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Review

UNVEILING THE ROLE OF HYPERTROPHIC CHONDROCYTES IN ABNORMAL CARTILAGE CALCIFICATION: INSIGHTS INTO OSTEOARTHRITIS MECHANISMS

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Abstract

Osteoarthritis (OA) is a chronic degenerative disease that affects the whole joint, especially the knee joint. Its main features include articular cartilage defects and osteophyte formation, and it is common in middle-aged and elderly people. Although the pathogenesis of OA is not fully understood, mechanical factors, inflammation and immune abnormalities can affect joint tissue metabolism and destroy cartilage and bone homeostasis. Cartilage calcification is closely related to chondrocyte hypertrophy, differentiation and bone sclerosis in OA, which is manifested as pathological calcification of cartilage matrix. Chondrocytes in OA may change from a state of maintaining cartilage matrix balance to a state of promoting cartilage destruction and calcification. Inflammatory factors such as TNF-α and IL-1β promote this phenotypic shift, accelerating matrix degradation and calcium salt deposition. The change of calcium signal and an important factor of angiogenesis and promote cartilage calcification. Chondrocyte hypertrophy plays a crucial role in the pathogenesis and progression of OA, characterized by complex interactions with cartilage calcification, subchondral bone sclerosis, as well as chondrocyte proliferation, apoptosis, matrix remodeling, and signaling cascades. The degree of chondrocyte hypertrophy exhibits a positive correlation with the severity of OA. Furthermore, structural changes in the articular cartilage are associated with factors including reduced cartilage collagen synthesis or the activation by degradative enzymes. Regulatory mechanisms governing chondrocyte hypertrophy and cartilage calcification, alongside the identification of pertinent genes, represent pivotal areas for future investigation. This research will further elucidate the pathogenesis of OA and lay the groundwork for devising therapeutic strategies.

Keywords: Osteoarthritis, chondrocytes, hypertrophic chondrocyte, cartilage calcification.

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Introduction

Osteoarthritis (OA) affects the entire joint, encompassing the articular cartilage, subchondral bone, synovium, meniscus, and ligaments, and is primarily characterized by articular cartilage defects and osteophyte formation (Zhou *et al.*, 2023). OA commonly affects the knee, making it one of the most prevalent chronic degenerative diseases among middle-aged and elderly individuals (Geng *et al.*, 2023). As age increases, both the prevalence and severity of OA incrementally rise, reaching an incidence rate as high as 50.3 % among elderly individuals with knee OA (Bank *et al.*, 2024). Influencing factors comprise environmental, genetic, endocrinological, metabolic, biomechanical, and traumatic elements, with prior joint trauma and obesity being significant contributors to OA onset (Golightly *et al.*, 2024).

The pathogenesis of OA remains incompletely understood; mechanical factors, inflammation, and immunological abnormalities are all implicated in driving joint tissue metabolism and disrupting the homeostasis of cartilage and

bone (He *et al.*, 2020). This can result in a spectrum of manifestations, including chondrocyte hypertrophy, apoptosis, degradation of the cartilage matrix, angiogenesis and calcification in hyaline cartilage, tide mark duplication, and osteophyte formation. The tideline is a prominent banded area in the soft iliac, which marks the boundary between the superficial layer of the soft iliac (touching the joint cavity) and the deep layer (near the bone) (LongFei *et al.*, 2023). This limits under normal circumstances is a single line, but in certain pathological conditions possible copy or repeat, this is called tidemark duplication (Henrotin *et al.*, 2012). Physiological calcification and pathological calcification are two distinct biomedical processes that involve the deposition of calcium salts in the body. Physiological calcification is a normal and necessary biological process, while pathological calcification refers to the process of abnormal deposition of calcium salts in the soft tissues of the body, which is usually associated with disease or tissue damage (Yan *et al.*, 2020). Chondrocyte hypertrophy may occur during the physiological process of endochondral ossification in normal cartilage development and under pathological conditions (Xiao *et al.*, 2018). Hypertrophied chondrocytes can expand to more than ten times the size of normal cartilage cells (Chen *et al.*, 2015). The hypertrophy may be attributed to alterations in intra- and extracellular osmotic pressure, degradation and remodeling of the extracellular matrix (ECM), and an increase in intracellular organelle numbers (Hodgkinson *et al.*, 2022). Metabolic activation of chondrocytes is closely associated with early-stage OA changes in articular cartilage (Zhai, 2019). Animal studies indicate that chondrocyte proliferation and hypertrophy do not facilitate articular cartilage healing, but rather exacerbate matrix degradation and contribute to cartilage calcification (Li *et al.*, 2022c; Wang *et al.*, 2021b). Chondrocyte-mediated formation and deposition of calcium crystals, such as basic calcium phosphate (BCP) and calcium pyrophosphate dihydrate (CPPD), cause cartilage calcification. The mechanism of calcification involves regulatory enzymes and pathways for PPi, Pi, and Ca^{2+} levels (Peng *et al.*, 2024). In conclusion, inhibiting chondrocyte hypertrophy may control cartilage calcification and thus delay OA progression.

Cartilage Calcification in OA

Cartilage calcification, closely associated with chondrocyte hypertrophy differentiation and bone sclerosis, highlights the complex interplay between cellular processes and structural alterations in OA (Coaccioli *et al.*, 2022). Normally, calcification is a critical component of bone tissue formation. However, in OA, pathological calcification of the extracellular matrix in cartilage and soft tissues can manifest (Lee *et al.*, 2020). In the early stages of OA, patients often have no obvious symptoms, but changes in the subchondral bone and calcified cartilage have already begun (Shang *et al.*, 2024), underscoring the importance of early detection and intervention in managing this degenerative joint disease (Meyer *et al.*, 2021).

