossification.

Nepal et al. identified that Kaempferol, a flavonoid which is abundant in plants, induces chondrogenic differentiation in ATDC5 cells through activation of the EPK/BMP-2 signalling pathway, and that Tranilast increased Ihh and Pthrp expression in ATDC5 cells [92]. Through screening a library of plant compounds, Choi et al. determined that genkwadaphnin stimulates chondrocyte differentiation in ATDC5 cells via the ERK and JNK signalling pathways. β-catenin mRNA expression was also upregulated after genkwadaphnin treatment [93].

Canonical Wnt signalling has been shown to promote progenitor cell differentiation towards the osteoblast and chondrocyte lineage in developing skeletal elements, whereas Hedgehog signalling regulates chondrocyte hypertrophy during EC ossification [49,94]. An inhibition of Wnt signalling would be predicted to cause an acceleration of both chondrogenic and osteogenic differentiation at the fracture sites [44]. Many processes observed in development are also recapitulated during bone fracture repair, including periosteal cells and osteoblasts near the fracture site, mature chondrocytes of the fracture callus and osteocytes at cortical bone 5 days post-fracture The cartilage anlagen is also enlarged by chondrocyte proliferation and hypertrophy, and ECM production which, in turn, drives bone growth. The rate of bone elongation is also driven by the orientation of the hypertrophic chondrocytes, which is regulated by Hedgehog signalling. [95]. Growth factors play an important role in the rapid regeneration of bone and cartilage that takes place during fracture repair. The complexity of the repair process requires coordination of a number of the signalling pathways that regulate skeletal cell proliferation, ECM synthesis and tissue differentiation. SULF2, unlike SULF1, is also expressed in the calcified matrix of hypertrophic chondrocytes, which suggests that its expression may be associated with inhibition of HSPG ligand by the desulfurization effect. Increased SULF2 expression along with increased Hedgehog signalling in the healing bone may be closely related.

The basic helix-loop-helix transcription factor Hand1, which is expressed in the cartilage primordia, is involved in the proper osteogenesis of the bone collar via its control of Ihh production. Hand1 downregulated Ihh gene expression *in vitro* by inhibiting Runx2 transactivation of the Ihh proximal promoter. These results demonstrate that Hand1 in chondrocytes regulates EC ossification, at least in part through the Runx2/Ihh axis [96]. Furthermore, Ihh and Ptch1 are upregulated during the initial stage of fracture repair [97-100].

Chondrocytes are derived from the chondrogenic differentiation of MSCs. This process includes six phases: mesenchymal cells (chondroprogenitors), condensed mesenchymal cells, chondrocytes, proliferating chondrocytes, pre-hypertrophic chondrocytes and hypertrophic chondrocytes. A number of transcription factors and cytokines influence discrete steps in the chondrocyte differentiation pathway. These include members of the SOX family (SOX9, SOX5 and SOX6), BMPs, connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family protein 2 (CCN2) as well as others [101].

For example, lack of MMP-9 expression can disrupt degradation of the cartilage matrix, leading to an increased cartilage hypertrophy region, which is the result of an abnormal regulation of the apoptotic process. In addition, in MMP-9−/− mice, the fracture healing process is delayed, which can be corrected by the addition of exogenous VEGF. These results show that angiogenesis is closely associated with cartilage apoptosis [62], and the molecular regulation of angiogenesis is connected with the cartilage removal during EC ossification.

Oxidative stress plays important roles in bone formation, growth and remodelling [17-20], especially in the terminal differentiation of MSCs that drives growth plate chondrocyte hypertrophy and apoptosis. This is necessary for lengthening long bones and forming structures of the appropriate shape. Apoptosis has been assessed by caspase-3 (CASP3), and a report by Giganti et al. [23] implicated ASK1 as an upstream activator of mitochondrial-dependent CASP3 activation. In addition, ASK1 is activated by the JNK and p38 MAPK pathways, which have important roles in cartilage and bone formation and turnover. Specifically, the JNK signalling pathway enhances cartilage by activating the transcription factor activated protein-1 (AP-1), which increases TGF-β expression [24]. However, long-term activation and differentiation via p38 MAPK signalling are required for chondrocyte maturation and are necessary for EC ossification.

We have shown that deletion (mouse mutants) or inhibition (nqdi-1) of ASK1 enhances the survival of hypertrophic chondrocytes and thus increases EC bone formation. The role of ASK1 in the growth plate is newly appreciated, but inhibition of other genes that cause a loss of hypertrophic chondrocyte apoptosis has also been reported to increase HZ length [30,31]. Specifically, TNFR1−/− mice that showed an absence of death-activated TNF alpha receptor showed a similar phenotype [32]. ASK1 activation is required for TNFR1 signalling, which suggests that the TNF pathway may be a major driving force for the death of hypertrophic chondrocytes. Interestingly, CASP3 knockout cells produced opposite results, including delayed ossification, decreased bone density and shorter HZ [33]. This suggests that the ASK1 pathway is likely to be as important as CASP3.

