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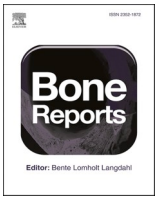

The role of hypertrophic chondrocytes in regulation of the cartilage-to-bone transition in fracture healing

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ABSTRACT

Endochondral bone formation is an important pathway in fracture healing, involving the formation of a cartilaginous soft callus and the process of cartilage-to-bone transition. Failure or delay in the cartilage-to-bone transition causes an impaired bony union such as nonunion or delayed union. During the healing process, multiple types of cells including chondrocytes, osteoprogenitors, osteoblasts, and endothelial cells coexist in the callus, and inevitably crosstalk with each other. Hypertrophic chondrocytes located between soft cartilaginous callus and bony hard callus mediate the crosstalk regulating cell-matrix degradation, vascularization, osteoclast recruitment, and osteoblast differentiation in autocrine and paracrine manners. Furthermore, hypertrophic chondrocytes can become osteoprogenitors and osteoblasts, and directly contribute to woven bone formation. In this review, we focus on the roles of hypertrophic chondrocytes in fracture healing and dissect the intermingled crosstalk in fracture callus during the cartilage-to-bone transition.

1. Introduction

Mammalian bones have high regenerative capacity. After injury, bones undergo a complex process of repair and eventually return to their original form once repair succeeds. Two paths of bone formation occur during fracture repair: intramembranous bone formation and endochondral bone formation. In intramembranous bone formation, stem/progenitor cells differentiate directly into osteoblasts, which lay down mineralized bone matrix. In endochondral bone formation, stem/progenitor cells differentiate into chondrocytes, making a cartilaginous mass which is eventually replaced by bone. Intramembranous ossification is predominant in stable fractures, whereas endochondral bone formation is more often observed in unstable fractures or fractures with large bone defects (Hagiwara et al., 2015). Endochondral bone formation can expand the soft callus volume to connect fracture ends and fill the gap by a series of changes in chondrocytes including rapid proliferation, maturation, and hypertrophy. Rapid formation of the soft callus by endochondral ossification is an advantage in fracture healing because it provides initial mechanical stability. Another advantage is seen in the healing of malaligned fractures. It has been demonstrated that in malaligned fractures, the soft callus on the concave side forms growth-plate-like cartilage which elongates the callus to correct the malalignment (Li et al., 2007; Li et al., 2004; Long et al., 2010; Rot et al., 2014), suggesting that endochondral bone formation may help correct the angulation, albeit to a limited extent. Thus, the prompt response of the cartilaginous callus-to-bone injury is beneficial for fractures with instability, large defects, and/or malalignment.

Pathological observations have traditionally divided the endochondral fracture healing process into four stages, (1) inflammation, (2) soft callus formation, (3) hard callus formation, and (4) bone remodeling. During the inflammation stage, a hematoma is formed at the fracture site containing degranulated platelets and inflammatory cells, followed by growing capillaries and reorganization of the granulation tissue. In the callus stages, osteochondral stem/progenitor cells begin committing into specific lineage cells responsible for bone formation and fracture repair. However, these stages largely overlap. The temporal sequence of the four-stage model is still useful to capture the entire picture of fracture healing, but more flexible models are proposed to understand the molecular mechanisms in this complex process. A metabolic model has been proposed that considers fracture healing as an anabolic phase

Abbreviations: VEGF, Vascular endothelial cell growth factor; BMP, Bone morphgentic protein; MMP, matrix metallopoteinase; IHH, Indian hedgehog; PTHrp, Parathyroid hormone-related peptide.

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involving chondrogenesis and osteogenesis, followed by a catabolic phase of remodeling of the cartilaginous and bony callus (Little et al., 2007; Schindeler et al., 2008). In contrast, Bahney et al. (Bahney et al., 2019) performed a comprehensive review of the fracture process from a cellular perspective and noted the various overlapping cells during fracture healing. The inflammatory cells exist not only at the inflammatory phase but also at the soft callus formation phase. Osteoblasts, chondrocytes, progenitor cells, and vascular cells contribute to the healing process through multiple stages with the transition from soft callus (cartilage) to hard callus (bone) being the most complicated phase in which numerous types of cells coexist and interact.

The cartilage-to-bone transition is essential to obtain firm stability and rigidity of the fractured bones. Failure or delay in this process causes an impaired bony union such as nonunion or delayed union (Cheng et al., 2020; Colnot et al., 2003; Julien et al., 2020; Wang et al., 2015). The cartilage-to-bone transition consists of multiple events including the degradation of cartilaginous matrix, vascular invasion, and bone formation. These events are bidirectional, interdependent and indispensable for fracture healing, and involve several populations of cells including chondrocytes, endothelial cells, osteoclasts, and bone cells. Among them, hypertrophic chondrocytes that reside in the border between the soft callus and the hard callus have an intermediary role. The degradation of the avascular cartilaginous matrix is initiated by hypertrophic chondrocytes, which allows the migration of other types of cells and vessels. Hypertrophic chondrocytes also express vascular endothelial growth factor (VEGF) which induces vascularization, and in turn accelerates cartilage matrix degradation by invading vessels. Vascularization results in the migration of hematopoietic lineage osteoclasts and osteoprogenitors, leading to new bone formation. Hypertrophic chondrocytes may also stimulate osteogenesis via their production of growth factors such as bone morphogentci protein 2 (BMP-2). Furthermore, hypertrophic chondrocytes proceed toward differentiation into osteoprogenitors, contributing directly to bone formation. In this review, we will discuss these diverse molecular and cellular interactions in the process of the cartilage-to-bone transition by focusing on the interaction of hypertrophic chondrocytes with osteoclasts, bone cells, and vessels.

