

Review

EMERGING ROLE OF HYPERTROPHIC CHONDROCYTES IN TISSUE REGENERATION AND FRACTURE HEALING: A NARRATIVE REVIEW

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Abstract

Fracture healing is a complex event that involves the coordination of various different processes, including intramembranous and endochondral bone formation. When facing fracture nonunion or delayed union, few organizational engineering structures can achieve the desired results. The main reason for this is that they cannot recapitulate the cellular morphology, biology, and mechanical functions of natural tissues. Ten years ago, the term Development Engineering was coined to refer to the use of developmental processes as a blueprint for designing and developing engineered live implants. Different sources of cells have been used as seed cells in developmental engineering. Among them, hypertrophic chondrocytes have attracted worldwide attention. Hypertrophic chondrocytes are the terminal state of growth plate chondrocytes, leading to degenerative maturation. Hypertrophic chondrocytes mediate crosstalk by regulating cell-matrix degradation, vascularization, osteoclast recruitment, and osteoblast differentiation. Furthermore, hypertrophic chondrocytes can transdifferentiate into osteoprogenitors and mature osteoblasts, and directly promote woven bone formation. In summary, elucidating the role of hypertrophic chondrocytes will contribute to understand of the physiological mechanism of fracture healing, research and development of novel therapeutic modes of developmental engineering, and further promotion of fracture healing.

Keywords: Hypertrophic chondrocytes, fracture healing, tissue regeneration, endochondral bone formation, developmental engineering, transdifferentiation.

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Introduction

Fractures are the most common types of large organ trauma in humans (Einhorn and Gerstenfeld, 2015). Fractures heal through two different processes: primary (direct) or secondary (indirect) healing. In primary healing, mesenchymal cells directly differentiate into osteoblasts and form strong bone callus in the area near the fracture and under the periosteum (intramembranous ossification). In secondary healing, chondrocytes form cartilage callus in the middle of the fracture and connect the two ends of the fracture (endochondral ossification) (Salhotra *et al.*, 2020). The process of endochondral bone formation is similar to long bone development. Mesenchymal precursor cells aggregate, differentiate into chondrocytes and sequentially undergo proliferation, maturation, hypertrophy, and terminal differentiation in sequence (Hu *et al.*, 2017; Kronenberg,

2003; Tsang *et al.*, 2015). The transition from cartilage to bone is essential for fracture stability and rigidity in fractures. A delay or failure of this process results in impaired bone healing, such as nonunion or delayed union (Cheng *et al.*, 2020; Julien *et al.*, 2020; Wang *et al.*, 2015).

Multiple processes are involved in cartilage-to-bone transition, such as cartilage matrix degradation, vascular invasion, and bone formation. Several cell populations, including chondrocytes, bone cells, osteoclasts, and endothelial cells are involved in these events. Hypertrophic chondrocytes located at the border between the soft and hard callus play an intermediary role. Degradation of the avascular cartilage matrix is initiated by hypertrophic chondrocytes, recruitment of other types of cells and vascular migration. Hypertrophic chondrocytes also express vascular endothelial growth factor (VEGF), which induces vascularization

and accelerates the degradation of the cartilage matrix. In addition, hypertrophic chondrocytes promote osteogenesis by producing growth factors, such as bone morphogenesis protein 2 (BMP-2) (Kodama *et al.*, 2022; Long *et al.*, 2022). Hypertrophic chondrocytes can also differentiate into osteoprogenitors that directly promote bone formation. Thus, hypertrophic chondrocytes play an important role in endochondral bone formation and are critical in all stages of fracture healing.

The classic tissue engineering paradigm seeks to develop live implants for the repair, maintenance, or replacement of damaged or missing tissues and organs. Live implants are formed by binding cells, scaffolds, or chemical stimuli. After nearly 40 years, many clinical trials involving tissue engineered products are ongoing. However, in terms of commercial products, the actual results to date have been limited. More than a decade ago, a paradigm shift from classical tissue engineering to developmental engineering was proposed, using the developmental process as a blueprint for tissue and organ regeneration (Lenas *et al.*, 2009). The biomimetic strategies can not only restate the successful formation of tissues and organs of interest, but also achieve this by restating the required process-related features, including regulation, autonomy, and robustness. Since the term ‘developmental engineering’ was coined more than a decade ago, research on this strategy has flourished in all organ systems.

Bone Development

Skeletogenesis begins at the fetal stage. Bones are derived from three different embryonic structures: (1) somite, which generates axial bones, (2) lateral plate, which generates limb bones, and (3) nerve crest, which generates craniofacial bones (Teti, 2011). Skeletogenesis begins when mesenchymal cells from these embryonic lineages migrate to sites of future bones (Helms and Schneider, 2003). They then form condensations of high cellular density. In condensations, mesenchymal cells differentiate into chondrocytes, forming an avascular cartilaginous template (endochondral ossification), or osteoblasts, directly forming bone (intramembranous ossification).

Intramembranous Ossification

Intramembranous ossification is the primitive form of ossification. The flat bones are formed by intramembranous ossification, involving the clavicles, mid-shaft of the long bones, frontal and parietal of the skull bones, mandible, maxilla and parts of the temporal and occipital bones (Teti, 2011). The mesenchyme derived from the cranial neural crest condenses into compact nodules that contribute to the ossification of the skull. Unlike the process of endochondral ossification, bone is formed without a cartilaginous intermediate. While some mesenchymal stem cells lead to capillaries, others generate osteoprogenitors, which become the osteoblast lineage. Osteoid components are then

secreted into the mesenchyme and evolve into mineralized osteoblasts in the lacunar spaces, where they evolve toward an osteocytic phenotype. For the flat bones of the skull, the external and internal tables are formed by the initial spicules, and the diploe is formed by the inner spicules. In the mid-shaft of the long bones, the cortical bones are formed by the apposition of a new matrix by osteoblasts (Teti, 2011).

Endochondral Ossification

Endochondral bone formation is the basic process of hard tissue biology. This is the process by which many bones are formed and is also the primary way to increase the length of long bones. The replacement of cartilage with mineralized bone during endochondral ossification is a complex process triggered by the differentiation of proliferating chondrocytes into a non-proliferative hypertrophic state in the center of cartilage anlagen. Osteoblast progenitors, osteoclasts, vascular endothelial cells, and hematopoietic cells then invade hypertrophic cartilage from the perichondrium. Hypertrophic cartilage is absorbed, the penetrating osteoblast progenitors differentiate into osteoblasts, and hematopoietic and endothelial cells establish the bone marrow where they become the primary ossification center (Maes *et al.*, 2010; Rolian, 2020). Osteoblast progenitors in the perichondrium differentiate into osteoblasts, which deposit the cortical bone around the cartilage anlage (Berendsen and Olsen, 2015; Pitsillides and Beier, 2011; Zhang *et al.*, 2023). With age, growth plates become thinner and are eventually replaced with bone at various times after puberty. The growth plates consist proliferative (specifically expressing *Sox9* and *Col2a1*), prehypertrophic (specifically expressing *Sox9*, *Foxa2/3*, *Mef2c* and *Ihh*), and hypertrophic (specifically expressing *Mmp13*, *Spp1* and *Runx2/3*) chondrocytes. The process of endochondral bone formation is usually triggered by programmed cell death of hypertrophic chondrocytes, and matrix metalloproteinase-13 (MMP13) and MMP9 then initiate absorption of the transverse mineralized matrix, along with the invasion of angiogenic cells and osteoblasts from the underlying bone marrow (Mackie *et al.*, 2011). In this process, chondrogenesis serves mainly as a means of producing hypertrophic chondrocytes, which in turn initiate subchondral bone formation by other cells. The transition of hypertrophic chondrocytes to osteoblasts also plays a major role in endochondral bone formation.

Developmental Engineering and Hypertrophic Chondrocytes

Bone fracture healing is a postnatal regenerative process that recapitulates the ontological events of embryonic skeletal development. Fracture healing is one of the few postnatal processes that are truly regenerative, restoring the fractured bones to their pre-injury cellular composition, structure, and biomechanical function. However, previous

Table 1. The advantages and disadvantages of different cell sources for developmental engineering.

