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The hypertrophic chondrocyte: To be or not to be.

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Abstract

Hypertrophic chondrocytes are the master regulators of endochondral ossification; however, their ultimate cell fates cells remain largely elusive due to their transient nature. Historically, hypertrophic chondrocytes have been considered as the terminal state of growth plate chondrocytes, which are destined to meet their inevitable demise at the primary spongiosa. Chondrocyte hypertrophy is accompanied by increased organelle synthesis and rapid intracellular water uptake, which serve as the major drivers of longitudinal bone growth. This process is delicately regulated by major signaling pathways and their target genes, including growth hormone (GH), insulin growth factor-1 (IGF-1), indian hedgehog (Ihh), parathyroid hormone-related protein (PTHrP), bone morphogenetic proteins (BMPs), sex determining region Y-box 9 (Sox9), runt-related transcription factors (Runx) and fibroblast growth factor receptors (FGFRs). Hypertrophic chondrocytes orchestrate endochondral ossification by regulating osteogenic-angiogenic and osteogenic-osteoclastic coupling through the production of vascular endothelial growth factor (VEGF), receptor activator of nuclear factor kappa-B ligand (RANKL) and matrix metallopeptidases-9/13 (MMP-9/13). Hypertrophic chondrocytes also indirectly regulate resorption of the cartilaginous extracellular matrix, by controlling formation of a special subtype of osteoclasts termed “chondroclasts”. Notably, hypertrophic chondrocytes may possess innate potential for plasticity, reentering the cell cycle and differentiating into osteoblasts and other types of mesenchymal cells in the marrow space. We may be able to harness this unique plasticity for therapeutic purposes, for a variety of skeletal abnormalities and injuries. In this review, we discuss the morphological and molecular properties of hypertrophic chondrocytes, which carry out important functions during skeletal growth and regeneration.

Keywords

growth plate; hypertrophy; chondrocyte; chondroclast; osteoblast; primary spongiosa; transdifferentiation; apoptosis; type X collagen; vascular endothelial growth factor; matrix

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S.A.H. performed the literature search and drafted the literature review. S.A.H., W.O. and N.O. revised and finalized the manuscript.

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metalloproteinase 9; insulin like growth factor-1; bone morphogenetic protein; SRY-Box transcription factor 9; runt-related transcription factor 2; fibroblast growth factor receptor 3

Introduction

Chondrocyte hypertrophy is a process by which cells undergo a 10 to 20-fold enlargement due to rapid volumetric increases and distinct metabolic and molecular changes. This process facilitates sustained endochondral ossification and plays an instrumental role in the explosive longitudinal bone growth observed among diverse mammalian species. Historically, hypertrophic chondrocytes have been considered as the terminal state of growth plate chondrocytes resulting in degenerative maturation, denoted by cell cycle exit, nuclear condensation and apoptosis (Bonucci et al., 2020). Yet, there is evidence that hypertrophic chondrocytes undergo "transdifferentiation" and directly become osteoblasts at the primary spongiosa (Yang et al., 2014a,b; Zhou et al., 2014; Park et al., 2015; Tsang et al., 2015; Hu et al., 2017). Thus, the "terminal" state of hypertrophic chondrocytes should be more accurately described as a "transient" state, denoted by the ability to be reprogrammed into an osteoblast-like state in response to external stimuli. Additionally, hypertrophic chondrocytes are a source of receptor activator of nuclear factor kappa-B ligand (RANKL) required to induce osteoclastogenesis and formation of the marrow space during endochondral ossification, and to maintain the balance between bone resorption and formation (Xiong et al., 2011). RANKL-mediated multinucleated "chondroclasts" are highest within the cartilaginous mineralized matrix of the hypertrophic zone (Odgren et al., 2016). Hypertrophic chondrocytes also express vascular endothelial growth factor (VEGF), a cytokine that induces angiogenesis and vascularization of the ossification center (Gerber, et al., 1999a; Harper and Klagsbrun, 1999; Zelzer et al., 2004). Thus, hypertrophic chondrocytes possess multifaceted roles to orchestrate endochondral ossification, beyond what was initially described as the terminal state of growth plate chondrocytes that are destined to apoptose. Here, we discuss the morphological properties of hypertrophic chondrocytes, as well as the molecular mechanisms underlying their diverse functions in skeletal development, growth and regeneration (Figure 1).

The growth plate: The fountain of bone growth

Hypertrophic chondrocytes are the descendants of chondrocytes in the resting zone of the growth plate. The growth plate is organized into three distinct layers classified by cell morphology, function and molecular signature (Hallett et al., 2019). At the top, resting chondrocytes possess stem-like properties associated with infrequent cell division and the ability to feed their daughter cells into the adjacent proliferating zone. The notion that the resting zone houses a population of stem cells was first postulated by autotransplantation experiments in rabbits (Abad et al., 2002) and subsequently by in vivo clonal analyses (Newton et al., 2019) and lineage-tracing studies in mice (Mizuhashi et al., 2018). The resting zone is maintained through the parathyroid hormone-related protein (PTHrP)–Indian Hedgehog (Ihh) feedback loop, which directs the organization and activity of the growth plate (Kronenberg, 2003). The resting zone has two functions dictating chondrocyte hypertrophy: (1) to provide a source of growth plate chondrocytes; (2) to

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coordinate chondrocyte differentiation into proliferative and hypertrophic cells in a non-cell autonomous manner.

Below the resting zone, proliferating chondrocytes organize vertically into columns. Once proliferative chondrocytes exhaust their mitotic capabilities, they differentiate into pre-hypertrophic chondrocytes and express *Ihh*. Through PTHrP–Ihh feedback regulation, IHH secreted by pre-hypertrophic cells functions in a paracrine manner to stimulate mitosis of adjacent chondrocytes in the proliferating layer, thus regulating the rate of hypertrophy (Lanske et al., 1996; Vortkamp et al., 1996). Further, pre-hypertrophic chondrocytes undergo rapid volumetric increases due to cell swelling and differentiate into hypertrophic chondrocytes.

Hypertrophic chondrocytes: morphological changes to apoptosis or transdifferentiation

Hypertrophic chondrocytes: Morphological transformation

Hypertrophic chondrocytes are the only bone cells that undergo multiple phases of volumetric increase due to hydration-induced cell swelling (Figure 2). Two classical theories for bone growth exist: (1) it is the result of mitotic activities of proliferating chondrocytes, or (2) it is the result of their cell synthetic activities, including increases in cell volume and height (Hunziker and Schenk, 1989). Early studies indicated that hypertrophic chondrocyte enlargement most significantly contributes to longitudinal bone growth (Hunziker et al., 1987), denoted by increases in absolute volume of the cellular matrix, Golgi apparatus, endoplasmic reticulum (ER) and mitochondria and an 8-fold increase in cytoplasmic water intake (Buckwalter et al., 1986). During chondrocyte hypertrophy, cell volume and height increase linearly, until the cell occupies its greatest volumetric state. This may also be the result of increased synthesis of ultrastructural components, such as hyaluronic acid and proteoglycans (Farnum et al., 1984).