2. Hypertrophic chondrocytes mediate the crosstalk between cartilage and bone

Hypertrophic chondrocytes play multiple roles in the cartilage-to-bone transition including the degradation of cartilage matrix, induction of vascularization, and recruitment of osteoclasts.

2.1. Matrix degradation

The first step of the transition is the rapid degradation and resorption of the cartilaginous tissue. Two matrix metalloproteinases (MMPs), MMP13 and MMP9, are essential for the degradation of cartilage and vascularization. In murine fracture models, the number and size of chondrocytes in the callus reach their maximum from day 9 to day 14 postfracture (Henle et al., 2005). As chondrocytes mature into hypertrophy, they express MMP13 (D'Angelo et al., 2000; Uusitalo et al., 2000). *Mmp13*-deficient mice have an expanded hypertrophic zone in the growth plate with an absence of collagenase-mediated collagen cleavage and a delay of vascularization, indicating that MMP13 expression of hypertrophic chondrocytes -in the growth plate during embryonic and postnatal development is essential for cartilage resorption and (Stickens et al., 2004). In the fracture callus, MMP13 is expressed by hypertrophic chondrocytes and osteoblasts, and its deficiency causes increased cartilage volume and delayed cartilage resorption in the callus. The delay of cartilage resorption is not caused by hindered vascularization or osteoclast recruitment, but caused by impaired degradation of cartilaginous substrate such as aggrecan (Behonick et al., 2007; Kosaki et al., 2007). MMP9 is another key enzyme for cartilage degradation and vascularization which is expressed by endothelial cells (Romeo et al., 2019), inflammatory cells, osteoclasts and other bone marrow cells (Colnot et al., 2003; Uusitalo et al., 2000; Wang et al., 2013). *Mmp9*-deficient mice exhibit larger soft callus and delayed fracture healing associated with decreases in vascularization and cartilage degradation in long bone fractures (Colnot et al., 2003), indicating that MMP9 mediates the vascular invasion into hypertrophic cartilage callus.

Research has demonstrated that MMP13 works synergistically with MMP9 to degrade cartilaginous matrices such as type II collagen and aggrecan, since the mice lacking both MMP13 and MMP9 exhibit more severe impairment of endochondral bone formation (Stickens et al., 2004). Interestingly, bone marrow transplantation from wildtype mice can rescue the defect in cartilage resorption in *Mmp9*-deficient mice (Vu et al., 1998; Wang et al., 2013) but not in *Mmp13*-deficient mice (Behonick et al., 2007). This finding suggests that MMP9 and MMP13 production relies on distinct sources, and that hypertrophic chondrocyte derived MMP13 is indispensable for the cartilage-to-bone transition in fracture repair. In contrast, *Mmp9*-deficient mice upregulate MMP13 expression in terminal hypertrophic chondrocytes, but this upregulation will not sufficiently rescue the growth plate phenotype (Kojima et al., 2013). In addition, *Mmp10*-deficiency causes a similar phenotype in Mmp9 knockouts (Valdés-Fernández et al., 2021), suggesting that MMP10 participates in vascularization of the growth plate, possibly in callus. Taken together, these findings indicate that MMPs play synergic, but non-compensable, roles in coupling cartilage degradation and vascularization during the cartilage-to-bone transition in fracture healing.

2.2. Vascularization

Vascularization is inevitable throughout the entire fracture healing process. The invasion of vessels into the cartilaginous callus starts in the final step of endochondral ossification, and is mutually coupled with matrix degradation in the soft callus and bone formation during the cartilage-to-bone transition and hard callus remodeling. Hypertrophic chondrocytes in the callus play a role in stimulating vascularization and interact with endothelial cells and osteoprogenitors that reach hypertrophic chondrocytes through neovascularization.

Hypertrophic chondrocytes produce VEGF which induces angiogenesis into the avascular cartilage region. Sequestering VEGF by its antagonist (Flt-(1-3)-IgG) induces shortened limbs with stagnated hypertrophic chondrocytes in the growth plate (Gerber et al., 1999). Similarly, when *Vegf* is ablated in *Col2a1*-expressing chondrocytes, invasion of blood vessels into cartilage is disturbed, and cell death of hypertrophic chondrocytes is increased (Zelzer et al., 2004). Studies using conditional knockout of *Runx2*, a stimulator of VEGF in chondrocytes, have indirectly investigated the specific role of VEGF in hypertrophic chondrocytes. A lack of vascularization in the growth plate is found in *Runx2*-deficient mice associated with the absence of *Vegf* expression (Zelzer et al., 2001). When *Runx2* is deleted in hypertrophic chondrocytes by using *Col10a1-Cre;Runx2*$^{flox/flox}$ *mice*, as expected, *Vegf* expression is absent in hypertrophic chondrocytes and osteoblasts in the primary spongiosa. However, the vascularization of the cartilage is not significantly impaired despite the fact that the transition from hypertrophic chondrocytes to osteoblasts in the primary spongiosa is interrupted, leading to a delayed formation of primary spongiosa. Notably, the expression of *Vegf* in osteoblasts in the bone collar is strongly upregulated (Qin et al., 2020). These results indicate that vascularization in the growth plate is regulated by VEGF from multiple sources, including chondrocytes, hypertrophic chondrocytes, and osteoblasts.