Cell sources	Advantages	Disadvantages
Bone marrow-derived mesenchymal stem cells	High potential to differentiate into hypertrophic chondrocytes most frequently used	Donor variability, invasive harvesting protocols, difficulty expanding cells <i>in vitro</i>
Adipose-derived stem cells	Multilineage potential, easily accessible, minimally invasive procedure	Complex processing protocol, long-term <i>in vitro</i> expansion procedures
Embryonic stem cells	Potential to differentiate into many cell types	Ethical objections, immune rejection, teratoma formation
Hypertrophic chondrocytes	The constitution of cartilage, the essential process of endochondral ossification, stimulation of both osteogenesis and vasculogenesis	Phenotype stability, <i>in vitro</i> expansion

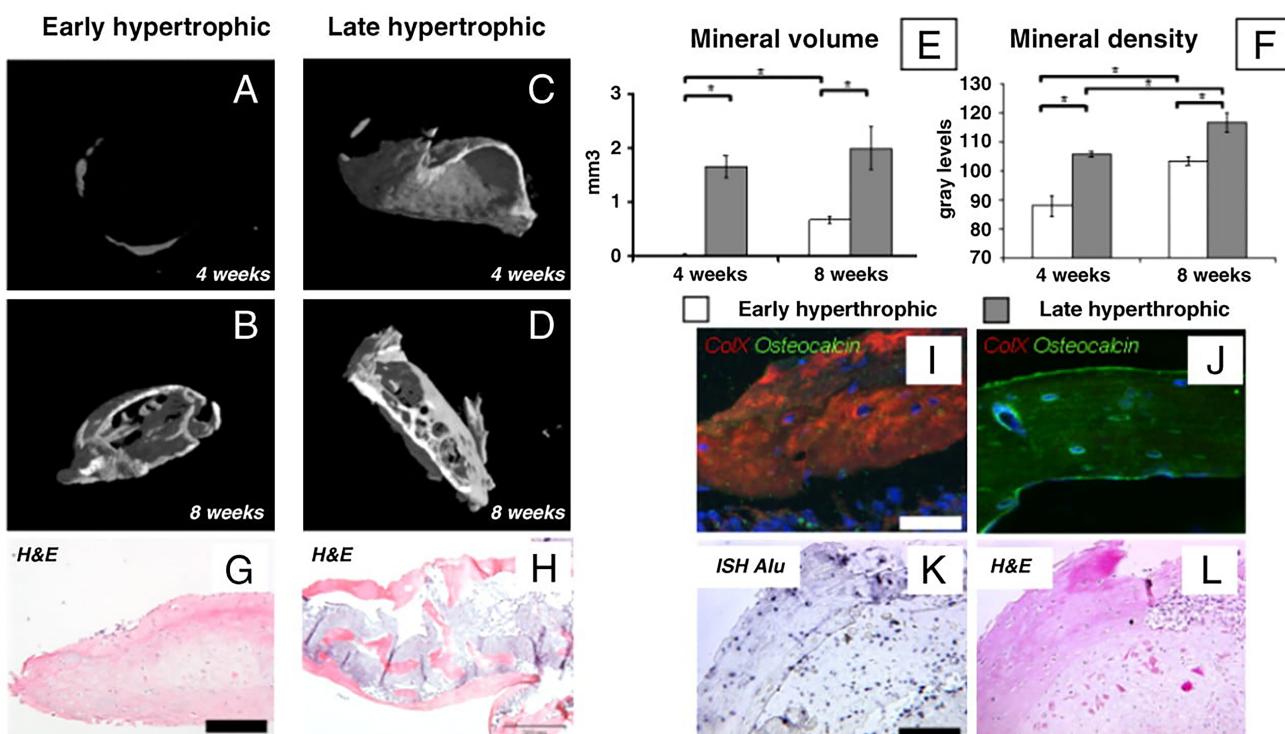


Fig. 1. The successful implantation of hypertrophic cartilage tissues *in vivo*. (A-F) Three-dimensional CT reconstructions and quantitative data indicate higher bone quantity of late hypertrophic samples. (G-L) Trabecular-like structures and positive staining of OCN and COL X can be found in late hypertrophic samples. (* indicates significant differences; $p < 0.01$.) [Panels A-L are from (Scotti *et al.*, 2010), reprinted with permission. Copyright © PNAS].

studies have mostly focused on generating bone substitutes by emulating the physical and biochemical properties of bone. This process, which is based on intramembranous ossification is much simpler than endochondral ossification. However, this often results poor vascularization and limited bone regeneration. In recent years, a new tissue engineering method, called developmental engineering, has been developed based on endochondral bone formation. Unlike traditional regenerative engineering which seeks to mimic the functional and mechanical properties of bone tissue, developmental engineering emphasizes the simulation of *in vivo* developmental processes and takes advantage of the natural mechanisms regulating cell differentiation and osteogenesis. Briefly, mesenchymal stem cells (MSC) are induced to differentiate into chondrocytes and further into hyper-

trophic chondrocytes to initiate the process of endochondral ossification. This strategy overcomes the disadvantages of traditional regenerative engineering techniques.

The main advantage of tissue engineering based on endochondral ossification is the possibility of regenerating a fully-functional bone organ with a mature vascularized mineralized matrix. Successful implantation of different types of stem cells can recreate the functional hematopoietic niches *in vivo*. Scotti *et al.* (2013) demonstrates that human bone marrow-derived mesenchymal stem/stromal cells pushed through endochondral ossification can engineer an ossicle with bone organs features, including physiologically remodeled bone, mature vasculature, and a fully-functional hematopoietic compartment (Scotti *et al.*, 2013). The ideal cell source for developmental engineering should possess

the capacity to differentiate into hypertrophic chondrocytes and synthesize a hypertrophic cartilage-specific extracellular matrix. Bone marrow-derived mesenchymal stem cells (BMSC) (Bei *et al.*, 2021; Freeman *et al.*, 2015; Huang *et al.*, 2018; Li *et al.*, 2016; Longoni *et al.*, 2020), adipose-derived stem cells (ASCs) (Huang *et al.*, 2020; Li *et al.*, 2018; Mazini *et al.*, 2019; Zhou *et al.*, 2019), periosteum-derived cells (PDCs) (Debnath *et al.*, 2018; Nilsson Hall *et al.*, 2019), embryonic stem cells (ESCs) (Verbeeck *et al.*, 2019; Wang *et al.*, 2016) and hypertrophic chondrocytes (Bahney *et al.*, 2014; Bardsley *et al.*, 2017) have been applied to developmental engineering (Table 1) (Fig. 1).

Chondrocyte hypertrophy is a process involving a rapid increase in chondrocyte volume and significant metabolic and molecular changes, such that the cell volume undergoes a 10 to 20-fold increase. This process promotes endochondral ossification and plays a significant role in longitudinal bone growth in different mammalian species (Chawla *et al.*, 2022; Hallett *et al.*, 2021). Hypertrophic chondrocytes characteristically express MMP-13, MMP-10, collagen X (COL X), and VEGF (Chen *et al.*, 2023a; Jahanmir *et al.*, 2020; Kamakura *et al.*, 2023). COL X is a non-fibril protein specifically secreted by hypertrophic chondrocytes and is associated with the calcification process of cartilage in the growth plate (Kamakura *et al.*, 2023; Knuth *et al.*, 2019). Hypertrophic chondrocytes synthesize VEGF (Gerber *et al.*, 1999), PDGF (Andrew *et al.*, 1995) and placental growth factor (PIGF) (Maes *et al.*, 2006) and facilitate the second phase of vascular invasion. Hypertrophic chondrocytes then lose the expression of sex-determining region Y-box 9 (Sox9) transcription factor and relieve the repression of runt-related transcription factor 2 (Runx2) and β -catenin. Hypertrophic chondrocytes then express bone-specific genes (Gerstenfeld and Shapiro, 1996). Furthermore, hypertrophic chondrocytes are the main source of receptor activator of nuclear factor-kappa B ligand (RANKL). During endochondral ossification, RANKL is required to induce osteoclastogenesis and formation of the bone marrow space, as well as to maintain a balance between bone resorption and formation (Xiong *et al.*, 2011). RANKL-mediated multinucleated “chondroclasts” are most abundant in the cartilaginous matrix of the hypertrophic zone (Ferrao Blanco *et al.*, 2021; Odgren *et al.*, 2016). The activation of nuclear factor-kappa B (NF- κ B) can also impede the transdifferentiation of hypertrophic chondrocytes and lead to an extended hypertrophic zone in growth plates of mice.

Previous studies have suggested that hypertrophic chondrocytes are the terminal state of growth plate chondrocytes, leading to degenerative maturation, which is characterized by cell cycle exit, nuclear consolidation, and apoptosis (Tsang *et al.*, 2015). However, recent studies have shown that hypertrophic chondrocytes can undergo “transdifferentiation” and become osteoblasts directly at the primary spongiosa (Park *et al.*, 2015; Zhou *et al.*, 2014).