Effects of Apoptosis and Angiogenesis on Fracture Healing

Effects of apoptosis on bone fracture healing

When undifferentiated mesenchymal cells differentiate into chondrocytes, chondrocyte apoptosis begins. In the chondrogenic lineage different cells differentiate to different areas of the epiphyseal cartilage from the resting cartilage zone, and then follow the proliferation of hypertrophic chondrocytes. The region of cell death is located in the central region of the skeletal element, starting at a joint surface and continuing through the rest of the proliferating zone of the cartilage cells. Col2a1 expression marks the quiescent and proliferating cells, while in the hypertrophic zone chondrocytes expression Col10A1. Hypertrophic chondrocytes further develop characteristics of terminally differentiated cells and upregulate Col10A1 and the terminal differentiation markers osteopontin and MMP13, which also mark bone cells [102]. Ossification occurs when terminally differentiated chondrocytes undergo apoptosis and the calcified cartilage is invaded by blood vessels, osteoclasts, osteoblasts, and mesenchymal precursor cells [102].

Chondrocyte apoptosis is an essential process for replacing cartilage with bone during fracture healing and the growth of long bones [103]. Previous reports have shown the presence of TUNEL-positive apoptotic cells in hypertrophic chondrocytes [5]. Unregulated FGF signalling can directly induce apoptosis; whereas, apoptosis has been also reported to be dependent on the induction of pro-apoptotic

molecules by FGF [104]. FGF signalling decreases PTHrP receptor expression in the growth plate [105], and the down regulation of PTHrP signalling could also play a role in promoting apoptosis by decreasing BCL2 expression [106]. FGFR3-mediated STAT1-p21 signalling has also been reported to induce apoptosis in hypertrophic chondrocytes during EC ossification and in fracture repair [107]. However, another study showed that STAT1 is crucial in mediating FGF-induced apoptosis in proliferating chondrocytes, but not in the hypertrophic chondrocytes [104]. According to this model, mast cells are programmed for death [108-111], then osteoblasts or bone cartilage progenitor cells can form bone matrix through blood vessels, instead of cartilage [10]. This long held view is standard in the literature of the growth plate biology and fracture repair.

Induced apoptosis in chondrocytes during EC ossification may involve Fas- and caspase-mediated signalling pathways [112]. Other studies have shown that in the process of EC ossification, β-catenin is required upstream of Ihh signalling for chondrocyte survival and apoptosis inhibition [113].

Effects of angiogenesis on bone fracture healing

Bones are highly vascularized tissues and vascularization plays an important role in normal physiology, including regulating balance, allowing bone homeostasis, and providing a hematopoietic niche. Therefore, reconstructing bone vessels and the bone marrow cavity is necessary for complete bone regeneration and to restore the full function of the bone marrow.

During the repair process, the fracture callus in the initial soft callus stage has no blood vessels. However, as the callus matures, it becomes a potent stimulator of angiogenesis and vascular invasion through the secretion of VEGF [114-116], PIGF [117], and PDGF [118]. The importance of angiogenesis in fracture healing has been experimentally demonstrated by inhibiting VEGF through the delivery of soluble VEGF receptor (Flt-IgG), which delayed the transition of cartilage to bone after vascular invasion [115,119]. In addition, vascular endothelial cells secrete MMP-9, which has high specificity for collagen degradation, thereby accelerating cartilage degradation and vascular invasion. Moreover, cartilage matrix degradation can be enhanced by osteoclasts transferred to the cartilage matrix through the newly formed blood vessels. MMP-9 expression can be found in the vasculature of calcified cartilage that is recruited to the bone [120]. These results are similar to those found in animals receiving the angiogenesis inhibitor rapamycin, which showed a significant delay in EC repair [121].

SULF2 expression in hypertrophic chondrocytes may indicate their different roles in vascularization as has been suggested by the anti-angiogenic activities of SULF1 [122,123], but pro-angiogenic activity of SULF2 in some mammary tumors [124]. A study by Ueng et al. suggested that one mechanism is smoking, which causes a reduction in angiogenesis [125].

The ossification of cartilage to bone requires cell proliferation and angiogenesis. Vascular supply of a number of circulatory factors such as parathyroid hormone (PTH), insulin and vitamin D is important for normal fracture healing. Importantly, vascular invasion is also associated with cartilage matrix calcification and the transition to bone. Changes in calcium concentration are sufficient to induce hypertrophic chondrocytes to become mineralized, but it remains unclear what the source of calcium is, and which cells detect these changes.

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