In a murine tibial cortical defect model, suppression of *Vegf* expression in hypertrophic chondrocytes and osteoblastic precursors in *Osx-Cre;Vegfa*$^{flox/flox}$ mice caused delayed vascularization and cartilage turnover (Hu and Olsen, 2016). Buettmann et al. used *Osx-CreERT2; VegfaF*$^{flox/flox}$ mice with full femoral fracture to also demonstrate that VEGF from early osteoblastic cells is necessary for vascularization in the

fracture callus (Buettmann et al., 2019). However, the *Cre*-recombinase driver (*osterix*) used in these studies are not specified to hypertrophic chondrocytes. The importance of vascularization in the cartilage-to-bone transition is paramount during fracture repair, and VEGF produced by hypertrophic chondrocytes likely contributes to the stimulation of vascularization. However, whether the production of VEGF by hypertrophic chondrocytes is dominant in the cartilage-to-bone transition remains unanswered. Future investigations focused on *Col10a1*-drived ablation of *Vegf* should be explored.

Interestingly, endothelial cells have been shown to act on cartilage and chondrocytes. Romeo et al. demonstrated that endothelial cells form Type H capillaries which express PECAM-1 and Endomucin, produce MMP9, and are essential for cartilaginous matrix absorption in the growth plate (Romeo et al., 2019). Endothelial cells also act as a modulator between chondrocytes and bone cells. The endothelial cells require Notch signaling for their proliferation and vessel growth (Ramasamy et al., 2014). Notch positively regulates the expression of Noggin in endothelial cells, and Noggin controls osteoblastic differentiation of perivascular osteoprogenitor cells. The Notch-Noggin axis also supports chondrocyte maturation and hypertrophy, which establishes a positive loop for angiogenesis by increasing the expression of *Vegf* from hypertrophic chondrocytes (Ramasamy et al., 2014).

Furthermore, vascularization is also reported to be related to the fate decision of osteochondroprogenitors through the action of lipid availability. Physical blocking of vessel intrusion by a filter in a bone graft model induces chondrogenesis rather than osteogenesis (van Gastel et al., 2020). This study has demonstrated that the lack of lipids, because of impaired vascularization, causes upregulation of forkhead box O (FOXO) in skeletal progenitors and promotes upregulation of the expression of *Sox9*, a master regulator of chondrogenesis (van Gastel et al., 2020), suggesting that the fate determination of skeletal progenitor cells is under the control of the lipid transported by blood vessels. This scenario may hold true in the vascularized cartilaginous callus, and the environment rich in lipids may inhibit excess formation of the cartilaginous callus during fracture repair.

2.3. Recruitment of osteoclasts

Once vessels start invading the hypertrophic zone in the growth plate, mono or multinuclear cells appear and resorb the mineralized matrices. These cells are generally named osteoclasts. However, the cells can be divided into two types of clast cells according to spatial distribution: chondroclasts attaching to the cartilage surface and osteoclasts attaching to bone surface. Both types express digestive enzymes such as Cathepsin K, MMP9, and tartrate-resistant acid phosphatase (TRAP); however, the cells differ in the morphology of the ruffled border and in the secretion of TRAP (Nordahl et al., 1998). Because osteoclasts and chondroclasts have not been studied distinctly in the fracture repair process, both clast cells are termed ‘osteoclasts’ in this review.

Hypertrophic chondrocytes play a role in the recruitment of osteoclasts. In the murine growth plate, the last two to three layers of hypertrophic chondrocytes express RANKL (receptor activator of nuclear factor-kappa B ligand) which is a strong inducer of osteoclasts from hematopoietic monocytes (Kishimoto et al., 2006). Ablation of RANKL in *Col10a1-Cre* mice impairs cartilage resorption in the primary spongiosa (Xiong et al., 2011), suggesting RANKL produced by hypertrophic chondrocytes is important for cartilage-to-bone transition. Interestingly, hypertrophic chondrocytes also express OPG (osteoprotegerin), a decoy receptor for RANKL, which competes with RANK and suppresses osteoclastogenesis (Silvestrini et al., 2005). *Opg*-deficient mice show accelerated fracture union in the osteochondral junction because of a higher number of osteoclasts and faster resorption of the cartilaginous callus (Ota et al., 2009). Of note, the expression patterns of *Opg* and *Rankl* differ during fracture healing. The dual peaks of *Rankl* expression are observed in an early phase (day 3) and an intermediate phase (day 14) after the transverse fracture in the murine tibia bones, possibly corresponding to initial inflammation and later cartilage resorption/bone remodeling, respectively, while the expression peak of *Opg* is found in the cartilaginous callus day 7 postfracture (Kon et al., 2001). The different expression profiles of RANKL and OPG in the callus may contribute to the fine-tuning of osteoclastogenesis in fracture healing.