Therefore, the “transient” state more accurately describes hypertrophic chondrocytes than the “terminal” state. This is the ability to respond to external stimuli and be reprogrammed into an osteoblast-like state.

The Role of Hypertrophic Chondrocytes in Tissue Regeneration and Fracture Healing

Hypertrophic Chondrocytes Mediate the Crosstalk between Cartilage and Bone

Hypertrophic chondrocytes play various roles in the transition of cartilage to bone. The first step is the rapid degradation and resorption of cartilaginous tissue. Two matrix metalloproteinases, MMP-13 and MMP-9, play critical roles in cartilage degradation and vascularization. In murine fracture models, the number and size of chondrocytes in the callus reaches a maximum from days 9 to 14 after the fracture. MMP-13 is expressed when chondrocytes mature into hypertrophic chondrocytes (D’Angelo *et al.*, 2000). MMP-9 is a key enzyme involved in cartilage degradation and vascularization and is expressed by endothelial cells, inflammatory cells, osteoclasts, and other bone marrow cells (Romeo *et al.*, 2019; Wang *et al.*, 2013). *Mmp13*-deficient mice exhibit an expanded hypertrophic zone in the growth plate and an absence of collagenase-mediated collagen cleavage and delayed vascularization, indicating that during embryonic and postnatal development, the expression of MMP13 by hypertrophic chondrocytes in the growth plate is necessary for cartilage resorption (Stickens *et al.*, 2004). In the fracture callus, the deficiency of MMP13 causes delayed cartilage resorption and increases cartilage volume (Behonick *et al.*, 2007; Kosaki *et al.*, 2007). Studies have shown that MMP13 and MMP9 play a synergistic role in degrading cartilaginous matrices, such as type II collagen and aggrecan, as mice lacking both MMP13 and MMP9 exhibit more severe endochondral bone formation impairment (Stickens *et al.*, 2004). However, bone marrow transplantation from wildtype mice repairs the cartilage resorption defects in *Mmp9*-deficient mice, but there is no obvious effect on *Mmp13*-deficient mice (Behonick *et al.*, 2007). This result shows that the MMP9 and MMP13 originate from different sources and that MMP13 derived from hypertrophic chondrocytes is essential for the transition of cartilage to bone during fracture healing. Bai *et al.* (2020) identifies a lncRNA, hypertrophic chondrocyte angiogenesis-related lncRNA (HCAR). HCAR upregulates the expression of MMP13 and VEGF, which promotes the endochondral bone repair (Bai *et al.*, 2020). In contrast, the expression of MMP13 in *MMP9*-deficient mice is upregulated in hypertrophic chondrocytes, which cannot sufficiently rescue the defects in growth plate phenotype (Kojima *et al.*, 2013). In summary, during the transition from cartilage to bone in fracture healing, MMPs secreted by hypertrophic chondrocytes play a collaborative but irreplaceable role in the coupling of cartilage degradation and vascularization (Fig. 2).

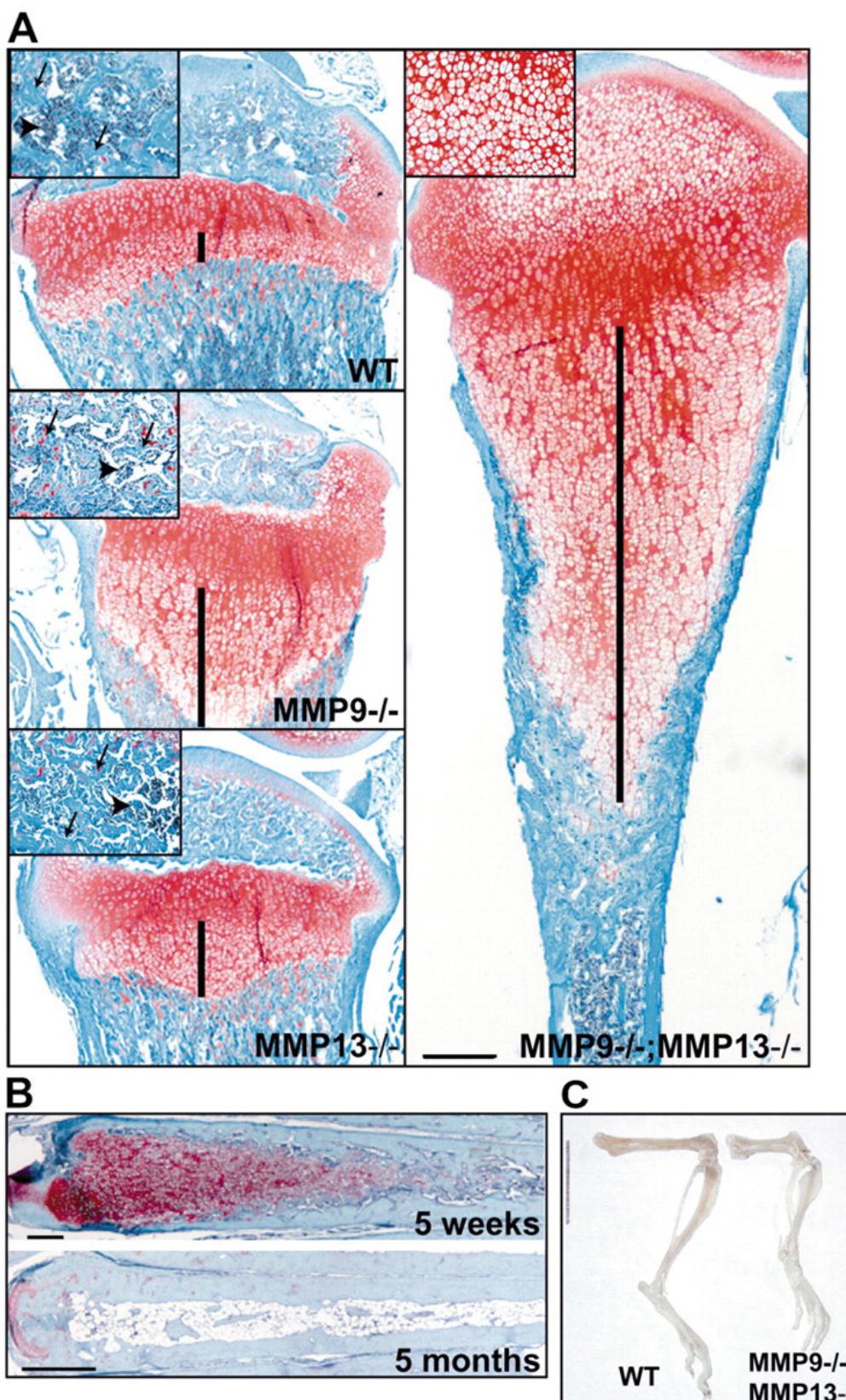


Fig. 2. Examination of $MMP9^{-/-}$, $MMP13^{-/-}$ and $MMP9^{-/-}; MMP13^{-/-}$ endochondral bones. (A) Safranin-O staining shows a dramatically expanded hypertrophic chondrocyte zone in $MMP9^{-/-}; MMP13^{-/-}$ tibia. (B) Safranin-O staining shows a closed growth plate in 5-month-old $MMP9^{-/-}; MMP13^{-/-}$ metatarsals. (C) 12-month-old $MMP9^{-/-}; MMP13^{-/-}$ mice shows a shortening of hind limbs. [Panels A-C are from (Stickens *et al.*, 2004), reprinted with permission. Copyright © Development].