Recently, diffraction phase microscopy was utilized to show that mammalian chondrocytes undergo three phases of volumetric increase due to swelling versus dry mass production (Cooper et al., 2013). “Dry mass” is defined as the total amount of solid substances in a cell (Ginzberg et al., 2015). During Phase 1, there is a 3-fold increase in dry mass and fluid uptake, suggesting that intracellular components of chondrocytes rapidly accumulate. Yet, during Phases 2 and 3, there are 2- and 4-fold increases in dry mass and fluid uptake, respectively, leading to stabilization of dry mass density. This was confirmed by 3D dry mass density index mapping using tomographic phase microscopy in small high-density and large low-density cells. Large chondrocytes had 60% less dry mass density in the cytoplasm. Using an independent conditional knockout study in the hindlimb, the authors demonstrated that Phase 3 entry is regulated by insulin-like growth factor 1 (IGF-1). Through Phases 1–3, hypertrophic chondrocytes increase their volume 10- to 20-fold. Therefore, hypertrophic cell size is not limited due to physical constraint but rather adaptive regulation within its environment. Thus, swelling facilitates hypertrophic cell enlargement while minimizing energetic cost. These studies shed light on the cellular characteristics enabling hypertrophic chondrocyte swelling.

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Chondrocyte apoptosis: terminal differentiation followed by cell death

A group of hypertrophic chondrocytes undergoes apoptosis, as defined by physiological cell death due to sporadic or programmed cellular events leading to cytoplasmic shrinkage and maintenance of membrane integrity (Nagata, 2018). Cell cycle checkpoint proteins, p53 and Caspase proteases, play significant roles in the regulation of apoptosis (Galluzzi et al., 2018). In the articular surface, chondrocyte apoptosis is associated with degenerative musculoskeletal diseases, such as osteoarthritis (Hwang and Kim, 2015). Further, external inorganic phosphate ions are released during hydroxyapatite resorption and induce apoptosis of hypertrophic chondrocytes in vitro via nitrosative stress (Mansfield et al., 2001). Thus, hypertrophic chondrocyte apoptosis may be mediated by extrinsic factors.

Apoptosis of hypertrophic chondrocytes is also intrinsically regulated. When cultured with Caspase inhibitors, hypertrophic chondrocytes fail to undergo apoptosis, but maintain ColX synthesis (Roach et al., 2004). The morphological features of chondrocyte apoptosis differ from traditional definition, due to a lack of apoptotic bodies within the lacunae (Roach and Clarke, 2000). Observations of chick and horse terminal hypertrophic chondrocytes noted these cells are “paralyzed” or “dark”, denoted by digestions of organelles within enclosed “islands” formed by expanded or hydrated lumens of ER or vacuoles, respectively (Ahmed et al., 2007; Roach et al., 1999). Only a fraction of hypertrophic chondrocytes is labeled by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), thus, non-labeled cells with morphologically distinct DNA breaks may undergo active gene transcription (Aizawa et al., 1997; Ohyama et al., 1997). Due to the morphological and biochemical differences between classical versus chondrocyte apoptosis, “chondroptosis” has been proposed as an alternative method by which hypertrophic chondrocytes undergo combined apoptotic and autophagic processes (Roach et al., 2004; Luo et al., 2019).

A recent study has assessed morphometric parameters for classifying apoptotic hypertrophic chondrocytes, with contradictory findings (Pazzaglia et al., 2020). Using transmission electron microscopy, the authors found no evidence for expanded cytoplasm containing increased mitochondria, ribosomes, ER or Golgi apparatus in hypertrophic chondrocytes. Rather, these cells possess morphological properties similar to terminally differentiated hypertrophic cells, denoted by nuclear fragmentation and chromatin disappearance. Below the vascular invasion front, macrophages remove the degraded material produced by “hypertrophic ghosts”. These cells have been described during secondary necrosis in hypertrophic chondrocytes in response to metabolic inhibition (Pazzaglia and Congiu, 2013). Thus, there exist discrepancies in the interpretation of chondroptosis, both in terms of morphology and frequency of apoptosis-like events. Resultantly, there is a need to establish quantitative metrics and biochemical assays to accurately define hypertrophic chondrocyte state during this transition.

Hypertrophic chondrocyte transdifferentiation: the bony dilemma

Death is not the only fate of hypertrophic chondrocytes. For centuries, the idea that cells within a committed lineage can undergo alternative fates has been suggested. One example of this is transdifferentation, or the conversion of one differentiated cell type into another due to intrinsic or extrinsic factors (Merrell and Stanger, 2016).

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In endochondral bones, early analyses of mouse and rat growth plates suggest that hypertrophic chondrocytes take on multiple cellular fates: apoptosis or transdifferentiation into osteoblasts (Farnum et al., 1990). Additional early investigations showed that hypertrophic chondrocytes derived from murine rib explants or bone rudiments are metabolically active, denoted by incorporation of [^{3}H] thymidine (Crelin and Koch, 1967). EdU-labeling morphometric studies by Roach suggest similar results and postulates that terminally differentiated hypertrophic chondrocytes re-enter the cell cycle and differentiate into osteoblasts at the ossification front (Erenpreisa and Roach, 1996). Further, hypertrophic chondrocytes can undergo an osteogenic fate in response to extrinsic factors from the bone microenvironment, such as gradients of signaling molecules and high concentrations of peptides, ions and glycans (Ishizeki et al., 1996; Bianco et al., 1998; Zerega et al., 1999). These studies provided evidence that not all hypertrophic chondrocytes are destined to die and may have the potential to transdifferentiate into osteoblasts (Aghajanian and Mohan, 2018; Wolff and Hartmann, 2019; Jing et al., 2020). Yet, further investigation into the molecular and morphological changes of hypertrophic cells are required to better define chondrocyte-to-osteoblast transdifferentiation.

Hypertrophic chondrocytes as a supporter for chondroclasts

Hypertrophic chondrocytes are closely intertwined with unique matrix-resorbing cells, “chondroclasts”. Chondroclasts are a subset of osteoclasts dedicated to resorbing mineralized matrix in the hypertrophic zone (Knowles et al., 2012; Odgren et al., 2016). Chondroclasts are morphologically similar to osteoclasts, denoted by multinucleation, polarization and “ruffled bordered” membranes (Feher, 2017). Chondroclasts have been observed in the hyaline cartilage erosion area surrounding the knee joint in patients with osteoarthritis (Bromley and Woolley, 1984). Chondroclasts regulate osteogenic-angiogenic coupling by degrading extracellular matrix (ECM) in the hypertrophic zone, thus enhancing bioavailability of MMP-9 and VEGF in the ossification center (Vu et al., 1998; Gerber et al., 1999b). Chondroclasts share similar transcriptomic profiles with osteoclasts (Khan et al., 2020), but possess higher and lower levels of intracellular and extracellular Tartrate-Resistant Acid Phosphatase (TRAP), respectively (Nordahl et al., 1998). Similar genetic perturbations reduce osteoclasts and chondroclasts in the hypertrophic zone (Odgren et al., 2003). Chondroclasts regulate osteoclastic-angiogenic coupling in the ossification center, as terminal differentiation of hypertrophic chondrocytes coincides with chondroclast-mediated resorption of mineralized matrix and vascular invasion (Farnum and Wilsman, 1989; Lewinson and Silbermann, 1992). Thus, chondroclasts, a unique osteoclast subtype, resorb calcified hypertrophic cartilage, thereby maintaining balance between matrix deposition and resorption in the ossification center adjacent to the hypertrophic zone.