Anti-resorption therapies such as anti-RANKL, bisphosphonates, and alendronates are shown to affect cartilage resorption in the callus, both preclinically and clinically (Gerstenfeld et al., 2009; Lin and O’Connor, 2017). A larger callus is observed in anti-resorption treatment groups caused by delayed cartilage resorption and bone remodeling, but these medications do not seem to impair final healing status (Gerstenfeld et al., 2009; Jalan et al., 2021). These observations suggest the role of RANKL and osteoclasts in the resorption of cartilaginous callus, although we need to understand the control significance of cartilaginous callus resorption by osteoclasts in fracture repair.

2.4. Crosstalk between hypertrophic chondrocytes and osteogenic cells

Hypertrophic chondrocytes are located in the border between cartilage and bone, and exhibit crosstalk with osteoprogenitors and osteoblasts during the cartilage-to-bone transition. This crosstalk is mediated by multiple signaling molecules such as Hedgehog, Wnt, and BMP.

Hedgehog signaling is broadly conserved among species and has important roles in skeletal development and metabolism (Iwamoto et al., 1999). In the murine growth plate, Indian Hedgehog (Ihh) is expressed in prehypertrophic chondrocytes and in some hypertrophic chondrocytes, forms a negative feedback loop with Parathyroid hormone-related protein (PTHrP), and regulates chondrocyte proliferation and differentiation in the growing growth plate (Kronenberg, 2003). Ihh has been demonstrated to have an important role in osteoblast differentiation. *Ihh*-deficient mice lack a bone collar flanking the pre and hypertrophic zone (Chung et al., 2001; St-Jacques et al., 1999). Conditional knockout of *Ihh* in chondrocytes results in reduced Wnt signaling in osteoblastic cells and the loss of trabecular bone (Maeda et al., 2007). When Ihh signaling is inhibited by conditional ablation of Smo, an essential co-receptor for Ihh, in perichondral cells, the perichondral cells keep expressing chondrocyte marker genes and fail to form a bone collar. Similarly, *Smo*-deficient cells cannot contribute to bone formation in the primary spongiosa (Long et al., 2004). In contrast, another group has demonstrated that *Ihh*-deficiency is not required for osteoblast differentiation in ex vivo, but that it causes negative impacts on perichondrium and blood vessel formation (Colnot et al., 2005), suggesting the importance of Ihh in bone collar formation. Altogether, Ihh produced by prehypertrophic chondrocytes directly or indirectly regulates the osteoblastic differentiation of the surrounding osteoprogenitors in bone formation.

Similar to the growth plate, Ihh also has an important role in bone formation during fracture healing. Ihh expression is detected during fracture healing. Prehypertrophic chondrocytes start to express Ihh proteins at approximately day 7 postfracture in adult rat femurs, and Ihh protein is detected in hypertrophic chondrocytes 2 weeks postfracture. As new bone formation proceeds, osteoblasts also express Ihh protein (Murakami and Noda, 2000). The expression of *Ihh* largely overlaps with *Col10a1* expression in callus. The cells surrounding *Ihh*-expressing cells exhibit high levels of expression of the hedgehog-responsive genes, *Ptch1* and *Gli1*, suggesting an active status of Ihh signaling in the endochondral fracture repair (Vortkamp et al., 1998). Wang et al. have shown that in woven bone, various types of cells, such as mesenchymal cells, chondroprogenitors, proliferating chondrocytes, endothelial cells, and osteoblasts are responsive to the hedgehog signal using *Ptc1-LacZ* reporter mice and that the ablation of this signal by deleting Smo impairs new bone formation (Wang et al., 2010). Several recent functional studies have further supported the participation of hedgehog signaling in fracture healing. Increasing hedgehog signaling by low-intensity pulsed ultrasound stimulation or systemic administration of Smo

agonist promotes callus formation and angiogenesis during fracture healing (Matsumoto et al., 2018; McKenzie et al., 2019). Administration of a Smo inhibitor only causes delayed callus mineralization in femur fractures, but does not affect chondrogenesis, angiogenesis, and the eventual healing (Liu et al., 2017). In a different stress fracture model, the same treatment impairs osteoblastic differentiation and angiogenesis, and consequently, caused reduced bone formation (Kazmers et al., 2015). Collectively, hedgehog signaling produced by hypertrophic chondrocytes might benefit fracture healing by coupling chondrogenesis and osteogenesis, but further investigation of the precise mechanism with different fracture models is required.