In OA, chondrocytes may undergo a phenotypic shift from cells that maintain cartilage matrix balance to cells that promote cartilage destruction and calcification (Zaki *et al.*, 2020). This transition includes the ability of chondrocytes to proliferate, differentiate into osteocyte-like cells, and produce calcification-promoting factors such as phosphorylated proteins and alkaline phosphatase (ALP) (Lu *et al.*, 2014). Chondrocytes produce and maintain ECM, a major component of cartilage, but in OA, ECM undergoes changes such as increased degradation of type II collagen (Col2) and increased expression of non-cartilaginous collagens such as types I and X, which may promote the calcification process (Dennis *et al.*, 2020). Increased activity of matrix metalloproteinases (MMPs) also leads to ECM breakdown, providing space for calcium salt deposition (Nicodemus *et al.*, 2011). Inflammation also plays an important role in the development of OA. Inflammatory factors such as tumor necrosis factor-α (TNF-α) and interleukins (IL-1β, IL-6, etc.) can affect the behavior of chondrocytes, promote the phenotypic transformation of chondrocytes and the degradation of ECM, and then promote cartilage calcification (Charlier *et al.*, 2016). Calcium ions have important signaling functions inside and outside the cell. In addition, the calcium signaling of chondrocytes may also be altered, resulting in abnormally elevated intracellular calcium concentrations, which can further promote chondrocyte differentiation and calcification by activating calcium-sensitive signaling pathways (Guasto and Cormier-Daire, 2021). Finally, the cartilage calcification process is closely related to angiogenesis. Chondrocytes can promote vascular invasion by producing pro-angiogenic factors such as vascular endothelial growth factor (VEGF), which not only provides the necessary nutritional support for calcification, but also may be directly involved in the calcification process (Hu and Olsen, 2016). These mechanisms interact and work together to promote cartilage calcification and the progression of OA. Cartilage calcification not only aggravates cartilage degradation, but also may aggravate joint pain and dysfunction by affecting the biomechanical properties of the joint (Peng *et al.*, 2021b) (Fig. 1).

Chondrocytes in OA

Under normal physiological conditions, chondrocytes are the only cell type in cartilage tissue and are responsible for maintaining the structure and function of cartilage. Cartilage calcification is generally considered to be a process under pathological conditions, especially in osteoarthritis and cartilage damage (Bernabei *et al.*, 2023). However, under certain physiological conditions such as chondrocytes in growth plate cartilage also participate in limited calcification processes during normal bone growth and development (Kazemi and Williams, 2021). This physiologic cartilage