New insights into the molecular regulation of hypertrophic chondrocytes

Chondrocyte hypertrophy is regulated by several major signaling pathways. Here, we discuss regulatory pathways that direct chondrocyte hypertrophy, including HIF1-α, GH, IGF-1, Ihh, BMPs, Sox9, Runx2 and FGFRs (Figure 1).

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Metabolic regulation of hypertrophic chondrocytes by HIF1-α signaling

Chondrocytes adapt to hypoxic environments by shifting metabolic catabolysis to anaerobic/glycolytic modes (Shapiro and Srinivas, 2007). The Crabtree effect allows cells in avascular environments with high glucose content to decrease O_2 consumption through oxidative phosphorylation while maintaining low ATP production through the Pasteur effect (Hochachka and Lutz, 2001). Hypoxia inducible factor 1-alpha (HIF1-α), a transcription factor that regulates genes involved in glucose transport and the Pasteur effect in mammalian cells, is expressed by hypertrophic chondrocytes and is a survival factor for hypoxic chondrocytes by elevating expression of *SRY-Box transcription factor 9* (*Sox9)* and glycolytic enzymes *in vivo* (Semenza, 2000). HIF1-α knockout mice display hypo-cellularization in the center of the hypertrophic zone associated with disorganization at the chondro-osseous junction (Pfander et al., 2003; Amarilio et al., 2007). Therefore, HIF1-α signaling uniquely regulates metabolism of hypoxic hypertrophic chondrocytes.

GH and IGF-1: Direct regulators of chondrocyte hypertrophy

Two important regulators of chondrocyte hypertrophy are growth hormone (GH) and IGF-1. Subcutaneous administration of GH and IGF-1 into rats and rabbits, respectively, stimulates [^{3}H] thymidine incorporation into hypertrophic chondrocytes, denoting their metabolic responsiveness following GH and IGF-1 treatment (List et al., 2019). GH treatment also stimulates growth plate elongation and restores *Igf1* mRNA levels in the hypertrophic zone of hypophysectomized rats, indicating that GH regulates IGF-1 expression in the growth plate (Racine and Serrat, 2020). GH-deficient mice also have decreased body length compared to controls in a sex-independent manner (Alba and Salvatori, 2004).

IGF-1 regulates endochondral bone growth by promoting chondrocyte proliferation and hypertrophy (Yakar et al., 2018). IGF-1 is one of the major hormones required for skeletal growth and is used to treat pediatric skeletal disorders, such as limb-length discrepancy and short stature (Giustina et al., 2008). *Igf1* haploinsufficient mice are 10–20% smaller than controls due to decreased organ, muscle and bone mass and serum IGF-1 (Powell-Braxton et al., 1993). *Igf1* knockout mice display a 35% reduction in long bone growth due to specific reductions in the linear length of hypertrophic chondrocytes, suggesting that IGF-1 regulates chondrocyte hypertrophy (Wang et al., 1999a).

IGF-1 receptor (*Igf1r*) deletion in mice causes delayed endochondral ossification, abnormal chondrocyte proliferation and differentiation and dwarfism (Bikle et al., 2001). Deletion of *Igf1r* in *type II collagen alpha 1 chain* (*Col2a1*) expressing chondrocytes caused dwarfism, expansion of the proliferating zone and increased apoptosis of hypertrophic chondrocytes (Wang et al., 2011). IGF1R signaling interacts with the PTHrP–Ihh feedback loop; in which PTHrP prolongs chondrocyte proliferation and delays their hypertrophic differentiation, thereby delaying IHH expression (Vortkamp et al., 1996). Thus, IGF-1 is necessary for skeletal growth and development due to its role as a regulator of chondrocyte hypertrophy.

Pre-hypertrophic IHH as an indirect regulator of chondrocyte hypertrophy

IHH regulates chondrocyte differentiation and skeletal morphogenesis (Lanske et al., 1996; Vortkamp et al., 1996; Chung et al., 1998; St-Jacques et al., 1999; Kobayashi et al., 2002,

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2005). IHH expressed by pre-hypertrophic chondrocytes works in a concerted manner with PTHrP expressed by resting chondrocytes to maintain growth plate structure and longitudinal bone growth (Kronenberg, 2003). *Ihh*-deficient mice lack proper chondrocyte differentiation and mineralization due to delayed expression of *type 10 collagen alpha 1* (*Col10a1*), a marker of hypertrophic chondrocytes (Linsenmayer et al., 1991; St-Jacques et al., 1999). Activation of Hedgehog signaling via loss of Patched-1 (PTCH1) receptor causes delayed chondrocyte hypertrophy (Mak et al., 2006). Thus, Ihh indirectly regulates chondrocyte hypertrophy through interactions with chondrocytes in the adjacent layers.

BMPs mediate chondrocyte hypertrophy via independent and complimentary mechanisms

BMPs regulate chondrocyte hypertrophy both directly and indirectly. BMP2 and BMP4 are expressed in pre-hypertrophic and hypertrophic chondrocytes (Nilsson et al., 2007) and stimulate chondrocyte hypertrophy in limb explants (de Luca et al., 2001; Hatakeyama et al., 2004). In vitro administration of BMP2 in cultured chondrocytes and limb explants targets hypertrophic chondrocytes, resulting in an increase in *Rankl* expression in $ColX^+$ cells as well as *Ihh* and *Col10a1* expression in the pre-hypertrophic and hypertrophic zones, respectively (Valcourt et al., 2002; Zhou et al., 2016). Canonical BMP signaling directly regulates chondrocyte hypertrophy, as BMP2 administration inhibits chondrocyte hypertrophy via Smad1/5/8 (Valcourt et al., 2002; Canalis et al., 2003). *Col2a1-cre*-specific deletion of BMP2 causes shortened long bones due to delayed formation of the hypertrophic zone (Shu et al., 2011). BMP2 induces Runx2 expression at the transcriptional and post-transcriptional levels via phosphorylation of CDK4, which inhibits chondrocyte hypertrophy via Runx2 degradation (Zhang et al., 2009). This is important, since Runx2 activation is necessary for hypertrophic chondrocyte differentiation (Ding et al., 2012) and transdifferentiation (Qin et al., 2020). Deletion of BMP signaling members, *Smad6* and *Bmpr1a/b*, leads to chondrodysplasia due to premature hypertrophic differentiation and smaller hypertrophic zones (Yoon et al., 2005). Thus, BMP2 and members of the canonical BMP signaling pathway regulate chondrocyte hypertrophy through Runx2.