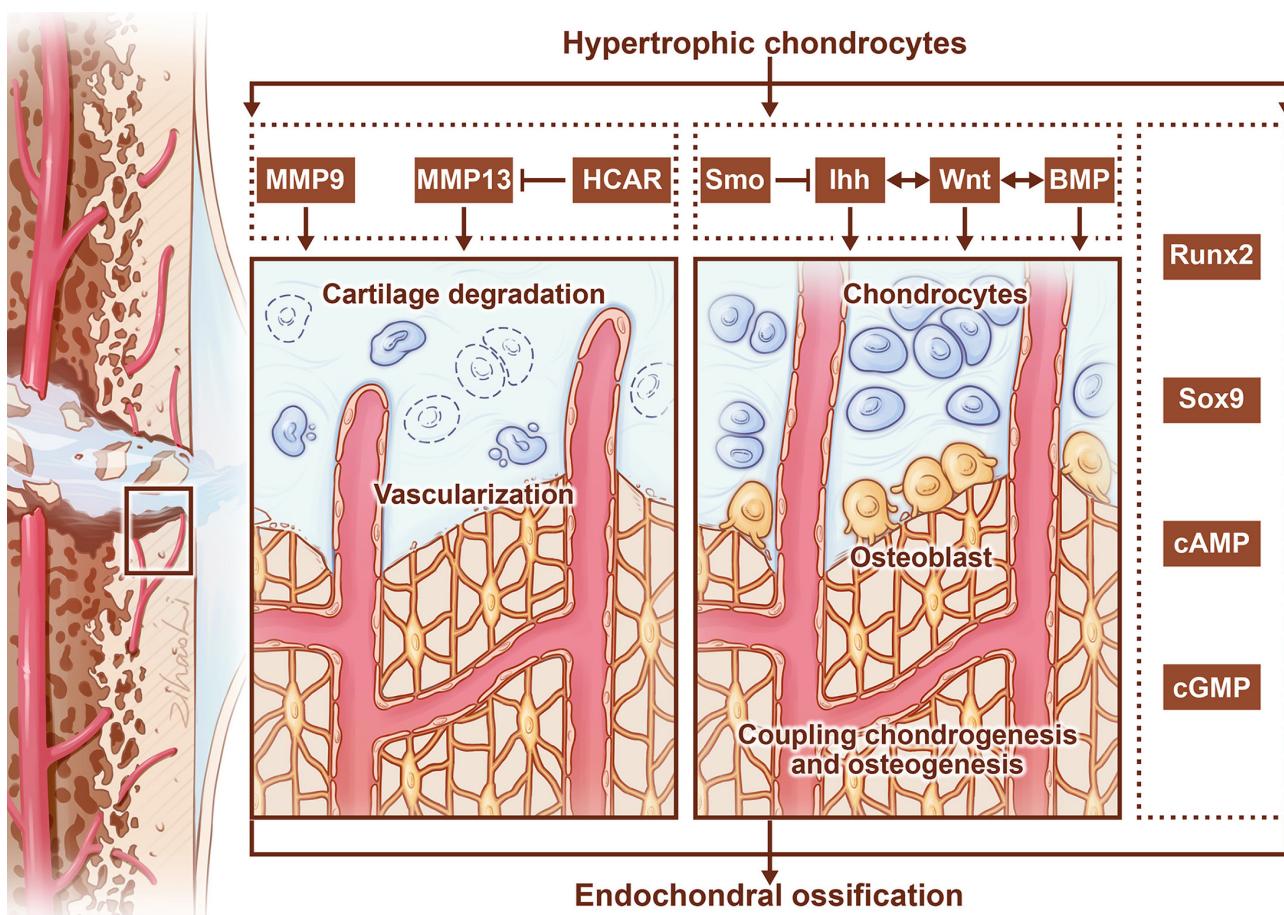


Fig. 3. The main pathways of hypertrophic chondrocytes mediating the crosstalk between cartilage and bone.

Hypertrophic chondrocytes are located at the junction of cartilage and bone and are associated with osteoprogenitors and osteoblasts during the transition from cartilage to bone. This crosstalk is mediated by multiple signaling molecules, such as Wnt, BMP, and Hedgehog (Aghajanian and Mohan, 2018; Chen *et al.*, 2023b; Du *et al.*, 2023; Garcia de Vinuesa *et al.*, 2021; Li and Dong, 2016; Lui *et al.*, 2019; Singh *et al.*, 2021). The Hedgehog signaling pathway is pivotal in skeletal development and metabolism (Iwamoto *et al.*, 1999). Indian Hedgehog (Ihh) is expressed in pre-hypertrophic and hypertrophic chondrocytes located in the murine growth plate. Ihh plays an important role in regulating the proliferation and differentiation of chondrocytes as well as osteogenic differentiation (Kronenberg, 2003). *Ihh*-deficient mice lack the bone collar located in pre and hypertrophic zones (Chung *et al.*, 2001). Conditional knockout of *Ihh* in chondrocytes causes a decrease in Wnt signaling in osteoblasts and the loss of bone trabeculae (Maeda *et al.*, 2007). However, another study shows that *Ihh*-deficiency is not necessary for osteoblast differentiation *in vitro*, but adversely affects perichondrium and blood vessel formation, suggesting that the Ihh pathway plays a dominant role in bone collar formation (Colnot *et al.*, 2005). In summary, Ihh produced by hypertrophic chondrocytes

directly or indirectly regulates the osteogenic differentiation of the surrounding osteoprogenitor cells during bone formation.

Similar to growth plates, Ihh regulates bone formation during fracture healing. The expression of Ihh is measured during fracture healing. Approximately seven days after femur fracture in adult rats, pre-hypertrophic chondrocytes initiate Ihh protein expression. Two weeks post-fracture, Ihh protein can be detected in hypertrophic chondrocytes. As new bone formation progresses, osteoblasts express Ihh protein (Murakami and Noda, 2000). In callus, the expression of *Ihh* greatly overlaps with that of *Col10A1*. The cells surrounding *Ihh*-expressing cells express high levels of the hedgehog response genes patched homologue 1 (*Ptch1*) and glioma-associated oncogene homologue 1 (*Gli1*), suggesting that Ihh signaling is active during endochondral fracture repair (Vortkamp *et al.*, 1998). Inhibition of this signaling impairs new bone formation by deleting smoothened homologue (*Smo*) (Wang *et al.*, 2010). Low-intensity pulsed ultrasound stimulation or the systemic application of *Smo* agonists can increase the hedgehog signaling and promote callus formation and angiogenesis during fracture healing (Matsumoto *et al.*, 2018). The application of a *Smo* inhibitor leads to delayed callus mineralization in femoral

Table 2. Additional research on the role of hypertrophic chondrocytes in the crosstalk between cartilage and bone.

Pathway	Target	Effect
Ihh	p21	p21 deficiency in HCs causes delayed differentiation of HCs and delayed endochondral ossification (Kikuchi <i>et al.</i> , 2022)
BMP	BMP2 and BMP4	Deletion of BMP2 and BMP4 causes delayed formation of the primary ossification center (Shu <i>et al.</i> , 2011)
BMP	Bmpr1a and Bmpr1b	Ablation of Bmpr1a and Bmpr1b causes severe chondrodysplasia (Jing <i>et al.</i> , 2017)
BMP	Wnt7b	Wnt7b is suppressed by BMP4 stimulation and ablation of Wnt7b inhibits endochondral ossification (Tsukamoto <i>et al.</i> , 2023)
Wnt/ β -catenin	β -catenin	Ablation of β -catenin in chondrocytes causes deficiency of mature osteoblasts and loss of subchondral bone (Golovchenko <i>et al.</i> , 2013); up-regulation of β -catenin promotes the transdifferentiation of chondrocytes and hypertrophic core mineralization (Houben <i>et al.</i> , 2016); the co-transcriptional activity of β -catenin is required in hypertrophic chondrocytes to promote developmental bone modeling (Wolff <i>et al.</i> , 2021)
Wnt/ β -catenin	Iroquois homeobox-containing transcription factors 3 and 5 (IRX3 and IRX5)	Ablation of IRX3 and IRX5 in HCs reduced HC-derived osteoblasts (Tan <i>et al.</i> , 2020)
Runx2	Runx2	Deleting Runx2 in HCs increases the apoptotic activity of HCs and results in a lack of osteoblasts (Qin <i>et al.</i> , 2020)
Sex determining region Y-box 9 (Sox9)	Double-sex and mab-3 related transcription factor 2 (DMRT2)	Sox9 induces the expression of Dmrt2 in HCs and promotes the transition of endochondral bone formation (Ono <i>et al.</i> , 2021)
Sox9	Sox9	Ablation of Sox9 reduces chondrocyte hypertrophy associated with Col10a1 expression (Lui <i>et al.</i> , 2019)
Sox9	Zinc finger and BTB domain-containing protein 20 (Zbtb20)	Disruption of Zbtb20 in HCs inhibits the expression of Sox9 and causes delayed endochondral ossification and postnatal growth retardation (Zhou <i>et al.</i> , 2015)
Cyclic adenosine monophosphate (cAMP)	C-type natriuretic peptide (CNP)	CNP activates the cAMP/PKA pathway in HCs and promotes endochondral bone formation (Riegger <i>et al.</i> , 2020)
Cyclic guanosine monophosphate (cGMP)	Cyclic AMP responsive element-binding protein (CREB)	CREB activation in HCs causes skeletal overgrowth (Yamamoto <i>et al.</i> , 2019)
Endoplasmic reticulum	4-phenylbutyric acid	Restores the endoplasmic reticulum structure of HCs and promotes the transition of HCs to osteoblasts (Scheiber <i>et al.</i> , 2022)

fractures, but has no effects on chondrogenesis, angiogenesis, and eventual healing (Liu *et al.*, 2017). Ablation of *Smo* in hypertrophic chondrocytes did not result in phenotypic changes. Ablation of *Ptch1* in hypertrophic chondrocytes disrupts the formation of primary spongiosa and activates proliferating osteogenic cells derived from hypertrophic chondrocytes. Activation of Ihh signaling blocks the differentiation of hypertrophic chondrocyte-derived osteoblasts into osteocytes (Wang *et al.*, 2022b). In summary, Ihh signaling induced by hypertrophic chondrocytes may promote fracture healing by coupling chondrogenesis and osteogenesis. However, further research on the precise mechanisms of different fracture models is needed.