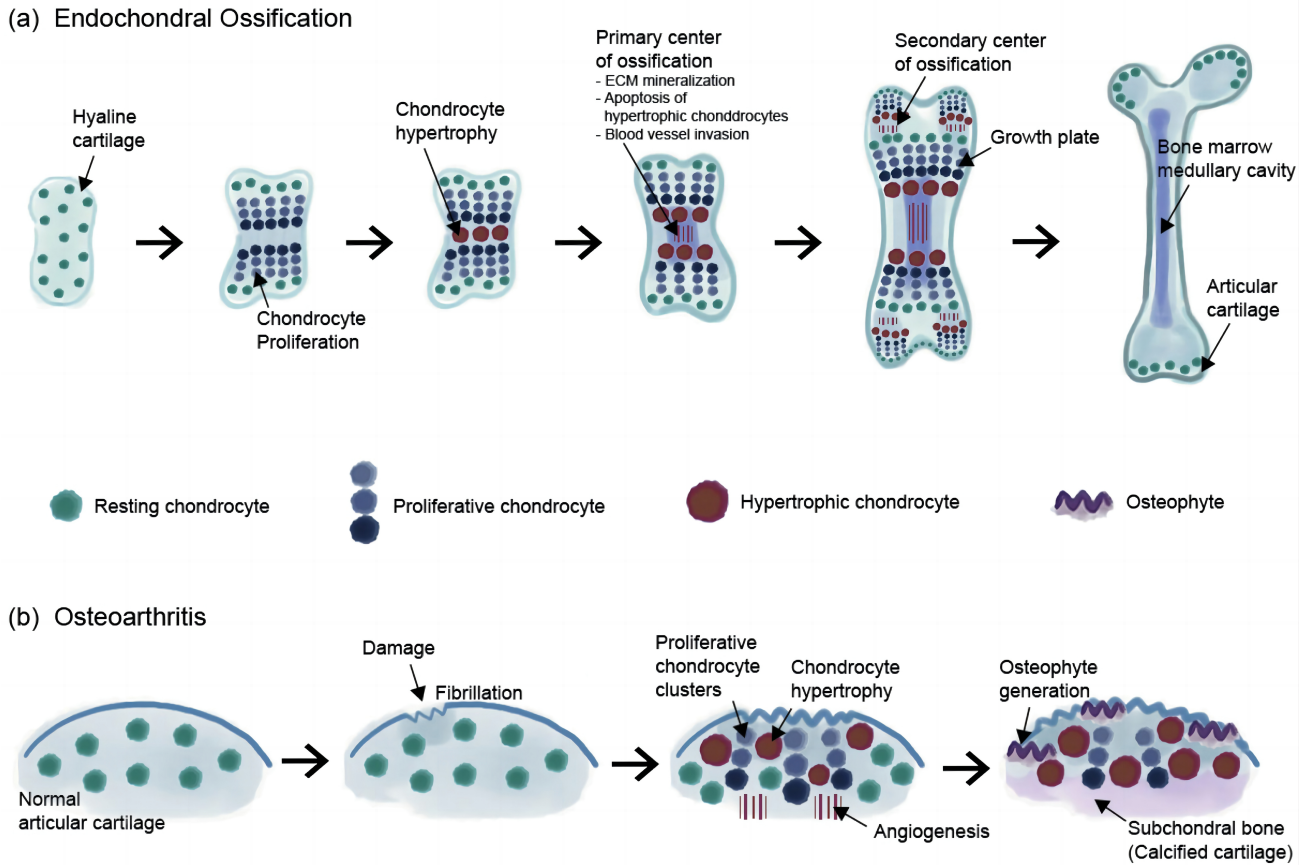

Fig. 1. Schematic image of endochondral ossification in the embryonic cartilage and progression of osteoarthritis in the articular cartilage. (a) Embryonic cartilage ossification development process. (b) Cartilaginous degeneration in osteoarthritis.

calcification occurs primarily in the growth plate region and is an essential component of bone growth and maturation. Cell types distributed in cartilage and subchondral bone include chondrocytes, osteoblasts, osteoclasts, and bone marrow mesenchymal cells (Kangari *et al.*, 2020). During bone development, chondrocytes first form cartilage templates, and then a subset of chondrocytes differentiate into ossified chondrocytes, which promote cartilage calcification and bone formation. Osteoblasts and osteoclasts participate in bone growth and repair through the process of bone remodeling to ensure the strength and health of bone (Fan *et al.*, 2021).

Under OA conditions, chondrocytes may undergo a phenotypic shift that leads to accelerated cartilage matrix degradation. With the development of single-cell sequencing technologies, such as single-cell RNA sequencing (scRNA-seq), researchers are able to reveal the cellular heterogeneity present in these tissues at unprecedented resolution (Wang *et al.*, 2021a). To further understand the role of various types of cells in developmental, physiological and pathological states. At present, the known types of chondrocytes mainly include cartilage progenitor cells, effector chondrocytes, fibrocartilage chondrocytes, homeostatic chondrocytes, hypertrophic chondrocytes, prehypertrophic chondrocytes, proliferative chondrocytes and regulatory chondrocytes. Proliferative chondrocytes are cells located in the proliferative zone, prehypertrophic chondrocytes could induce cell differentiation into hypertrophy, and Hypertrophic chondrocytes could regulate the mineralization of cartilage matrix. Chondrocyte hypertrophy and matrix lesions can cause cartilage destruction in OA. Proliferative chondrocytes, prehypertrophic chondrocytes and Hypertrophic chondrocytes, these groups have specific markers, And the potential transport relationship between them. The transition from Proliferative to more active Hypertrophic phenotype promotes chondrocyte apoptosis and calcium deposition, and eventually attracts vascular and osteocyte invasion (Hu *et al.*, 2022).

The dedifferentiation of chondrocytes into the hypertrophic stage is driven by autocrine and paracrine factors and the extracellular matrix microenvironment, and the regenerative capacity of cartilage is limited (Horváth *et al.*, 2023). However, it may utilize an intrinsic stem cell source for repair. In the early stages of joint diseases, cartilage tissue does exhibit some capacity for self-repair, potentially harnessing these intrinsic stem cells. However, this regenerative ability is limited and often insufficient to fully counteract the progressive damage seen in conditions like

OA (Trengove *et al.*, 2022). As the disease progresses, the capacity for cartilage regeneration diminishes significantly, highlighting the need for effective therapeutic interventions. In OA, chondrocytes are subjected to a variety of stimuli (such as mechanical stress, inflammatory factors, etc.) and will show differentiation (Fang *et al.*, 2021). The mechanism includes the activation of a variety of signaling pathways by external stimuli, such as Wnt/β-catenin, Notch, TGF-β and NF-κB pathways. These signaling pathways regulate gene expression and cell behavior of chondrocytes. The composition of the ECM changes, such as the change of collagen fiber type from type II to type I. These changes affect the maintenance of chondrocyte phenotype. Dedifferentiation process is accompanied by the change of the epigenetic modifications, such as DNA methylation, histone modifications, results in the decrease of cartilage cells specific gene expression (Zhou *et al.*, 2016). Articular cartilage can be repaired with targeted interventions that focus on inhibition of pathways leading to cartilage degradation (Muthu *et al.*, 2023). The process of dedifferentiation is also influenced by antigenic cellular reactions with the synovium, highlighting the importance of factors such as subchondral circulation and synovial fluid nutrition in the survival and potential repair of articular cartilage (Peng *et al.*, 2021a). In OA, chondrocytes often transdifferentiate into fibroblast-like or osteoblast-like cells. Sox9 is an important transcription factor for the maintenance of chondrocyte phenotype, while Runx2 is a transcription factor specific to osteoblasts. In OA, the expression of Sox9 decreased and Runx2 increased, which promoted the transformation of chondrocytes into osteoblast-like phenotypes (Lefebvre and Smits, 2005). Inflammatory factors such as IL-1β and TNF-α promote the transdifferentiation of chondrocytes into fibroblast-like cells, which exhibit high expression of stroma-degrading enzymes such as MMPs and further destroy articular cartilage. Efficient gene delivery for the expression of abnormal cellular phenotypes at pathological stages of the joint offers the potential for cartilage repair by targeted gene transfer, further highlighting the complexity of dedifferentiation and transdifferentiation processes (Li *et al.*, 2022a; Li *et al.*, 2022b). Chondrocytes may also undergo dedifferentiation in response to the stimulation of chondrocyte death, leading to the appearance of chondroprogenitor cells (Phull *et al.*, 2016). This endogenous mechanism is used to repair cartilage defects and prevent progressive cartilage loss, suggesting a capacity for self-repair in the early stages of joint pathology. Regarding the reversal of phenotypic alterations, this area remains a significant challenge for regenerative medicine and tissue engineering (Xu *et al.*, 2020). Strategies may include gene therapy, cell reprogramming, or the use of specific growth factors or cytokines to encourage cells to return to a more "native" phenotype (Vo *et al.*, 2012). However, the feasibility, efficiency, and long-term outcomes of these approaches require detailed study and depend on an understanding of the underlying mechanisms driving phenotypic changes (Fig. 2).