Sox9 downregulation induces hypertrophic chondrocyte transdifferentiation

Sox9 activation is required for mesenchymal condensation of the cartilaginous anlage during fetal development (Lefebvre and Smits, 2005). *Sox9* is expressed in chondroprogenitor cells and becomes isolated to resting, proliferating and pre-hypertrophic chondrocytes postnatally (Zhao et al., 1997). *Sox9* knockout mice have reduced chondrocyte hypertrophy due to absence of *Col10a1* expression in the hypertrophic zone (Ikegami et al., 2011; Dy et al., 2012). Sox9 activates *Col10a1* transcription in hypertrophic chondrocytes by binding to its promoter cooperatively with myocyte enhancer factor 2C (Mef2c) (Dy et al., 2012). Thus, Sox9-mediated *Col10a1* transcription is required for chondrocyte hypertrophy. *Sox9* misexpression in $Col10a1^+$ hypertrophic chondrocytes results in reduced bone marrow formation at P0, reduced bone growth and deficiencies in *Vegfa*, *Mmp13*, *Rankl* and *Opn* expression in hypertrophic cells (Hattori et al., 2010). Further, a recent study has demonstrated that persistent *Sox9* expression in the growth plate causes inhibition of chondrocyte-to-osteoblast transdifferentiation in trabecular bone associated with decreased expression of *Mmp9*, *Mmp13*, *Sp7* and *Col1a1* (Lui et al., 2019). Thus, downregulation of Sox9 in hypertrophic chondrocytes is necessary for vascular invasion and degradation

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of calcified hypertrophic cartilage in the growth plate in addition to transdifferentiation of hypertrophic chondrocytes into osteoblasts.

According to a recent study, Sox9 maintains growth plate architecture and safeguards the lineage fates of chondrocytes by preventing their dedifferentiation into mesenchymal progenitors while facilitating hypertrophic chondrocyte transdifferentiation into osteoblasts (Haseeb et al., 2021). Using an *Acan-creERT2; ROSA26R^{tdTomato}; Sox9$^{f/f}$*, chondrocyte-specific conditional knockout mouse, single cell RNA-sequencing analysis of chondrocytes extracted from control and mutant distal tibial and femur epiphyses discovered that transcriptomic profiles of mutant chondrocytes bypass late proliferative, pre-hypertrophic and hypertrophic stages, becoming prematurely terminally differentiated or osteoblast-like cells. These transcriptomic data were confirmed by immunohistochemical analyses, denoted by increased expression of terminal hypertrophic chondrocytes markers, *Col10a1* and *matrix GLA protein* (*Mgp*) and osteoblast markers, *Sp7*, *Col1a1* and *Bglap* at the transition zone of *Sox9*-deficient growth plates. Thus, *Sox9* expression in the postnatal growth plate regulates transdifferentiation of hypertrophic chondrocytes to osteoblast-like cells.

Runx-related genes are required for chondrocyte hypertrophy

The Runx transcription factors play important roles in chondrocyte hypertrophy. During fetal development, *Runx1* is expressed by early mesenchymal progenitor cells in condensations (Yamashiro et al., 2002; Smith et al., 2005). *Runx2/3* are expressed in pre-hypertrophic and hypertrophic chondrocytes, suggesting direct functional roles of Runx2/3 in chondrocyte hypertrophy (Inada et al., 1999a; Kim et al., 1999; Sato et al., 2008). Runx2 regulates osteoblast differentiation in the early stages of endochondral bone formation (Komori et al., 1997; Otto et al., 1997). Genetic ablation or expression of dominant negative RUNX2 leads to reduced chondrocyte hypertrophy (Inada et al., 1999b; Ueta et al., 2001). RUNX2 transcriptionally regulates genes critical for vascular invasion and ECM synthesis, including VEGF (Zelzer et al., 2001) and MMP13, respectively (Selvamurugan et al., 2000). *Runx2/Runx3* double knockout mice have loss of chondrocyte maturation due to failed formation of the hypertrophic zone and decreased *Col10a1* expression (Yoshida et al., 2004). Conversely, *Runx2* overexpression in chondrocytes causes premature chondrocyte hypertrophy and early induction of *ColX* expression in vitro (Enomoto et al., 2000) and in vivo (Takeda et al., 2001). Further, Runx2 regulates ColX transcription in hypertrophic chondrocytes (Drissi et al., 2003; Zheng et al., 2003).

A recent study shows that hypertrophic chondrocyte-specific conditional knockout of *Runx2* (*Col10a1-cre*; *Runx2$^{f/f}$*) causes decreased expression of *Vegfa* in hypertrophic chondrocytes, and *Mmp13*, *Col1a1* in the primary spongiosa, associated with increased apoptosis and failure of chondrocyte-to-osteoblast transdifferentiation (Qin et al., 2020). Using a *Col10a1-cre*; *Rosa26*-mTFP1; *Runx2$^{f/f}$*; *2.3Col1a1*-tdTomato compound mutant mouse, the authors demonstrated that hypertrophic chondrocyte-derived trabecular and endosteal osteoblasts were significantly reduced or absent in mutants at embryonic day 17.5 (E17.5), P0 and 1-week. Primary spongiosa formation was delayed in mutants, indicated by decreased expression of *bone sialoprotein 2* and *Col1a1*, hypertrophic chondrocyte and osteoblast markers, respectively, at E15.5. At birth, spongiosa development and trabecular bone volume

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were similar in wild-type and mutant mice. Thus, Runx2 is required for survival and transdifferentiation of hypertrophic chondrocytes during fetal development. Runx2, initially identified as a regulator of osteoblast formation, also plays roles in chondrocyte hypertrophy, transdifferentiation, vascular invasion and matrix deposition in the hypertrophic zone.

FGFRs play dual roles in chondrocyte hypertrophy and skeletal growth

Fibroblast growth factors receptors (Fgfr) play dual roles in promoting or inhibiting chondrocyte differentiation and endochondral bone growth. *Fgfr1* and *Fgfr2* are initially expressed in the embryonic perichondrium and become restricted to the hypertrophic and resting zones, respectively (Delezoide et al., 1998; Lazarus et al., 2007; Sheeba et al., 2010). *Fgfr3* is expressed after the pre-condensation stage in the cartilage anlage and becomes isolated to proliferating and pre-hypertrophic chondrocytes (Ornitz and Marie, 2015). FGFR1 overactivation in humans causes appendicular skeletal deformities and dwarfism (White et al., 2005), although a similar mutation in *Fgfr1* in mice had no effect on bone formation (Zhou et al., 2000). Mesoderm-specific deletion of *Fgfr1* (*Dermo1-cre; Fgfr1$^{f/f}$*) causes impaired chondrocyte hypertrophy in fetal stages (Hung et al., 2007). During postnatal development, chondrocyte-specific deletion of *Fgfr1* (*Col2a1-cre; Fgfr1$^{f/f}$*) causes hypertrophic zone expansion associated with delayed degradative maturation of hypertrophic chondrocytes (Jacob et al., 2006). Further, FGFR1 signaling delays hypertrophic differentiation of chondrocytes. Thus, *Fgfr1* expression is important for regulating chondrocyte hypertrophy through unknown mechanisms. FGFR2 functions in resting and proliferating chondrocytes in a redundant manner; *Dermo1-cre*; *Fgfr2$^{f/f}$* mice display normal chondrocyte proliferation and growth plate morphology (Yu et al., 2003).