The canonical Wnt/β-catenin pathway is another crucial factor regulating the interactions between hypertrophic chondrocytes and the surrounding cells. The role of Wnt/β-catenin signaling in the regulation of chondrocyte hypertrophy and endochondral bone formation remains controversial. Both gain-of-function and loss-of-function of Wnt/β-catenin signaling in *Col10a1* expressing chondrocytes caused impaired bone formation. The ablation of β-catenin causes an increased *Rankl/Opg* ratio resulting in higher bone resorption, whereas stabilization of β-catenin induces elongation of the hypertrophic zone with an increased number of Osterix^{+} and Runx2^{+} *Col10a1* lineage cells (Houben et al., 2016). Guo et al. have demonstrated that inactivation of Wnt/β-catenin signaling inhibits chondrocyte hypertrophy, independently of PTHrp signaling, whereas Wnt/β-catenin signaling enhances the terminal differentiation of hypertrophic chondrocytes through BMP-2 signaling (Guo et al., 2009). It remains unclear whether Wnts directly or indirectly mediate the interaction of hypertrophic chondrocytes and osteogenic cells.

The read-out of Wnt signaling with T cell factor (TCF)-reporter mice has revealed strong activation in osteoblasts and chondrocytes in the fracture callus. Interestingly, this pathway seems to work distinctly on cartilage and bone. Both genetic gain-of-function and loss-of-function of Wnt/β-catenin signaling induced locally in the cartilage callus impair fracture healing, whereas only the loss-of-function in *Col1a1* expressing osteoblasts suppresses fracture healing. This finding suggests that Wnt/β-catenin signaling enhances osteogenesis of osteoblasts, but needs to be controlled in a sophisticated manner for stimulation of fracture repair (Chen et al., 2007).

BMP signaling is an inevitable pathway for bone formation. BMP2 and BMP7 are known strong osteogenic inducers, and those recombinant proteins have been studied for bone tissue regeneration and fracture healing for clinical use (Krishnakumar et al., 2017). In mouse hypertrophic chondrocytes, expression of BMP agonists (*Bmp2* and *Bmp6*), BMP receptors (*Bmpr1a*, *Bmpr2*, *Acvr1b,* and *Acvr2a*), and an extracellular BMP antagonist (*Nog*) has been observed (Garrison et al., 2017). Phosphorylation of Smad1/5/8 decreases in hypertrophic chondrocytes but expression of Smad 7, an inhibitor of Smad, is upregulated, suggesting that BMP signaling declines in hypertrophic chondrocytes although production of BMP2 and BMP6 is high (Garrison et al., 2017).

Bmp6-deficient mice exhibit a decrease in cortical cross-sectional area, but no change in cancellous bone mass, and they fail to respond to estrogen by accelerating endochondral bone formation (Perry et al., 2008). The compound deficient mice (*Bmp2*$^{+/-}$*;Bmp6*$^{+/-}$), but not the single deficient mice (*Bmp2*$^{+/-}$ or *Bmp6*$^{+/-}$), demonstrate moderate growth retardation with a reduction in trabecular bone (Kugimiya et al., 2005). The bone marrow cells isolated from the *Bmp2*$^{+/-}$*;Bmp6*$^{+/-}$ compound mice do not have reduced osteogenic differentiation ability. These findings suggest that BMPs produced by hypertrophic chondrocytes support osteoblast differentiation and bone formation (Kugimiya et al., 2005).

Endogenous BMP2 expression and function in fracture callus differs temporally and spatially. In the early phase before callus growth, only a few primitive mesenchymal cells express BMP2. In rodent fracture models during the soft callus and woven bone formation phase, the expression of BMP2 increases in chondrocytes and osteoblasts, respectively (Bostrom, 1998; Bostrom et al., 1995). BMPs and most of the components in the BMP/Smad signaling pathway are expressed in hypertrophic chondrocytes at a high level in the fracture site healed by endochondral bone formation (Yu et al., 2010). In the human fracture callus, progenitors, mature chondrocytes, and active osteoblasts strongly express BMP2 (Kloen et al., 2003; Kloen et al., 2002). When human fracture healing proceeds toward malunion or nonunion, the expression of BMP2 in maturing chondrocytes significantly decreases (Kloen et al., 2012; Kwong et al., 2009).

When BMP2 is conditionally ablated in murine limbs (*Bmp2*$^{f/f}$*;Prx1-Cre*), spontaneous fractures are observed and the healing process cannot be initiated, whereas mice lacking Bmp4 do not exhibit defects in skeletogenesis and bone repair (Tsuji et al., 2008; Tsuji et al., 2006). When BMP2 is ablated just before fracture, the initiation of the reparative process by periosteal stem cells is substantially hindered (Wang et al., 2011). These findings indicate necessity of BMP2 in fracture repair. *Col2a1* driven deletion of Ggps1 (a suppressor of BMP2 signal) accelerated fracture healing (Dai et al., 2018). In contrast, BMP2 deletion in osteoblasts or endothelial cells did not affect fracture healing (McBride-Gagyi et al., 2015; Mi et al., 2013). These findings suggest that BMP signaling produced by chondrocytes supports bone formation in fracture repair. Future studies with conditional deletion of BMP2 and BMP6, specifically in hypertrophic chondrocytes, should be performed to dissect the role of BMP signaling in cartilaginous callus formation, stimulation of hypertrophy of chondrocytes and in the cartilage-to-bone transition.