Hypertrophic Chondrocytes and Cartilage Calcification

Regulation of physiological calcification is complex, necessitating coordinated actions between calcification inhibitors and promoters. Calcification progression is bifurcated into two phases, with the initial phase entailing the formation of calcium-containing crystal precursors within the chondrocyte extracellular matrix through diverse mechanisms. This process encompasses three pathways: chondrocyte hypertrophy and proliferative differentiation, mitochondrial autophagy, and apoptosis (Proudfoot, 2019). Research supports chondrocyte hypertrophy's role in cartilage calcification during OA. In healthy cartilage, chondrocytes in the surface layer, transitional layer and radiating layer, remain quiescent and uncalcified (Das Gupta *et al.*, 2020); conversely, those in the deep layer are calcified, facilitating an appropriate biomechanical transition from uncalcified upper layers to the highly calcified subchondral bone (Fan *et al.*, 2021). Pathological calcification transpires when quiescent chondrocytes transdifferentiate into hypertrophic proliferative chondrocytes, thereby producing calcium crystals and releasing calcification-initiating matrix vesicle (MV) (Semenistaja *et al.*, 2023). The transformation of crystal precursors into mature crystals and their growth includes both intrafibrillar and extrafibrillar crystal growth, governed by conditions for crystal formation and growth, ensuring appropriate calcification localization and extension (Rey *et al.*, 1991). Regulatory conditions comprise inflammation, reactive oxygen species (ROS), reactive nitrogen species (RNS), bone morphogenetic protein (BMP), Fetuin-A, diverse proteins, and ionic imbalances (Yang *et al.*, 2023). In summary, hypertrophic chondrocytes are terminally differentiated chondrocytes (Li and Dong, 2016). They play a key role in the process of ossification, but under pathological conditions, the abnormal behavior of hypertrophic chondrocytes can lead to pathological mineralization and crystal formation. Hypertrophic chondrocytes directly initiate and promote the mineralization process of the matrix by secreting matrix vesicles and mineralization factors. Thick abnormal cartilage cells secrete pyrophosphate, lead to the formation and deposition of calcium pyrophosphage crystal. These crystals can cause an inflammatory response that further destroys the joint structure. Recent animal studies have uncovered a new microanatomical structure termed concentric lamellar layers around chondrocytes, which seem to be systematically arranged with the advancement of the tide line, indicating their potential formation during the cartilage matrix calcification process, and suggesting a significant role in the pathogenesis of OA (Keenan *et al.*, 2019).

At birth, in both humans and mice, the articular cartilage of many joints remains indistinguishable from the epiphyseal growth plate (Chijimatsu and Saito, 2019). Shortly