During fetal development, FGFR3 activates chondrocyte proliferation. By early postnatal development, FGFR3 inhibits chondrocyte proliferation and hypertrophic differentiation (Iwata et al., 2000). Activating mutations in *FGFR3* in humans and mice cause impaired chondrocyte proliferation and premature hypertrophy, leading to achondroplasia (Wang et al., 1999b). Conversely, *Fgfr3* deficient mice present increased hypertrophic zone linear length and prolonged endochondral ossification (Colvin et al., 1996). FGFR3-mediated inhibition of chondrocyte proliferation and hypertrophy are regulated by STAT1-p21 and MAPK-ERK signaling, respectively (Su et al., 1997; Murakami et al., 2004; Raucci et al., 2004; de Frutos et al., 2007). FGFR3-mediated suppression of Sox9 decreases pre-hypertrophic chondrocyte differentiation (Zhou et al., 2015). Mesenchymal cell-specific overactivation of FGFR3 (*Prrx1-cre; Fgfr3$^{Y637C/+}$*) causes failure of chondrocyte-to-osteoblast transdifferentiation in a tibial fracture healing model, resulting in persistent fibrocartilages at the callus (Julien et al., 2020). In mutants, Col10a1^{+} cells fail to become osteoblasts, denoted by decreased vascularization and chondrocyte proliferation at the callus. The fracture defect in mutants is due to an inability for periosteal cells to differentiate into hypertrophic chondrocytes, causing an intrinsic reduction in transdifferentiation. Notably, when mutant-derived periosteal cells were transplanted to wild-type hosts, transdifferentiation occurred (Julien et al., 2020). Thus, Fgfr3 is important for chondrocyte proliferation, hypertrophy and transdifferentiation during skeletal regeneration. Yet, Fgfr3's role during physiologic hypertrophic chondrocyte-to-osteoblast transdifferentiation remains unknown.

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Hypertrophic chondrocytes regulate osteogenic-angiogenic and osteogenic-osteoclastic coupling

Hypertrophic chondrocytes as an important regulator of osteoclastogenesis

Hypertrophic chondrocytes express RANKL and regulate osteoclastogenesis. Coupling between bone-forming osteoblasts and bone-resorbing osteoclasts maintains skeletal homeostasis (Sims and Martin, 2014). RANKL is expressed by cells of the osteoblast lineage and facilitates osteoclast formation (Kong et al., 1999; Sobacchi et al., 2007). It has been known for decades that osteoblasts regulate osteoclastogenesis in vitro (Rodan and Martin, 1982; Takahashi et al., 1988). Yet, recent studies suggest that matrix-embedded osteocytes, not osteoblasts, are the primary source of RANKL (O'Brien, 2010). Ablation of osteoblasts *in vivo* and *in vitro* has no impact on *Rankl* expression or osteoclast number (Corral et al., 1998; Galli et al., 2009). Anabolic glucocorticoid administration in mice reduces osteoblasts and pre-osteoblasts, but not osteocytes (Weinstein et al., 1998, 2002). Conditional deletion of RANKL in limb bud mesenchyme causes significant reduction of osteoclasts below the hypertrophic zone (Xiong et al., 2011). In this study, conditional ablation of RANKL in osteoblasts (*Osteocalcin-cre* [*Ocn-cre; Rankl$^{f/f}$*]; *Osterix-cre* [*Osx-cre; Rankl$^{f/f}$*]) causes loss of *Rankl* expression in the hypertrophic zone. Further, *Col10a1-cre; Rankl$^{f/f}$*, *Osx-cre; Rankl$^{f/f}$* and *Ocn-cre; Rankl$^{f/f}$* knockout mice all prevented calcified cartilage resorption by reducing RANKL expression in hypertrophic cells. Osteocytes embedded in the trabecular lacunae highly express RANKL (Nakashima et al., 2011). Osteocyte-specific deletion of RANKL (*Dmp1-cre; Rankl$^{f/f}$*) causes decreased osteoclast number and increased trabecular bone volume, leading to osteopetrosis. Therefore, RANKL produced by hypertrophic chondrocytes and osteocytes is essential for osteoclastogenesis.

Hypertrophic chondrocytes as a central regulator of osteogenic-angiogenic coupling

Vascularization of the ossification center is an essential process to establish the marrow cavity. Capillary invasion into the cartilage template is followed by ossification. Growth factors VEGF, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) are expressed in the growth plate and regulate vascularization (Hu and Olsen, 2016). VEGF expressed by hypertrophic chondrocytes induces vascularization of the ossification center by recruiting blood vessels (Risau, 1995; Carmeliet et al., 1996; Ferrara et al., 1996; Gerber et al., 1999a). Inhibition of VEGF protein by chimeric VEGF–IgG decreases femur length and enhances *Col10a1* expression in the hypertrophic zone, associated with disorganization of metaphyseal blood vessels (Gerber et al., 1999b). VEGF-mediated metaphyseal vasculogenesis triggers apoptosis of hypertrophic chondrocytes (Gerber et al., 1999b; Harper and Klagsbrun, 1999). *Col2a1*-cre-specific deletion of *Vegfa* causes reduced cartilage formation and skeletal mineralization, delayed vascularization of the ossification center and removal of hypertrophic chondrocytes (Zelzer et al., 2004). Thus, VEGF is necessary for maintaining hypertrophic chondrocyte survival.

VEGF-mediated osteogenic-angiogenic coupling during skeletal growth has been extensively studied. Functioning cooperatively with VEGF, matrix metalloproteinase-9 (MMP-9), is expressed by hypertrophic chondrocytes and degrades cartilaginous ECM (Paiva and Granjeiro, 2017). Similar to the VEGF inhibition phenotype (Gerber et al.,

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1999b), *Mmp-9* knockout mice have an expanded hypertrophic zone (Vu et al., 1998; Ortega et al., 2005) associated with reduced chondrocyte apoptosis, vascularization and ossification. *Mmp9*-deficient mice have impaired skeletal regeneration, denoted by accumulation of hypertrophic cartilage and delayed endochondral ossification during healing (Colnot et al., 2003). This was confirmed by analysis of *Mmp-9*-deficient growth plates, in which *Mmp13* expression was elevated in the expanded hypertrophic zone (Kojima et al., 2013). Consistent with others, this suggests a role for MMP-13 to compensate for MMP-9 loss in hypertrophic cells to degrade ECM (Wu et al., 2002; Ortega et al., 2010). MMP-13-mediated ECM degradation of the hypertrophic zone also coincides with apoptosis of hypertrophic chondrocytes (Inada et al., 2004).

MMP-9-mediated ECM degradation increases bioavailability of VEGF, resulting in the recruitment of osteoclasts to the vascular front to facilitate ECM remodeling and hypertrophic chondrocyte turnover. Expansion of the hypertrophic zone and vascularization of the ossification center in *Mmp9* knockout mice are partially rescued by exogenous VEGF (Ortega et al., 2010). Thus, MMP9-driven resorption of the hypertrophic zone is synergistically coupled to VEGF-mediated vasculogenesis. Apoptosis of hypertrophic chondrocytes in *Mmp9*-deficient mice is observed within the center of the expanded hypertrophic zone (Vu et al., 1998). Furthermore, *Mmp9*-deficient hypertrophic chondrocytes delay release of pro-angiogenic factors, indicating that MMP-9-driven osteogenic-angiogenic coupling in the hypertrophic zone regulates apoptosis of hypertrophic chondrocytes, ECM degradation and vasculogenesis of the metaphysis.