3. Hypertrophic chondrocytes may directly become osteoblasts

It has long been thought that terminally differentiated hypertrophic chondrocytes in endochondral bone formation undergo apoptosis when cartilage is replaced by bone. However, accumulating evidence shows that a substantial number of hypertrophic chondrocytes can survive in the callus and become osteoblasts (Bahney et al., 2014; Scammell and Roach, 1996; Wong et al., 2021; Yang et al., 2014; Zhou et al., 2014). Bahney et al. (2014) demonstrated presence of host chondrocyte-derived bone cells in a bone grafting model. Yang et al. have traced the fate of hypertrophic chondrocytes using *Col10a1-Cre* or inducible *Col10a1-CreERT* mice with Cre reporter mice and demonstrated that hypertrophic chondrocyte-derived cells become *Col1a1* expressing osteoblasts and sclerostin-expressing osteocytes (Yang et al., 2014). Zhou et al. have also used *Col10a1-Cre* mice or *Aggrecan-CreERT2* mice, and demonstrated that chondrocytes from the growth plate become osteoblasts in the trabecular and cortical bones. The authors have also tracked the fate of chondrocytes in the facture callus using *Aggrecan-CreERT2; Rosa26-tdTomato;Col1a12.3 kb-GFP* mice. Induction of Cre recombination in aggrecan-expressing chondrocytes by tamoxifen at day 7 post-fracture demonstrates that their progenies have become Col1a1-expressing osteoblasts (Zhou et al., 2014). This transition of hypertrophic chondrocytes to osteoblasts is also observed in mandibular fractures in mice (Wong et al., 2021).

To track the osteogenic fate of hypertrophic chondrocytes, we also developed a mouse strain similar to that previously reported by Zhou et al. (Zhou et al., 2014) using *Col10a1-Cre* instead of *Aggrecan-CreERT2* system. When Cre recombinase is combined with the complete promoter of *Col10a1*, high efficiency and specificity of gene targeting in hypertrophic chondrocytes is achieved in the murine growth plate (Gebhard et al., 2007, 2008). This *Col10a1-Cre;Rosa26-tdTomato;Col1a12.3 kb-GFP* strain expresses tdTomato (red fluorescent protein) in hypertrophic chondrocytes and their descendent cells, and GFP in mature osteoblasts/osteocytes. When hypertrophic chondrocyte-derived cells become osteoblasts, they become yellow by expressing both tdTomato and GFP. Consistent with previous reports, in the developing bones of this strain with particularly high growth plate activity, a substantial number of osteoblasts in the trabecular area was yellow due to being double-positive for red and green fluorescence signals, indicating that the osteoblasts are derived from hypertrophic chondrocytes in the growth

plate (Scheiber et al., 2022; Yang et al., 2014; Zhou et al., 2014). When a tibial fracture was created in this model, the bony callus contained green osteoblasts beneath the thickened periosteum of the healing bone at day 14 postfracture (Fig. 1a, asterisks). These green osteoblasts were not derived from hypertrophic chondrocytes, indicating that the bony callus was formed by intramembranous ossification. In the center of the fracture site, the cartilaginous callus was visualized by Safranin O staining (Fig. 1b). The cartilaginous callus consisted of only red hypertrophic chondrocytes (and/or their descendants) (Fig. 1b, arrows) while few yellow (*Col10a1* lineage osteoblasts) and green osteoblasts were present in the adjacent bony callus (Fig. 1b, arrowheads). In the woven bone forming region (the right side of the fractured bone in Fig. 1a), a significant portion of osteoblasts are *Col10a1* lineage (Fig. 1c). These results suggest that hypertrophic chondrocytes are involved in woven bone formation in fracture healing and the cartilage-to-bone transition is arranged by directly differentiating into osteoblasts. However, this lineage tracing using the *Cre/loxP* system cannot exclude the possibility that this population of osteoprogenitors in the fracture site may be originated from *Col10a1* expressed cells in early development.

It remains unknown how hypertrophic chondrocytes become osteoblasts. Hu et al. (2017) have demonstrated that BrdU incorporation and Ki67 expression are observed in the *Col10a1* expressing hypertrophic chondrocytes close to vessels in the transition zone between soft and hard callus, suggesting some of these hypertrophic chondrocytes re-enter the cell cycle. In addition, these cells express stem cell characteristic molecules, Oct4 (Pou5f1), Sox2, and Nanog. Thus, the authors propose that hypertrophic chondrocytes adjacent to the vasculature in the transition zone de-differentiate into osteochondroprogenitor cells, gain multipotency, and subsequently give rise to osteoblasts and osteocytes in the hard callus (Bahney et al., 2014; Hu et al., 2017). In addition, Park et al. have identified small proliferating YFP positive cells that co-express Osx in the lowest layer of the hypertrophic zone in the growth plate of *Col10a1-Cre;YFP* mice and proposed that these small cells are the transitory cells from hypertrophic chondrocytes to osteoblasts (Park et al., 2015). A recent study showed that the progenies of *Col10a1*-expressing cells in murine bone marrow included a stem cell subpopulation which possesses osteogenic and adipogenic potency, by using transcriptomic assay and kidney capsule transplantation model in *Col10a1-Cre* and *Col10a1-CreERT* lines (Long et al., 2022). These findings suggest the possibility that hypertrophic chondrocytes serve as a reservoir of progenitors which give rise to osteoblasts and osteocytes, at least partially, during fracture healing. However, this concept still needs to be verified with more direct observations. In parallel, it is necessary to test the possibility of a subpopulation of undifferentiated progenitor cells present in the hypertrophic zone during the cartilage-to-bone transition and paving the osteogenic lineage.