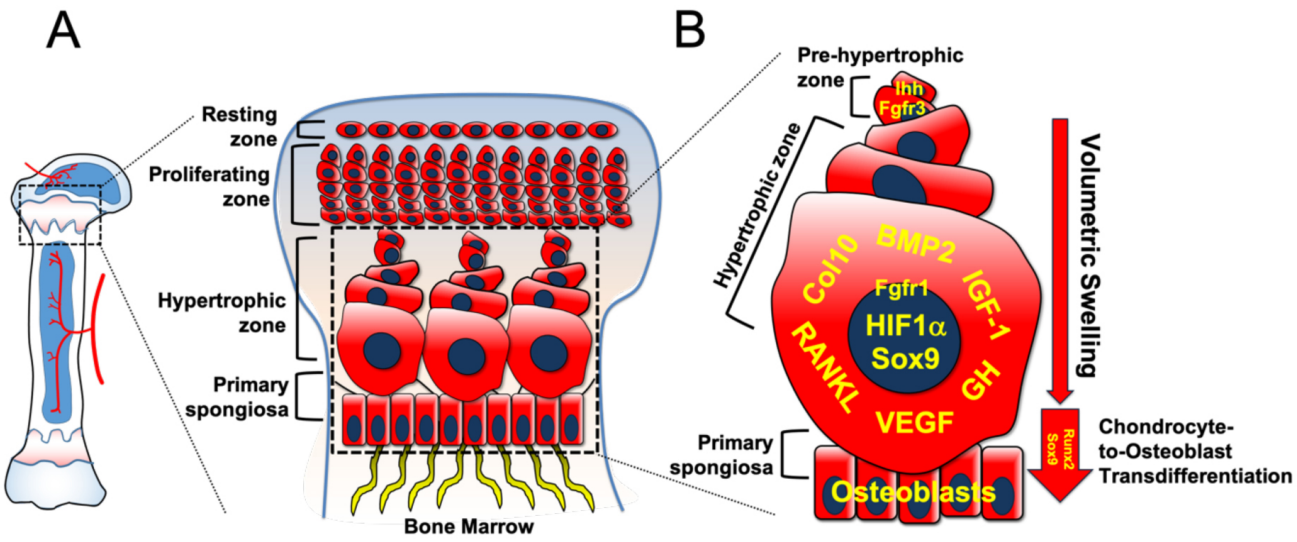

Fig. 2. Multifactorial roles of hypertrophic chondrocytes and their molecular regulation. (**A**) Magnified graphical representation of growth plate structure and morphology. (**B**) Enhanced cartoon of pre-hypertrophic and hypertrophic zones and primary spongiosa. Volumetric swelling due to increased synthesis of intracellular organelles and cytoplasmic water intake facilitates progressive hypertrophic chondrocyte enlargement. Growth hormone (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. ©The Author(s) 2021. HISTOLOGY AND HISTOPATHOLOGY. Reprinted with permission from ref (Hallett *et al.*, 2021).

after birth, secondary ossification centers (SOC) emerge within the epiphyseal cartilage, segregating it into the proximal future metaphyseal growth plate and the distal articular surface. The gradual thinning of cartilage at the distal end of the SOC suggests an endochondral ossification process akin to that occurring in the growth plate post-birth, in the subchondral regions adjacent to the joint (Xie and Chagin, 2021). Conversely, the process at the osteochondral interface must diverge significantly from that within the growth plate, given that ossification surrounding the joint must halt in a way that preserves cartilage on the joint surface (Singh *et al.*, 2021). Lastly, the bone quality of the subchondral plate, in comparison to the metaphyseal trabecular bone, exhibits differences in structure and mineral density, further supporting the idea that distinct mechanisms may govern ossification in these regions (Hu *et al.*, 2021). The cartilage-to-bone transition is pivotal for securing the skeletal bone's solid stability and rigidity (Campos *et al.*, 2019). Impediments or delays in this process can result in compromised bone healing, manifesting as nonunion or delayed union. This transition encompasses several events, notably cartilage matrix degradation, vascular invasion, and bone formation (Blumer, 2021). Hypertrophic chondrocytes initiate the degradation of the avascular cartilage matrix, facilitating the migration of other cell types and vessels. Moreover, hypertrophic chondrocytes express VEGF, inducing angiogenesis and thus accelerating cartilage matrix degradation via vascular invasion. Vascularization prompts the migration of hematopoietic lineage osteoclasts and osteoprogenitor cells, culminating in new bone formation. Additionally, hypertrophic chondrocytes can stimulate osteogenesis through the production of growth factors like BMP-2, highlighting their central role in the complex interplay of cellular and molecular events driving the ossification process (Halloran *et al.*, 2020).

Regulatory Factors of Calcification in Hypertrophic Chondrocytes

Although the mechanisms underlying chondrocyte hypertrophy remain elusive, Type X collagen, Runx2, VEGF, BMP, and Indian hedgehog (IHH) are recognized as principal markers associated with chondrocyte hypertrophy and cartilage calcification. These markers play a critical role in the complex biological processes leading to chondrocyte hypertrophy and subsequent cartilage calcification, highlighting the importance of ongoing research into their functions and interactions within the cartilage matrix (Thielen *et al.*, 2022). Show in Table 1.

Cytokine

Increased production of VEGF is a hallmark of hypertrophy and OA cartilage (Nagao *et al.*, 2017). VEGF facilitates endothelial cell migration and angiogenesis *in vivo* via chemotaxis. Additionally, VEGF stimulates angiogenesis in cartilage tissue, linked to chondrocyte calcification; such calcification may result in the dysregulation of normal cartilage ossification (Li *et al.*, 2019). VEGF, expressed by hypertrophic chondrocytes, induces the ossification center's vascularization by vessel recruitment (Su *et al.*, 2020). Inhibition of VEGF protein by chimeric VEGF-IgG results

Table 1. Major signaling factors involved in chondrocyte differentiation processes in cartilage calcification and OA.

Signaling factor	Effects on growth plate chondrocytes	Role in OA
Collagen X	Stimulates chondrocyte proliferation	Loss leads to cartilage calcification
Runx2	It positively regulated chondrocyte hypertrophy;	Induction of chondrocyte hypertrophy;
	Effect of VEGF upregulation on angiogenesis	Induction of MMP-13 expression
Fibroblast growth factors	Decrease proliferation;	Stimulation of ADAMTS-5
	Decrease hypertrophy;	
	Decrease matrix production	
IHH	Stimulates proliferation	Induction of ADAMTS-5 via Runx2
Bone morphogenic proteins	The proliferation of chondrocytes was induced;	Stimulation of MMP-13
	Chondrocyte hypertrophy was induced	
MMPs/ADAMTSs	It is important for remodeling the matrix;	Major factors in matrix degradation
	Influence bioavailability of VEGF	
CCAAT/enhancer binding	Inhibition of chondrocyte proliferation;	Mediates cartilage destruction protein beta
	Stimulation of chondrocyte hypertrophy;	
	Activation of collagen X expression	

OA, osteoarthritis; IHH, Indian hedgehog; MMPs, matrix metalloproteinases; VEGF, vascular endothelial growth factor.

in shortened femoral length and increased Col10a1 expression in the hypertrophic zone, associated with the disassembly of epiphyseal vessels (Li *et al.*, 2023). VEGF-mediated vascularization at the epiphyseal end triggers apoptosis in hypertrophic chondrocytes. Specific deletion of VEGFA in Col2a1-cre leads to reduced cartilage formation, skeletal mineralization, delayed ossification center vascularization, and elimination of hypertrophic chondrocytes (Qin *et al.*, 2020). Consequently, VEGF is essential for the survival of hypertrophic chondrocytes, underscoring its pivotal role in cartilage health and disease pathology.