Alternative osteogenic cell fates of hypertrophic chondrocytes

Col10a1-mCherry$^+$ cells are located in the metaphyseal marrow space

Type X collagen (ColX) is a short chain collagen that forms aggregates in the territorial matrix of hypertrophic chondrocytes (Schmid and Linsenmayer, 1990; Shen, 2005). *Col10a1*-deficient mice are viable and undergo normal bone formation (Rosati et al., 1994). *Col10a1* is expressed in hypertrophic chondrocytes, according to early immunohistochemical and molecular analyses (Schmid and Linsenmayer, 1985; Iyama et al., 1991; Gu et al., 2014) and mouse reporter models (Gebhard et al., 2008; Kong et al., 1993). Analysis of *Col10a1-mCherry* knock-in reporter mice revealed Col10a1$^+$ cells in the metaphyseal marrow space, in addition to in the pre-hypertrophic and hypertrophic zones (Maye et al., 2011). Yet, endogenous *Col10a1* mRNA is most abundant in pre-hypertrophic and hypertrophic chondrocytes. Thus, Col10a1-mCherry$^+$ cells in the marrow space may represent a population of apoptosis-evading chondrocytes or hypertrophic cells that have transdifferentiated.

Analysis of *Col10a1*-mCherry; Col3.6-Topaz; Col2.3-Emerald triple transgenic mice revealed distinct reporter activities within the growth plate and trabecular bone: *Col10a1*-mCherry$^+$ cells were found in the hypertrophic zone and surrounding trabecular osteoblasts, while Col2.3-Emerald$^+$ cells were localized to the growth plate and Col3.6-Topaz$^+$ cells were present in the trabecular bone. Interestingly, *Col10a1*-mCherry$^+$ trabecular osteoblasts do not overlap with Col3.6-Topaz$^+$ osteoblasts. Thus, Maye et al. conclude “no evidence of chondrocyte to osteoblast transdifferentiation” (Maye et al., 2011), although their analyses

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were limited to late embryonic and early postnatal stages therefore not addressing the possibility that Col10a1$^+$ cells may become osteoblast-like cells during late postnatal development. Additionally, others state that "*mCherry* expression fades before the onset of osteogenesis and expression of Col1a1-EGFP, and the fate of the [hypertrophic chondrocyte] cannot be traced" (Tsang et al., 2015). Could a subset of Col10a1-mCherry$^+$ cells represent a unique osteo-chondroprogenitor population that contributes to the trabecular compartment? To address this cell fate question, advances in lineage-tracing technology have facilitated the spatiotemporal analysis of hypertrophic chondrocyte cell fates through the use of *cre-loxP* system (Vanhorn and Morris, 2020).

Lineage-tracing findings from pan-chondrocyte *Col2a1-crER* and *Aggrecan-creER* lines

Yang et al. demonstrated that Col2a1$^+$ growth plate chondrocytes contributed to *Col1a1*$^+$ osteoblasts in the metaphysis, using a *Col2a1-creER; ROSA*^{EYFP} lineage-tracing model (Yang et al., 2014a). Analysis of *Col2a1-creER; ROSA*^{EYFP} and *Col2a1-creER; ROSA*^{Confetti} single and multicolor clonal lineage reporter mice demonstrated that Col2a1$^+$ chondrocytes give rise to metaphyseal osteoblasts at low frequencies (Yang et al., 2014a). Yet, because *Col2a1*-cre*ER* labels all chondrocyte subtypes in the growth plate, it is unknown if *Col2a1-creER*-lineage-traced osteoblasts are derived from hypertrophic cells or unidentified osteo-chondroprogenitor populations at the primary spongiosa. Additionally, analysis of a "chondrocyte-specific" *Aggrecan-creER (Acan-creER)* lineage-tracing model (Henry et al., 2009) discovers that Acan$^+$ cells contribute to osteoblasts at the primary spongiosa. This is also observed during skeletal regeneration, as Acan-creER$^+$ cells contribute to *2.3Col1a1*-GFP$^+$ osteoblasts at the repair callus (Zhou et al., 2014).

Col10a1-cre: is it the right tool to study hypertrophic chondrocyte transdifferentiation?

In vivo lineage-tracing studies have demonstrated that Col10$^+$ chondrocytes may transdifferentiate into osteoblasts and osteocytes in the trabecular and cortical bone (Yang et al., 2014a; Yang et al., 2014b; Zhou et al., 2014). In a study by Zhou et al., fetal-derived Col10a1$^+$ hypertrophic chondrocytes expressed *Col1a1* at the primary spongiosa and trabecular and endosteal surfaces during early and late postnatal development (Zhou et al., 2014). In a tandem analysis, a *Col10a1*^{int2}*-cre; ROSA*^{EYFP} reporter mouse shows that Col10a1$^+$ hypertrophic chondrocytes invade into the metaphysis and trabecular bone and express osteoblast markers *Col1a1*, *Ocn* and *Bsp* and eventually became matrix-embedded osteocytes in the diaphysis at P20. (Yang et al., 2014b). Consistent with early reports suggesting the metabolic capability of hypertrophic chondrocytes (Crelin and Koch, 1967), the authors found that *Col10a1-cre*$^+$ cells uptake BrdU in the metaphysis and are mitotically active. Further, *Col10a1*^{int2}*-cre; ROSA*^{EYFP}-marked cells became with perilipin$^+$ adipocytes.

An additional study using *Col10a1-cre; ROSA*$^{RYFP/LacZ}$ models demonstrated that descendants of Col10a1$^+$ hypertrophic chondrocytes contribute to osteoblast formation at the primary spongiosa and on the trabecular and endosteal surfaces (Yang et al., 2014a). *Col10a1-cre; Rosa*$^{LacZ+}$ cells became *Col1a1*$^+$ endosteal osteoblasts at P10 and at the chondro-osseous junction in cortical bone at 3 months, suggesting that Col10a1$^+$ hypertrophic chondrocytes may commit to an osteogenic lineage in adulthood. In the same study, fetal-derived *Col10a1-creERt; Rosa*^{LacZ}-marked hypertrophic chondrocytes

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gave rise to immature *Osx*$^{+}$ pre-osteoblasts at the primary spongiosa, *Col1a1*$^{+}$ metaphyseal osteoblasts and *Sclerostin*$^{+}$ osteocytes in the trabecular bone. Yet, these studies assess chondrocyte-to-osteoblast 'transdifferentiation' only in early postnatal time points, and therefore did not determine if conversion of *Col10a1*$^{+}$ hypertrophic cells to osteoblasts also occur in adulthood. These studies suggest that Col10a1^{+} lineage traced hypertrophic chondrocytes contribute to the osteogenic pool during early postnatal endochondral bone growth.