Molecules that control osteoblastic differentiation of hypertrophic chondrocytes have been actively investigated, but have not yet been fully elucidated. Wnt/β-catenin signaling in hypertrophic chondrocytes appears to be essential for osteogenic commitment (Golovchenko et al., 2013; Hill et al., 2005; Houben et al., 2016; Wong et al., 2020). Thyroid hormone activation of hedgehog signaling has also been shown to be indispensable for the chondrocyte-to-osteoblast transformation in the development of the murine secondary ossification center (Aghajanian et al., 2017; Xing et al., 2014); Runx1 and Runx2 are two potential regulators since their ablation in chondrocytes caused impaired transition (Qin et al., 2020; Tang et al., 2020). Detailed discussion on this topic can be found in other comprehensive reviews (Aghajanian and Mohan, 2018; Hinton et al., 2017; Wolff and Hartmann, 2019).

4. Conclusion

This review covered the endochondral ossification occurring in the callus during fracture healing, and focused on the process of hard bone callus replacing soft cartilaginous callus. We introduce multiple potential roles of hypertrophic chondrocytes in crosstalk with endothelial cells in blood vessels, osteoclasts, and osteoblasts (Fig. 2). The interaction of hypertrophic chondrocytes with endothelial cells, osteoclasts, and osteoblasts involving fracture repair is summarized as well.

Bone fracture healing is one of the most investigated fields; however, delayed union and nonunion are still significant issues. Future work should consider mutual and bidirectional interactions among these cells via local and systemic soluble factors, extracellular matrix proteins, and cell-cell contact. Interactions between these cells need to be well-

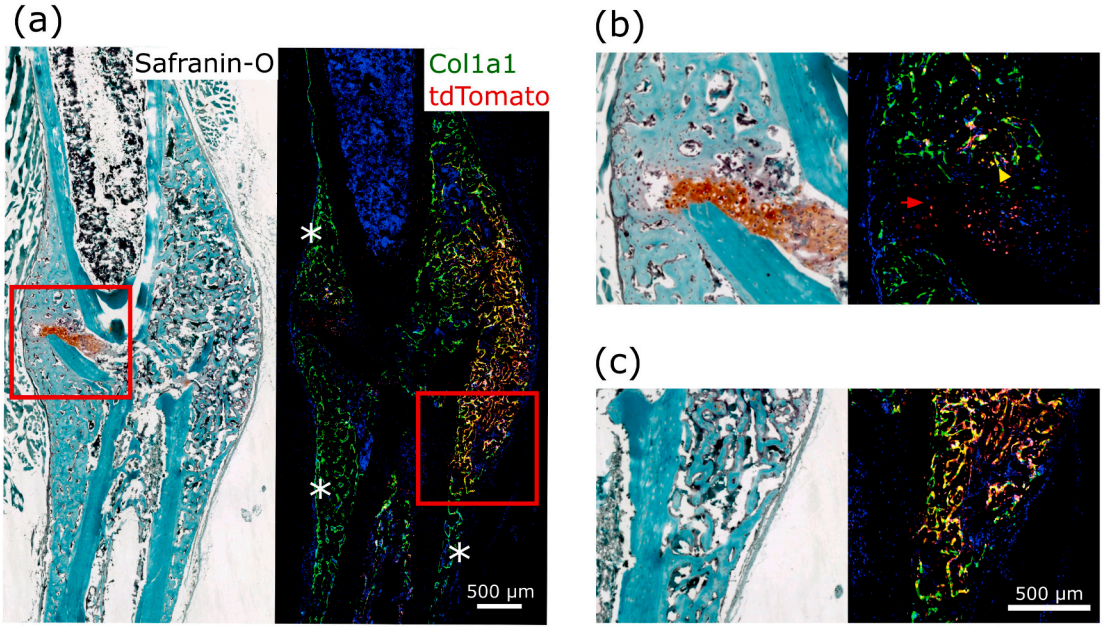

Fig. 1. Direct transition of hypertrophic chondrocytes into osteoblasts in fracture callus; 14 days after tibial fracture stabilization with intramedullary pin in *Col10a1-Cre (BAC);Rosa26-tdTomato;Col1a12.3 kb-GFP* mice. (a) Safranin-O staining and fluorescent photo of two serial sections. Asterisks indicate the GFP positive osteoblasts not derived from Col10a1-expressing hypertrophic chondrocytes. (b,c) Magnified images of the corresponding areas in (1a). The red arrow indicates a *Col10a1-Cre* labeled tdTomato positive cell. The yellow arrowhead indicates an osteoblast labeled with both GFP and tdTomato (Col10a1-derived osteoblast).