In patients with OA, serum and synovial fluid levels of pro-inflammatory cytokines, such as IL-1β, TNF, IL-6, and IL-8, along with response factors including S100 proteins, ATP, and HMGB1, are elevated (van den Bosch *et al.*, 2020). Research indicates that IL-6, TNF, and S100A11 contribute to the deposition of BCP crystals in cartilage tissue by promoting chondrocyte hypertrophy and increasing the expression of Type X collagen and TNAP. Moreover, IL-8 and S100A11 amplify chondrocyte mineralization via hypertrophy and apoptosis induced by transglutaminase 2 (Bernabei *et al.*, 2023). These findings underscore the complex interplay of inflammatory cytokines and response factors in the pathophysiology of OA, highlighting their roles in promoting cartilage degradation and calcification processes.

Transcription Factor

In skeletal development, mesenchymal stem cells aggregate and differentiate into chondrocytes, with Sox9 and Col2a1 expression marking the initial step of endochondral ossification (van Gastel *et al.*, 2020). Subsequently, differentiated chondrocytes enter a brief phase of cartilage differentiation, begin proliferating, and express IHH. Following this, cells expressing ColX and Runx family members, like Runx2, ultimately differentiate into osteogenic factors (Chan *et al.*, 2021). After these steps, vascular invasion from the subchondral bone into the cartilage template occurs, leading to widespread apoptosis of hypertrophic cells and ultimately remodeling the cartilage template into trabecular bone (Kazemi and Williams, 2021). Throughout OA progression, articular chondrocytes experience significant transcriptomic and phenotypic alterations. These phenomena, observed in human patients, suggest that targeting developmental pathways activated during endochondral ossification in various animal models could offer an effective strategy to hinder OA disease progression. This approach underscores the importance of understanding the molecular and cellular mechanisms underlying cartilage development and disease to develop novel therapeutic interventions (Ferrao Blanco *et al.*, 2021; Yao *et al.*, 2023) (Fig. 3).

Sox9, a pivotal transcription factor, is instrumental in the development and maturation of cartilage (Liang *et al.*, 2020). Expressed from the multipotent skeletal progenitor cell stage, Sox9's presence is sustained in the chondrocytes of healthy articular cartilage throughout life. Sox9 plays a regulatory role in the process of hypertrophic chondrocytes participating in cartilage calcification through a variety of mechanisms (Li and Dong, 2016). Its main by inhibiting Runx2 expression, adjust the IHH and parathyroid hormone-related protein (PTHrP) signaling pathways, affect the Wnt/β - catenin signaling pathway and the promotion of the ECM proteins expression to realize these functions. Recent studies have further revealed the complex interactions of Sox9 with other factors and pathways. For example, the interaction between Sox9 and YAP/TAZ signaling has also been found to play a key role in cartilage calcification (Kovar *et al.*, 2020). YAP/TAZ are effector molecules of the Hippo signaling pathway, and their activity is closely related to cell proliferation and differentiation (Setiawan *et al.*, 2021). Sox9 affects the fate of hypertrophic chondrocytes by regulating the activity of