Hypertrophic chondrocytes reenter the cell cycle and become osteoblast-like during skeletal regeneration

More recently, hypertrophic chondrocytes were confirmed to reenter the cell cycle, marked by BrdU incorporation and *Ki67* expression and undergo a pro-osteogenic fate during skeletal regeneration using combinatorial histomorphometric and gene expression analyses (Hu et al., 2017). In this study, as chondrocytes in the transition zone become osteoblast-like cells, they lose expression of chondrogenic signatures, *Sox9*, *Col2a1* and *Col10a1*, while beginning to express *Col1a1*. In addition to becoming osteoblast-like, transition zone hypertrophic chondrocytes express markers of cell pluripotency, *Oct4*, *Sox2* and *Nanog*, suggesting that hypertrophic cells may revert to a pluripotent-like state during transdifferentiation into osteoblasts. These findings denote unique morphological and gene expression signatures of hypertrophic chondrocytes in response to fracture healing.

Chondrocyte-derived osteoprogenitors become osteoblasts

In a tandem analysis, these results were confirmed using bacterial artificial chromosome (BAC)-generated *Col10-cre; ROSA*^{RYFP} and *Col10-cre; ROSA*^{LacZ} reporter models (Park et al., 2015). Col10^{+} chondrocytes overlap with Col1a1^{+} and Ocn^{+} osteoblasts in the primary ossification center during embryonic development and later in the primary spongiosa, suggesting that these cells may originate from *Col10a1*$^{+}$ hypertrophic chondrocytes in the growth plate. YFP^{+} trabecular cells isolated from the spongiosa of femoral heads of *Col10-cre; Rosa*^{RYFP} reporter mice were highly enriched for osteogenic markers, *Ocn*, *Osx*, *Col1a1* and *Runx2* at levels similar to cortical bone. According to flow cytometry analysis of cultured *Col10-cre; ROSA*^{RYFP}-derived endosteal osteoblasts at P7, 11% of these cells are YFP^{+}. Thus, the authors postulate that 11% of endochondral osteoblasts are derived from hypertrophic chondrocytes that have rapidly transdifferentiated into endosteal osteoblasts. Further, a novel chondrocyte-derived osteoprogenitor (CDOP) was identified using confocal microscopy, characterized by small, condensed chondrocytes with extensive cytoplasmic vascuolization at the bottom of the hypertrophic zone. In culture, CDOPs express *Col2a1*, *Col10*, *Col1a1*, *Osx*, are enriched for the stem cell markers, *Sca1*, *CD34*, *sox2* and *c-myc* and robustly incorporate BrdU (Park et al., 2015). These lineage tracing, morphometric and *in vitro* analyses suggest that Col10a1^{+} chondrocytes may represent "stem-like" cells that gives rise to pre-osteoblasts, osteoblasts and osteocytes at embryonic and postnatal times. We have also provided evidence of chondrocyte-to-osteoblast "transdifferentiation" based on a series of in vivo lineage-tracing experiments using a *Pthrp-creER* transgenic line that is specific to chondrocytes in the resting zone (Mizuhashi et al., 2018).

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Lack of morphometric evidence for hypertrophic chondrocyte transdifferentiation

Recently, however, a morphometric analysis of rabbit tibial hypertrophic chondrocytes supports no evidence of chondrocyte-to-osteoblast transdifferentiation (Pazzaglia et al., 2020). The authors stipulate that in order to constitute a transdifferentiation event, hypertrophic chondrocytes must undergo: 1) a 10-fold shrinkage of size, and 2) decreases in both number and density when compared to metaphyseal osteoblasts at the vascular invasion line. The latter observation suggests the incidence of increased mitoses at the chondrocyte-to-osteoblast transdifferentiation transition zone. The authors continue to suggest that lineage-tracing analyses of transdifferentiation (Yang et al., 2014b; Zhou et al., 2014) fail to consider the possibility that "unstructured substances of apoptotic chondrocytes were still present until cleared by macrophages and that these [cells] could account for the positive fluorescent staining observed in those analyses" (Pazzaglia et al., 2020). They conclude that, "distribution and density of hypertrophic chondrocytes, macrophages and osteoblasts were consistent with a committed function for each [cell type] in the general layout of the growth plate", based on their morphometric analyses.

Several questions remain regarding the fate of hypertrophic chondrocytes, including: (1) How often do descendants of Col10a1^{+} hypertrophic chondrocytes persist in adulthood and give rise to osteoblasts and osteocytes? (2) Are there unidentified osteo-chondroprogenitor populations at the interface of hypertrophic chondrocytes and newly formed bones? (3) Which molecular signals allow hypertrophic chondrocytes to alter their fate and differentiate into osteoblasts during skeletal regeneration? These outstanding questions represent future areas of investigation into the elusive nature of hypertrophic chondrocytes.

Conclusions

The ultimate cell fates of hypertrophic chondrocytes remain largely elusive due to their transient nature. Hypertrophic chondrocytes are the only skeletal cell type capable of increasing its intracellular volume through rapid water intake and increased metabolism due to accumulation of mitochondria, the Golgi apparatus and ER. Coupled with active proliferation of chondrocytes in the preceding layer, rapid enlargement of hypertrophic chondrocytes is a major driver of endochondral bone growth. Historically, hypertrophic chondrocytes have been considered as the terminal state of chondrocytes prior to apoptosis. Even this concept is debated as several varieties of "chondroptosis" denoted by "paralyzed" or "dark" cytoplasmic aggregates have been observed in hypertrophic chondrocytes. Hypertrophic chondrocytes are critical regulators of osteogenic-osteoclastic and osteogenic-angiogenic coupling activities during skeletal development, growth and regeneration. Lastly, the "terminal" state of hypertrophic chondrocytes may actually be transient; denoted by their ability to reenter the cell cycle and give rise to a newly identified, "chondrocyte-derived osteoprogenitor"-like cell, although details are not yet clear.

Chondrocyte-to-osteoblast transdifferentiation has been proposed for over a century. Early reports of this event are based on morphological characteristics, such as nuclear condensation and cellular shrinkage. More recent lineage-tracing experiments have substantially advanced our understanding of individual fates of hypertrophic chondrocytes. Hypertrophic chondrocytes represent a unique "terminally differentiated" cell type capable

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of giving rise to new cell types. Further investigations are required to unravel the molecular regulation of chondrocyte-to-osteoblast transdifferentiation under both physiological and pathological conditions. Resultantly, it may be possible to harness the amazingly diverse functions of hypertrophic chondrocytes in order to more effectively treat patients suffering from debilitating skeletal disorders, including skeletal abnormalities, chondrodysplasias and skeletal injuries.