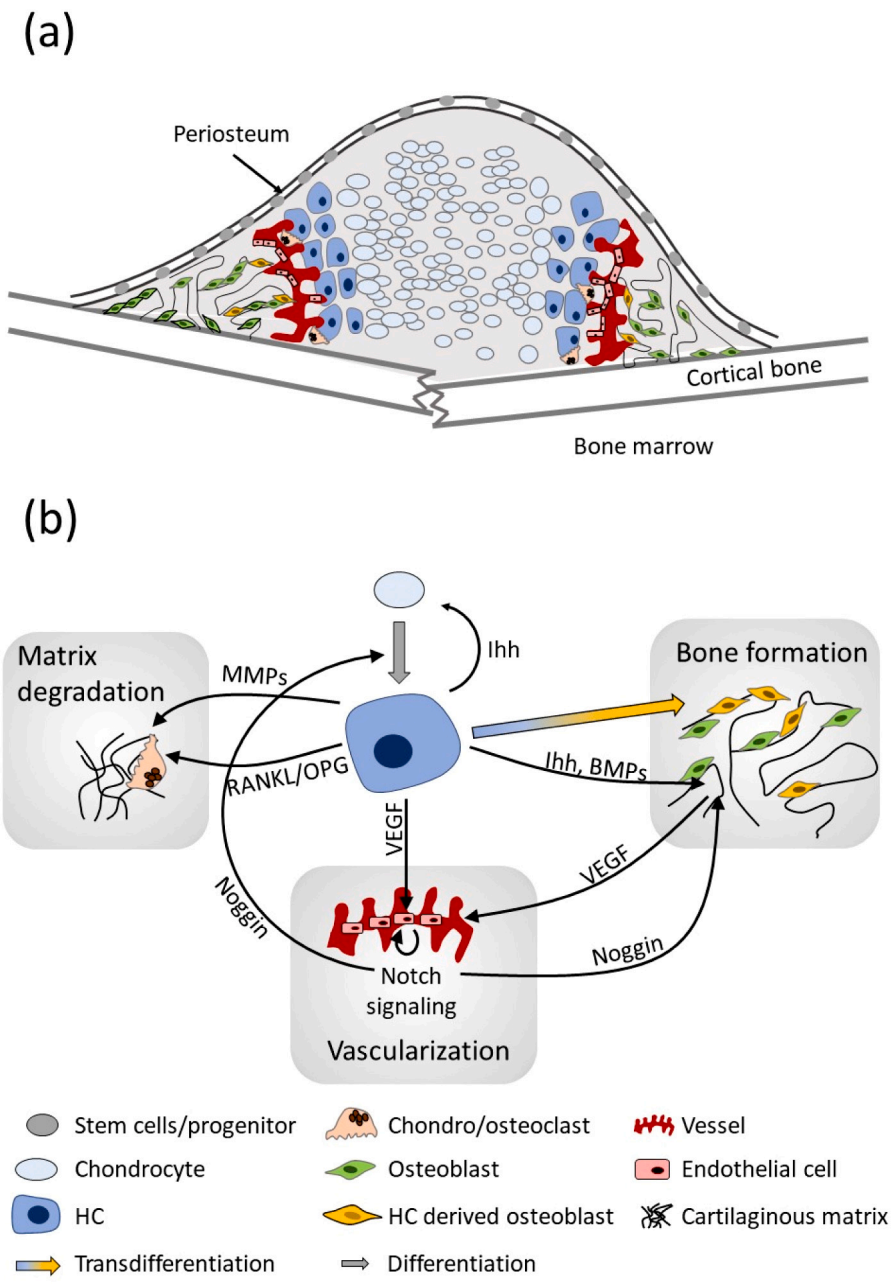

Fig. 2. Schematic diagram demonstrating crosstalk of the cells in the fracture callus. (a) The spatial relationship of tissue and cell components in the fracture callus. (b) The interactions among the cells involved in the process of cartilage-to-bone transition. Hypertrophic chondrocytes lie between the cartilage and bone components, secreting several factors which promote the cartilage-to-bone transition, including MMPs, Ihh, BMPs, VEGF, RANKL/OPG. Hypertrophic chondrocytes may also directly become bone cells, contributing to bone formation. Vascularization is another watershed for the cartilage-to-bone transition. Lipids brought by vessels are important for controlling the fate of osteochondral progenitors to an osteogenic lineage. Notch signaling in endothelial cells support their homeostasis and stimulate secretion of Noggin for the coupling of chondrocyte hypertrophy and osteoblast differentiation.

orchestrated temporally and spatially with the understanding that any disruptions of the interactions or malfunction of these cells end with delayed union or nonunion of fractured bones.

The cellular and molecular mechanism regulating cartilage-to-bone transition may be understudied at present. Further investigations would provide new therapeutic targets for prevention or amelioration of impairment of fracture healing including the stimulation of fracture repair).

CRediT authorship contribution statement

Joe Kodama: Conceptualization, Writing – original draft. **Kevin J. Wilkinson:** Conceptualization, Writing – review & editing. **Masahiro Iwamoto:** Conceptualization, Writing – review & editing. **Satoru Otsuru**: Conceptualization, Writing – review & editing. **Motomi Enomoto-Iwamoto**: Conceptualization, Writing – review & editing.

Declaration of competing interest

Joe Kodama: No conflict of interest to report.

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