The canonical Wnt/ β -catenin pathway is another prominent pathway that regulates the interaction between hypertrophic chondrocytes and surrounding cells (Melnik *et al.*, 2020; Sun *et al.*, 2019; Wang *et al.*, 2022a; Wang *et al.*, 2022d; Wang *et al.*, 2021). However, the effect of the Wnt/ β -catenin pathway on chondrocyte hypertro-

phy and endochondral bone formation is still controversial. In *Col10a1*-expressing chondrocytes, up-regulation and down-regulation of the Wnt/ β -catenin pathway can inhibit bone formation. Ablation of β -catenin causes an increase in the *RankL/OPG* (osteoprotegerin) ratio, leading to an increase in bone resorption, while stabilization of β -catenin can induce hypertrophic band extension and an increase in the number of *Osterix*⁺ and *Runx2*⁺ *Col10a1* lineage cells (Houben *et al.*, 2016; Yoon *et al.*, 2023). Inactivation of Wnt/ β -catenin signaling inhibits chondrocyte hypertrophy, while Wnt/ β -catenin signaling can promote the terminal differentiation of hypertrophic chondrocytes through the BMP-2 signaling (Guo *et al.*, 2009). Depletion of β -catenin *ctnnb1* in hypertrophic chondrocytes reduces the expression of *MMP13* and *Vegfa* while the size of the *Col10a1* positive hypertrophic zone is normal (Golovchenko *et al.*, 2013). Further research has demonstrated that the co-transcriptional activity of β -catenin is required in hypertrophic chondrocytes to suppress osteo-

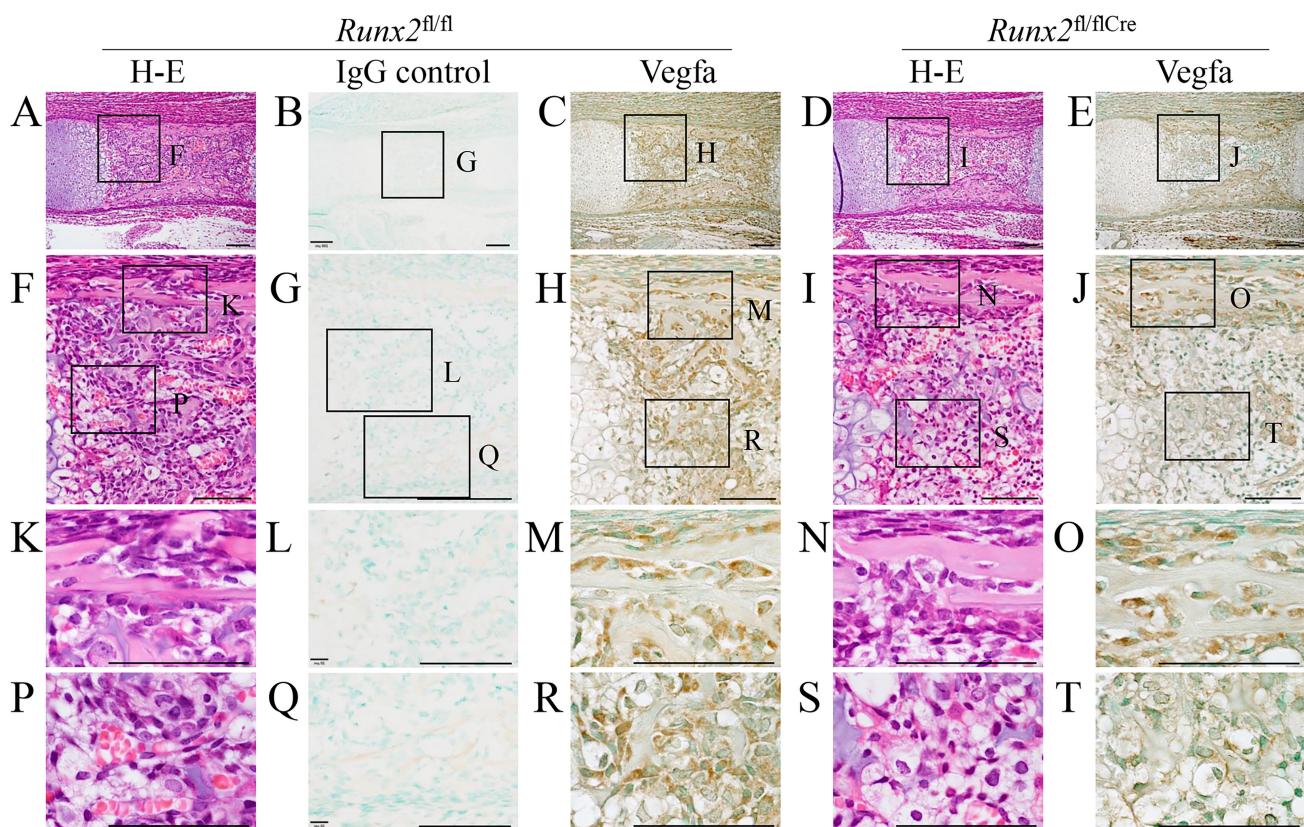


Fig. 4. VEGF expression in *Runx2^{fl/fl}* and *Runx2^{fl/flcre}* embryos. Hematoxylin and eosin staining and immunohistochemical staining of VEGF. H-E staining (A, D, F, I, K, N, P, S) and immunohistochemical analysis using anti-Vegfa antibody (C, E, H, J, M, O, R, T) or same working concentration of normal mouse IgG (B, G, L, Q) of femoral sections from *Runx2^{fl/fl}* (A-C, F-H, K-M, P-R) and *Runx2^{fl/flcre}* (D, E, I, J, N, O, S, T) mice. The boxed regions in A-E were magnified in F-J, respectively. The boxed regions in F-J were magnified in K and P, L and Q, M and R, and N and S, and O and T, respectively [Panels A-T are from, reprinted with permission (Qin *et al.*, 2020). Copyright © Plos Genetics].

clastogenesis and promote developmental bone modeling (Wolff *et al.*, 2021). It is currently unclear whether Wnt directly or indirectly mediates interactions between hypertrophic chondrocytes and osteoblasts.

Bone morphogenetic protein (BMP) signaling is another pathway necessary for bone formation. BMP agonists (*Bmp2* and *Bmp6*), receptors (*Bmpr1a*, *Bmpr2*, *Acvr1b*, and *Acvr2a*), and extracellular BMP antagonists (*Nog*) are expressed in mouse hypertrophic chondrocytes (Garrison *et al.*, 2017; Pérez-Lozano *et al.*, 2022; Thielen *et al.*, 2021). In hypertrophic chondrocytes, the phosphorylation levels of Smad1/5/8 are decreased, but the expression of Smad7, an inhibitor of Smad, is increased, indicating that although BMP2 and BMP6 are highly expressed in hypertrophic chondrocytes, BMP signaling pathway expression is decreased (Garrison *et al.*, 2017). *Bmp6*-deficient mice exhibit a decrease in the cross-sectional area of the bone cortex, but no change in the cancellous bone mass. They are unable to promote endochondral bone formation in response to estrogen (Perry *et al.*, 2008). The compound deficient mice (*Bmp2^{+/−}*; *Bmp6^{+/−}*) exhibit moderate growth retardation and trabecular bone reduction, whereas single

deficient mice (*Bmp2^{+/−}* or *Bmp6^{+/−}*) exhibit no significant phenotypes. There is no significant reduction in the osteogenic differentiation ability of bone marrow cells in the compound deficient mice (*Bmp2^{+/−}*; *Bmp6^{+/−}*). These results suggest that BMPs from hypertrophic chondrocytes promote osteoblast differentiation and bone formation (Kugimiya *et al.*, 2005) (Table 2) (Fig. 3).