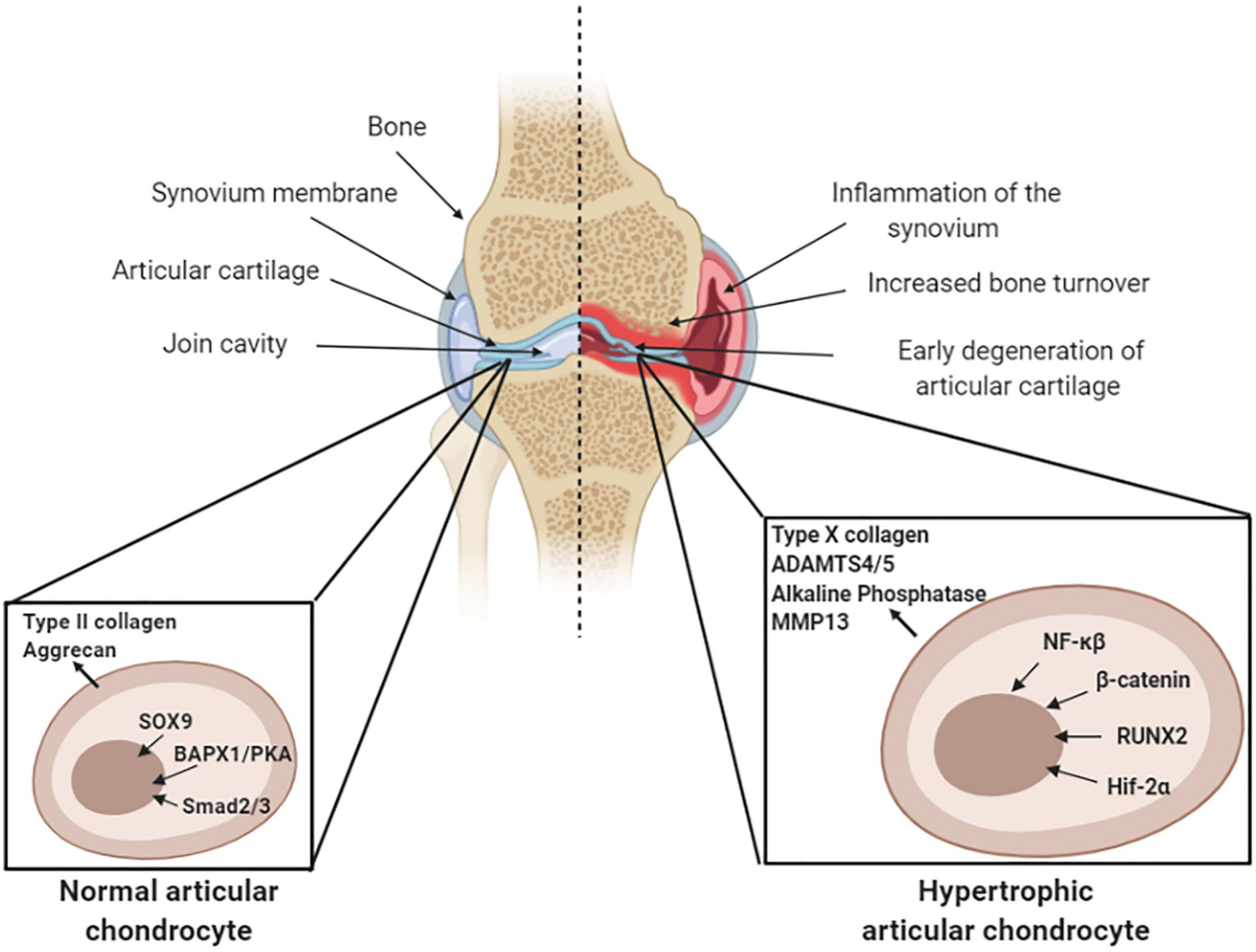

Fig. 3. Schematic representation of main characteristics of normal articular cartilage compared to an osteoarthritic cartilage. On the left, a normal articular chondrocyte is depicted together with the positive stimuli that induce chondrocyte homeostasis. Conversely, on the right a hypertrophic chondrocyte is shown, including the signaling and responses that occur during OA. © 2021 The Authors. Reprinted with permission from ref (Ferrao Blanco *et al*., 2021) Osteoarthritis Research Society International.

YAP/TAZ. This highlights the essential role of Sox9 in maintaining cartilage integrity and the complexity of its regulation, underscoring the potential therapeutic targets for cartilage-related disorders.

Runx2 is a key transcription factor in the differentiation of hypertrophic chondrocytes and early osteogenesis (Komori, 2020). Consequently, Runx2 is regarded as a primary transcription factor that directly regulates the expression of matrix-degrading enzymes in damaged articular cartilage (Gu *et al.*, 2014). Research indicates that Runx2 expression precedes chondrocyte differentiation into hypertrophic chondrocytes, implying its involvement in the early stages of cartilage formation. Yoon *et al.* (2023) suggested that regulating Runx2 expression could correct phenotypic changes induced by long noncoding RNA (lncRNA), thereby mitigating hypertrophic alterations during mesenchymal stem cells' chondrogenic differentiation. Originally identified as an osteoblastogenesis regulator, Runx2 also influences chondrocyte hypertrophy, transdifferentiation, vascular invasion, and matrix deposition within the hypertrophic zone. This underscores the multifaceted role of Runx2 in skeletal development and its potential as a therapeutic target in OA (Rashid *et al.*, 2021).

CCAAT/enhancer-binding protein β (C/EBPβ) is recognized as a transcription factor involved in chondrocyte terminal differentiation and OA. During chondrocyte hypertrophy, C/EBPβ and activating transcription factor 4 (ATF4) serve as coactivators for Runx2. Research indicates that C/EBPβ both directly and indirectly suppresses Col2a1's transcriptional activity by modulating Sox9 expression, thereby facilitating the phenotypic transition from proliferative to hypertrophic chondrocytes during chondrocyte differentiation (Nishimura *et al.*, 2017). However, other studies suggest that C/EBPβ's role in regulating chondrocyte hypertrophy is significant in the early stages of OA, yet diminishes in the terminal stages (Ushijima *et al.*, 2014). This highlights the complex regulatory mechanisms governing chondrocyte differentiation and the potential temporal dynamics of C/EBPβ's involvement in OA progression (Shimada *et al.*, 2011).

HIF-2α, a transcription factor, is markedly expressed in both human OA cartilage and animal OA models. It plays a crucial role in the terminal differentiation of chondrocytes. HIF-2α leads to cartilage destruction by stimulating chondrocyte terminal differentiation and upregulating catabolic enzymes (Zhou *et al.*, 2021). This underlines the pivotal role of HIF-2α in the pathology of OA, suggesting its potential as a target for therapeutic intervention to mitigate cartilage degradation (Zhang *et al.*, 2016b).