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Abbreviations

GH	Growth Hormone
IGF-1	Insulin-like Growth Factor-1
IGF-1R	Insulin-like Growth Factor-1 Receptor
Ihh	Indian Hedgehog
PTHrP	Parathyroid Hormone related Protein
BMP	Bone Morphogenetic Protein
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
Runx2	Runt-related Transcription Factor
Hdac4	Histone deacetylase 4
VEGF	Vascular Endothelial Growth Factor
PDGF	Platelet-derived Growth Factor
EGF	Epidermal Growth Factor
RANKL	Receptor Activator of Nuclear Factor Kappa-B Ligand
TRAP	Tartrate-Resistant Acid Phosphatase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
MMP-9	Matrix Metalloproteinase-9
MMP-13	Matrix Metalloproteinase-13

HIF1-α	Hypoxia Inducible Factor 1-alpha
Col2a1	Type II Collagen alpha 1 chain
Col10a1	Type 10 Collagen alpha 1 chain
Mef2c	Myocyte enhancer factor 2C
Mgp	Matrix GLA protein
ColX	Type 10 Collagen
Acan	Aggrecan
Adipoq	Adiponectin
PTCH	Protein patched homolog 1
IRX3/5	Iroquois Homeobox-containing Transcription Factors 3/5
Col1a1	Type I Collagen alpha 1
Ocn	Osteocalcin
Osx	Osterix
Sca1	Stem Cells Antigen-1
Sox2	Sex Determining Region Y-box 2
Sox9	Sex Determining Region Y-box 9
CSF-1	Colony Stimulating Factor-1
VCAM-1	Vascular Cell Adhesion Protein 1
MALP	Marrow Adipogenic Lineage Precursor
CDOP	Chondrocyte-derived Osteoprogenitor
CDK	Cyclin-dependent kinases
ER	Endoplasmic Reticulum
ECM	Extracellular Matrix
BAC	Bacterial artificial chromosome

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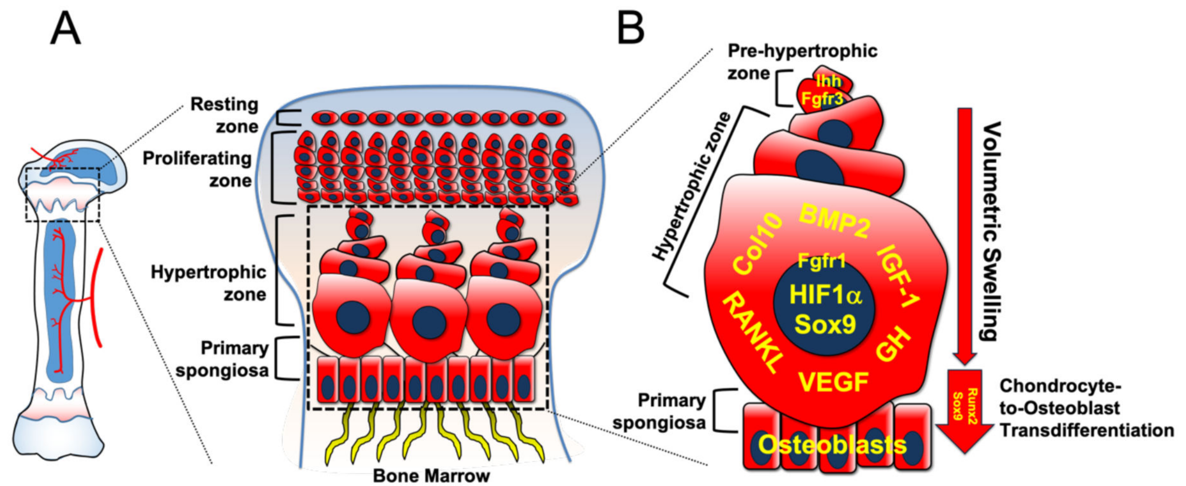

Figure 1. Multifactorial roles of hypertrophic chondrocytes and their molecular regulation.
A. Magnified graphical representation of growth plate structure and morphology.
B. Enhanced cartoon of pre-hypertrophic and hypertrophic zones and primary spongiosa. Volumetric swelling due to increased synthesis of intracellular organelles and cytoplasmic water intake facilitates progressive hypertrophic chondrocyte enlargement. GH, IGF-1, Sox9, BMP2, HIF1α and FGFRs regulate chondrocyte hypertrophy, swelling, metabolism and apoptosis. Col10 is a marker for hypertrophic chondrocytes. Runx2 and Sox9 are required for transdifferentiation of hypertrophic chondrocytes into osteoblasts.

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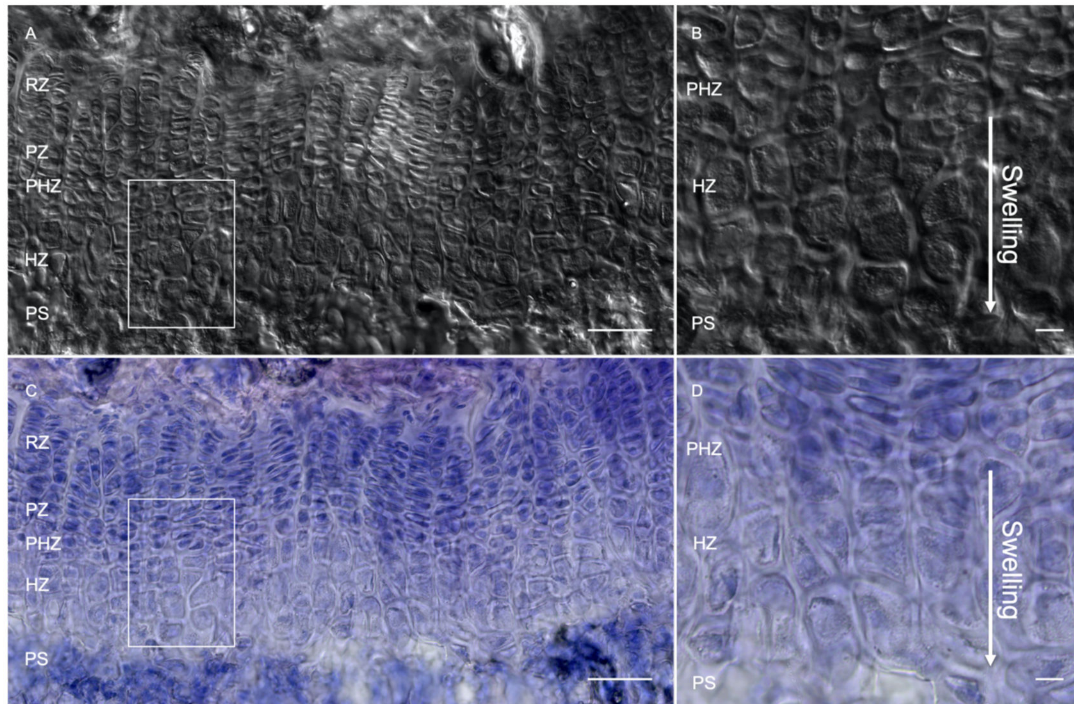

Figure 2. Morphological variation of hypertrophic chondrocytes.

Representative differential interference contrast (DIC) **(A,B)** and hematoxylin and eosin staining **(C,D)** of the growth plate at postnatal day 36 in a C57BL/6 mouse. Magnified images **(B,D)** denote cellular swelling and size variation of hypertrophic chondrocytes as they move towards the primary spongiosa. RZ: resting zone, PZ: proliferating zone, PHZ: pre-hypertrophic zone, HZ: hypertrophic zone, PS: primary spongiosa. Scale bars: 100 μM.