Hypertrophic Chondrocytes Regulate Osteogenic-Angiogenic Coupling

Vascularization of the ossification center is necessary to form the marrow cavity. Capillaries grow into a cartilage template, which in turn initiates the ossification process. Various growth factors such as VEGF, epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) are expressed in the growth plate and regulate angiogenesis (Buettmann *et al.*, 2019; Hu and Olsen, 2016b; Kishimoto *et al.*, 2006; Liu *et al.*, 2021). The invasion of vessels into the cartilaginous callus begins at the end of endochondral ossification. This process is coupled with degradation of the matrix in the soft callus, transition of cartilage to bone and remodeling of the hard callus. Hypertrophic chondrocytes

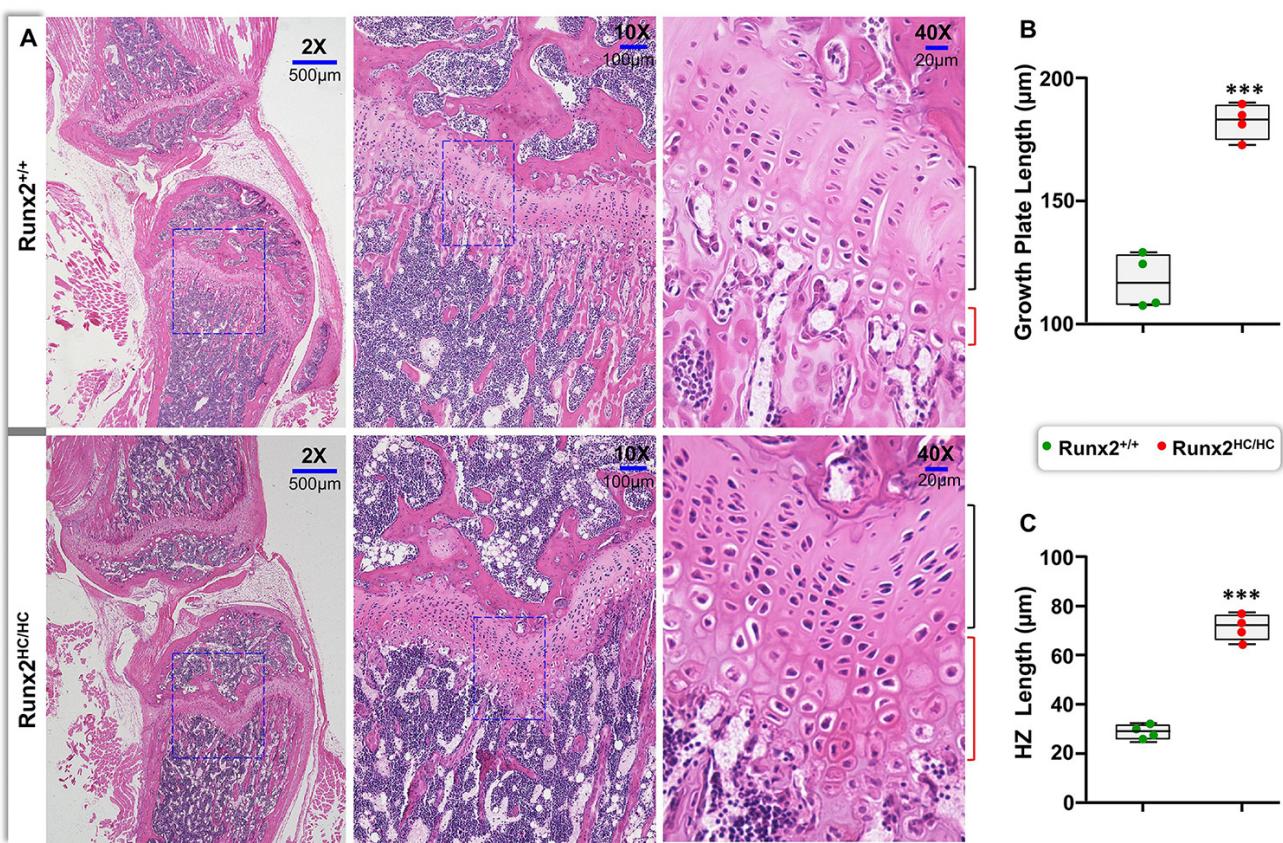


Fig. 5. Specific ablation of Runx2 in hypertrophic chondrocytes causes increase in the growth plate cartilage and hypertrophic zone. (A) Hematoxylin and eosin staining shows the distal femur and proximal tibia of the *Runx2*^{+/+} and *Runx2*^{HC/HC} mice. (B,C) Total length of the growth plate and the length of hypertrophic zones are measured. (*** indicates significant differences; $p < 0.001$) [Panels A-C are from, reprinted with permission (Rashid *et al.*, 2024). Copyright © Elsevier Inc.]

in the callus play an important role in promoting vascularization and interacting with endothelial cells and osteoprogenitors (Prein and Beier, 2019; Tuckermann and Adams, 2021).

VEGF expression in hypertrophic chondrocytes induces vascularization at the center of ossification by recruiting blood vessels. Inhibition of VEGF can lead to shortening of the femur, enhanced expression of *Col10A1* in the hypertrophic zone, and disorders of the metaphyseal blood vessels. Inhibition of VEGF by its antagonist causes hypertrophic chondrocyte stagnation and limb shortening. When VEGF is silenced in *Col2a1*-expressing chondrocytes, the invasion of blood vessels into the cartilage is inhibited, and cell death of hypertrophic chondrocytes is increased (Gerber *et al.*, 1999; Harper and Klagsbrun, 1999; Zelzer *et al.*, 2004). The specific role of VEGF in hypertrophic chondrocytes has been investigated using the conditional knock out of *Runx2*, a stimulator of VEGF in chondrocytes. In *Runx2*-deficient mice, the lack of vascularization in the growth plate is related to the absence of VEGF expression (Zelzer *et al.*, 2001). By deleting *Runx2* in hypertrophic chondrocytes using *Col10a1-cre; Runx2*^{flox/flox} mice, VEGF is not expressed in hypertrophic chondrocytes or os-

teoblasts in primary spongiosa. However, the vascularization of the cartilage is not significantly inhibited, whereas the formation of primary spongiosa is delayed, as the transition from hypertrophic chondrocytes to osteoblasts is impaired. The expression of VEGF in osteoblasts in the bone collar is significantly upregulated, indicating that vascularization in the growth plate is regulated by VEGF from various sources, including chondrocytes, hypertrophic chondrocytes, and osteoblasts (Qin *et al.*, 2020) (Fig. 4). Inhibition of VEGF expression in hypertrophic chondrocytes and osteoprogenitors in *Osx-cre; Vegfa*^{flox/flox} mice resulted in delayed vascularization and cartilage transition in a mouse tibial cortical defect model (Hu and Olsen, 2016a). However, it remains unknown whether VEGF secretion by hypertrophic chondrocytes is dominant during the transition from cartilage to bone. Further research should focus on *Col10a1*-derived deletion of *Vegf*.

Endothelial cells can also act on cartilage and chondrocytes. Romeo *et al.* (2019) confirmed that endothelial cells form type H capillaries expressing platelet endothelial cell adhesion molecule-1 (PECAM-1) and Endomucin can produce MMP9, which is essential for absorbing the cartilaginous matrix in the growth plate (Romeo *et al.*, 2019).

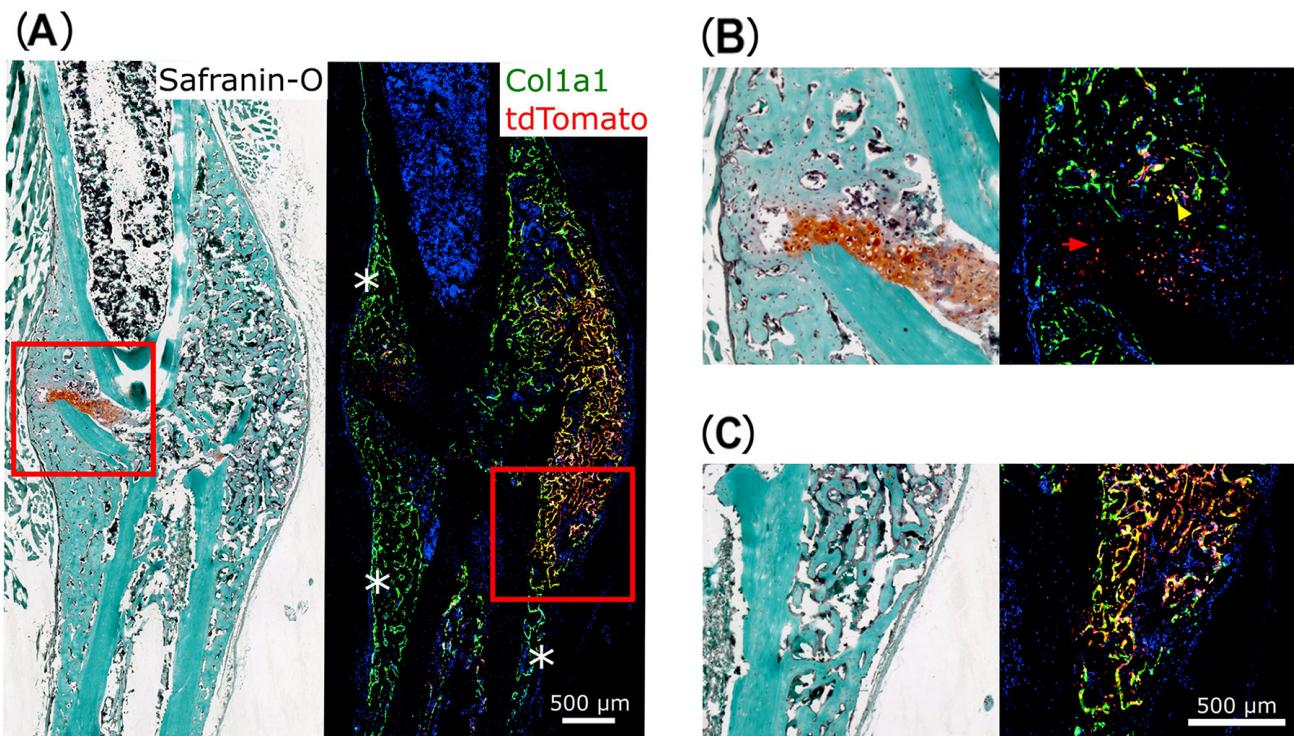


Fig. 6. Hypertrophic chondrocytes can directly transdifferentiate into osteoblasts. (A) Safranin-O staining and fluorescent photo of tibial cartilage. Asterisks indicate the GFP positive osteoblasts not derived from hypertrophic chondrocytes expressing Col10a1. (B,C) Magnified images of the labeled area in (A). The yellow arrow indicates an osteoblast labeled with both GFP and tdTomato (Col10a1-derived osteoblast). The red arrow indicates a *Col10a1-Cre* labeled tdTomato positive cell. [Panels A-C are from (Kodama *et al.*, 2022), reprinted with permission. Copyright © Elsevier Inc].

MMP9 mediates vascular invasion of hypertrophic cartilage callus. *Mmp9*^{-/-} mice have persistent cartilage, which leads to fracture nonunion and delayed union. Recombinant VEGF can also rescue it (Colnot *et al.*, 2003). Endothelial cells also directly regulate chondrocytes and bone cells. Notch signaling is necessary for endothelial cell proliferation and angiogenesis. Notch positively regulates the expression of Noggin in endothelial cells and Noggin regulates the osteogenic differentiation of osteoprogenitors. The Notch-Noggin pathway promotes chondrocyte maturation and hypertrophy, which establishes a positive loop for angiogenesis by increasing the expression of VEGF in hypertrophic chondrocytes (Ramasamy *et al.*, 2014).

Hypertrophic Chondrocytes Regulate Osteogenesis-Osteoclast Coupling

The coupling between bone-forming osteoblasts and bone-resorbing osteoclasts maintains bone homeostasis (Kong *et al.*, 1999; Liu-Bryan and Terkeltaub, 2015). In murine growth plates, the last two to three layers of hypertrophic chondrocytes express RANKL (Kishimoto *et al.*, 2006). RANKL is expressed by osteoblast lineage cells and promotes osteoclasts formation (Nakashima *et al.*, 2011; Sobacchi *et al.*, 2007). Conditional knockout of RANKL in osteoblasts results in loss of RANKL expression in the hypertrophic zone. Furthermore, *Col10a10-cre;Rankl*^{fl/fl}, *Osx-*

cre;Rankl^{fl/fl} and *Ocn-cre;Rankl*^{fl/fl} knockout mice inhibit the resorption of calcified bone by downregulating RANKL expression in hypertrophic cells. Deletion of RANKL in *Col10a1-Cre* mice inhibits the resorption of cartilage in the primary spongiosa (Liu *et al.*, 2021), indicating that RANKL produced by hypertrophic chondrocytes plays an important role in the transition of cartilage-to-bone (Xiong *et al.*, 2011). OPG is also expressed in hypertrophic chondrocytes and competes with RANK to inhibit osteoclastogenesis (Silvestrini *et al.*, 2005). Fracture union is accelerated in *Opg*-deficient mice, which have a higher number of osteoclasts and faster resorption of the cartilaginous callus (Ota *et al.*, 2009). Ablation of Runx2 in hypertrophic chondrocytes marked reduces both the number and surface area of osteoclasts and reduces the expression of RANKL and IL-17a (Rashid *et al.*, 2024). Therefore, RANKL expression in hypertrophic chondrocytes is indispensable for osteoclastogenesis (Fig. 5).

Fate of Hypertrophic Chondrocytes

Previous studies have shown that during endochondral bone formation, the process of cartilage replacement by bone is accompanied by apoptosis of terminally differentiated hypertrophic chondrocytes. Transforming growth factor β (Tgfb) signaling is essential in endochondral ossification. Deletion of the Tgfb typeII receptor gene

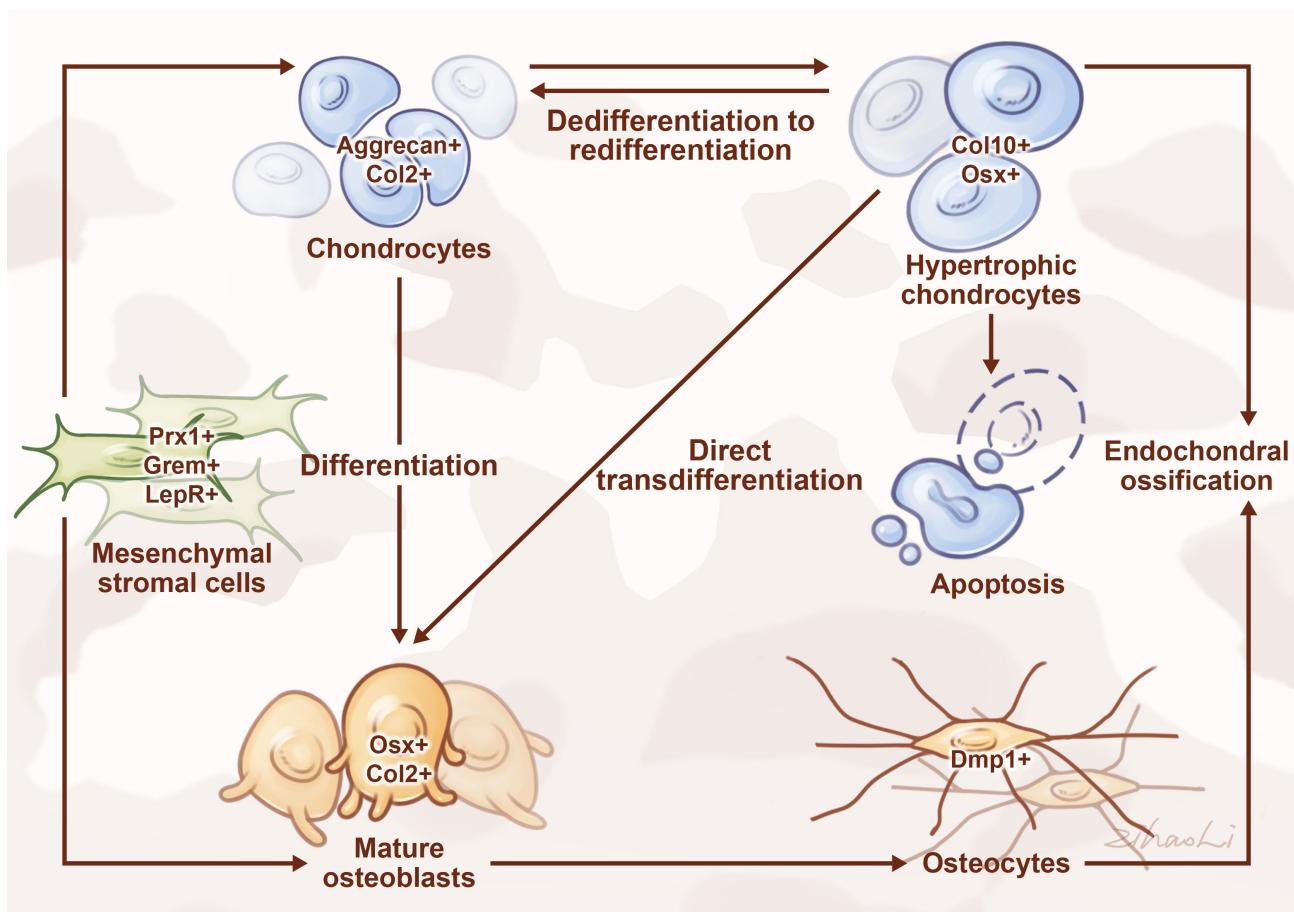


Fig. 7. The differentiation, transdifferentiation and apoptosis of hypertrophic chondrocytes. Osteochondroprogenitors can differentiate into chondrocytes and osteoblast progenitors. Chondrocytes can evolve into hypertrophic chondrocytes, which can further become terminal hypertrophic chondrocytes, transdifferentiate into mature osteoblasts or osteocytes, and undergo apoptosis.

(*Tgfb2*) in hypertrophic chondrocytes delays both the hypertrophic conversion of proliferating chondrocytes and subsequent terminal chondrocyte differentiation (Sueyoshi *et al.*, 2012). Recent studies using cell tracer techniques have shown that many hypertrophic chondrocytes can survive in the callus and become osteoblasts (Bahney *et al.*, 2014; Giovannone *et al.*, 2019; Knuth *et al.*, 2019; Wang *et al.*, 2022c; Wuelling *et al.*, 2021; Yang *et al.*, 2014). The presence of host chondrocyte-derived bone cells has been demonstrated in a bone-grafting model (Bahney *et al.*, 2014). Yang *et al.* (2014) have demonstrated that during fetal, postnatal endochondral bones and persisting into adulthood, hypertrophic chondrocyte-derived cells can become *Col10a1*-expressing osteoblasts and sclerostin-expressing osteocytes (Yang *et al.*, 2014). Using *Col10a1-Cre* or *Agc1-CreERT2* labeled hypertrophic chondrocytes, Zhou *et al.* (2014) shows that chondrocytes from growth plates can become osteoblasts in the trabeculae and cortical bones. The proportion of non-chondrocytic cells derived from labelled chondrocytes is functional osteoblasts. Osteoblasts derived from hypertrophic chondrocytes expressing *Col10a1* accounts for approximately 60 % of all mature osteoblasts

in the cartilage of one-month-old mice (Zhou *et al.*, 2014). The transition of hypertrophic chondrocytes to osteoblasts can also be observed in mouse models of mandibular fracture models (Wong *et al.*, 2021) (Fig. 6).

However, the mechanism underlying the transition of hypertrophic chondrocytes into osteoblasts is not well understood. BrdU and Ki67 expression can be observed in hypertrophic chondrocytes close to the vessels in the transition zone of soft and hard callus, indicating that some of these hypertrophic chondrocytes re-enter the cell cycle. Furthermore, these cells express molecules characteristic of stem cells, such as Oct4, Sox2, and Nanog. Therefore, hypertrophic chondrocytes close to vessels in the transition zone can de-differentiate into osteochondroprogenitor cells, gain pluripotency, and then become osteoblasts and osteocytes (Bahney *et al.*, 2014; Hu *et al.*, 2017). Park *et al.* (2015) identifies small proliferative YFP-positive cells co-expressing Osc in the lowest layer of the hypertrophic zone of a *Col10a1-cre; YFP* mouse growth plate. The authors proposed that these small cells are the transitory cells from hypertrophic chondrocytes to osteoblasts (Park *et al.*, 2015). The progenies of cells expressing *Col10a1* in bone

marrow include a subgroup of stem cells capable of osteogenic and adipogenic differentiation potency (Long *et al.*, 2022). These findings suggest that during fracture healing, hypertrophic chondrocytes may partially transit into osteoblasts and osteocytes as reservoirs of progenitor cells. However, this concept must be verified through more direct observation (Fig. 7).

Conclusion

Starting with bone development and the mechanism of endochondral ossification occurring in the callus during fracture healing, this paper summarizes the role of hypertrophic chondrocytes in fracture healing. Close links between hypertrophic chondrocytes with endothelial cells, osteoclasts, and osteoblasts are highlighted. Previous studies have shown that hypertrophic chondrocytes regulate endochondral ossification through various pathways that are critical for fracture healing. The formation of a cartilaginous callus during fracture healing is a recapitulation of endochondral ossification. Thus, the use of hypertrophic chondrocytes has been suggested as a promising developmental engineering strategy for treating fracture nonunion or delayed union.

Hence, future studies should focus on the mutual and bidirectional interactions of local and systemic soluble factors, extracellular matrix proteins, and cell-cell contacts between these cells to better clarify the role of hypertrophic chondrocytes and provide new ideas and methods for promoting fracture healing.

List of Abbreviations

ASC, adipose-derived stem cell; BMP, bone morphogenesis protein; BMSC, bone marrow derived mesenchymal stem cell; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CNP, C-type natriuretic peptide; COL, collagen; CREB, cyclic AMP-responsive element binding protein; DMRT, double-sex and mab-3 related transcription factor; EGF, epidermal growth factor; ESC, embryonic stem cells; Fox, forkhead box; Gli, glioma-associated oncogene homologue; HCs, hypertrophic chondrocytes; HCAR, hypertrophic chondrocyte angiogenesis-related lncRNA; Ihh, Indian hedgehog; IRX, Iroquois homeobox-containing transcription factors; Mef, myeloid Elf-1-like factor; MMP, matrix metalloproteinase; MSC, mesenchymal stem cells; NF- κ B, nuclear factor-kappa B; Osx, osterix; OPG, osteoprotegerin; PDC, periosteum-derived cells; PDGF, platelet-derived growth factor; PECAM, platelet endothelial cell adhesion molecule; PIGF, placental growth factor; Ptch, patched homologue; RANKL, receptor activator of nuclear factor-kappa B ligand; Runx, runt-related transcription factor; Smo, smoothened homologue; Spp, secreted phosphoprotein; Sox, sex determining region Y-box; Tgfb, transforming growth factor β ; VEGF, vascular endothelial growth factor; Zbtb, Zinc finger and BTB domain-containing protein.

tein; ASC, adipose-derived stem cell; BMP, bone morphogenesis protein; BMSC, bone marrow derived mesenchymal stem cell; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CNP, C-type natriuretic peptide; Col, collagen; CREB, cyclic AMP-responsive element binding protein; DMRT, double-sex and mab-3 related transcription factor; EGF, epidermal growth factor; ESC, embryonic stem cells; Fox, forkhead box; Gli, glioma-associated oncogene homolog; HC, hypertrophic chondrocyte; HCAR, hypertrophic chondrocyte angiogenesis-related lncRNA; Ihh, Indian hedgehog; IRX, Iroquois homeobox-containing transcription factors; Mef, myeloid Elf-1-like factor; MMP, matrix metalloproteinase; MSC, mesenchymal stem cells; NF- κ B, nuclear factor-kappa B; Osx, osterix; OPG, osteoprotegerin; PDC, periosteum-derived cells; PDGF, platelet-derived growth factor; PECAM, platelet endothelial cell adhesion molecule; PIGF, placental growth factor; Ptch, patched homolog; RANKL, receptor activator of nuclear factor-kappa B ligand; Runx, runt-related transcription factor; Smo, smoothened homolog; Spp, secreted phosphoprotein; Sox, sex determining region Y-box; Tgfb, transforming growth factor β ; VEGF, vascular endothelial growth factor; Zbtb, Zinc finger and BTB domain-containing protein.

Availability of Data and Materials

The data supporting the conclusions of this study are available from the corresponding author.

Author Contributions

DYZ and YSG planned the review and its outline. DYZ, GL and HYF did the primary literature research and drafted the manuscript. DYZ and HYF produced the figures. YSG edited the manuscript and provided funding. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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