Smads represent the pivotal transcription factors within the Transforming growth factor-β (TGF-β) family, encompassing TGF-β and BMP (Zou *et al.*, 2021). TGFs are activated by SMAD 2/3 and BMPs by SMAD 1/5/8, and, that there are suggestions that an imbalance of TGF/BMP signaling triggers transdifferentaition of articular chondrocytes to the epiphyseal phenotype (Scharstuhl *et al.*, 2002). These factors regulate chondrocyte hypertrophy through the modulation of Runx2 function. Smad proteins are phosphorylated by activin-like kinase, type I receptors of the TGF-β family (Tzavlaki and Moustakas, 2020). Activin-like kinases dictate which Smad proteins undergo phosphorylation. Activin-like kinases 4/5/7 phosphorylate Smad 2/3, whereas Smad 1/5/8 undergo phosphorylation by activin-like kinases 1/2/3/6. This phosphorylation process is crucial for the regulation of chondrocyte hypertrophy, illustrating the complex interplay between TGF-β signaling pathways and chondrocyte differentiation and growth (Shi *et al.*, 2022).

Signal Pathways

The Wnt signaling pathway is crucial for cartilage growth, development, and the hypertrophic differentiation of chondrocytes. Research indicates that inhibiting Wnt/β-catenin signaling suppresses rspo2-induced β-catenin accumulation and Lrp6 phosphorylation, a finding validated in an OA rat model that demonstrated reduced joint pathology (Okura *et al.*, 2019). Canonical Wnt signaling induces cartilage formation, whereas its abnormal activation promotes premature chondrocyte differentiation with increased Col10a1 expression, accelerating the onset of an OA-like phenotype. This underscores the dual role of Wnt signaling in cartilage physiology and pathology, highlighting potential therapeutic targets for OA intervention (Feng *et al.*, 2024; Feng *et al.*, 2022; Usami *et al.*, 2016). The synergistic activation of the two pathways, Wnt/β-catenin and NF-κB, exacerbates the inflammatory response and matrix degradation, leading to rapid destruction of cartilage tissue. In OA, the activation of Wnt signaling pathway promotes the activity of NF-κB and further amplifies the inflammatory response.

The extensive TGF-β family of ligands, upon binding to their corresponding receptors, induces phosphorylation changes. These mediate the progressive transmission of biological signals from the cell membrane to the nucleus, thereby activating or inhibiting the transcription of target genes (Jia and Meng, 2021). Research has established that the TGF-β signaling pathway plays a pivotal role in regulating chondrocyte proliferation and differentiation, thus impacting the onset and progression of OA. This highlights the critical function of TGF-β signaling in cartilage biology and its potential as a therapeutic target in managing OA (Du *et al.*, 2023). TGF-β and PI3K/Akt signaling together promote cartilage matrix production and cell survival (Sun *et al.*, 2020). TGF-β can promote the proliferation and matrix synthesis of chondrocytes through Akt pathway, which contributes to the repair and maintenance of cartilage tissue. However, Wnt/β-catenin and TGF-β pathways show antagonistic effects on chondrocyte differentiation and matrix metabolism. Activation of the Wnt signaling pathway inhibits TGF-β-mediated chondrogenesis, while TGF-β can inhibit the activity of the Wnt signaling pathway and alleviate chondrocyte dedifferentiation.

Parathyroid hormone-related protein (PTHrP) inhibits chondrocyte hypertrophy *in vivo*. PTHrP rapidly reduces HDAC4 phosphorylation levels in cultured cells, subsequently inhibiting Col10a1 expression (Nishimori *et al.*, 2019a). Mouse genetic models have demonstrated that PTHrP-mediated HDAC4 dephosphorylation facilitates its nuclear entry and inhibits the transcriptional activity of Salt-Inducible Kinase 3, thus preventing hypertrophic-like changes in chondrocytes. This underscores the significant role of PTHrP in modulating chondrocyte differentiation pathways and suggests a potential therapeutic mechanism for preventing chondrocyte hypertrophy and associated pathologies (Darling and Cohen, 2021; Jagannath *et al.*, 2023; Nishimori *et al.*, 2019b).

IHH expression in pre-hypertrophic chondrocytes regulates the rate of chondrocyte differentiation (Cong *et al.*, 2022). Within a paracrine-controlled feedback loop, chondrocyte-derived IHH prompts perichondrial cells to produce PTHrP, delaying late-stage differentiation during the late proliferative phase (Shen *et al.*, 2023). Furthermore, Hedgehog family proteins can expedite hypertrophic chondrocyte differentiation without PTHrP involvement, both *in vitro* and *in vivo* (Ohba, 2020). IHH, expressed by pre-hypertrophic chondrocytes, synergizes with PTHrP from resting chondrocytes to maintain growth plate integrity and longitudinal bone growth. Mice with IHH deficiency exhibit impaired chondrocyte differentiation and mineralization due to delayed Col10a1 expression (Fan *et al.*, 2022). Thus, IHH indirectly modulates chondrocyte hypertrophy via interactions with adjacent chondrocyte layers, highlighting its crucial role in skeletal development and potential as a therapeutic target in osteoarthritis management.

The ERK signaling pathway is essential for the hypertrophy and terminal differentiation of chondrocytes. Inhibition of the ERK signaling pathway activation suppresses matrix mineralization and accumulation. This highlights the pathway's pivotal role in chondrocyte development and suggests that targeting ERK signaling could be a therapeu-

tic strategy to mitigate pathological changes associated with cartilage diseases (Cheng *et al.*, 2020; Ibarra *et al.*, 2021).

Noncoding RNA

MicroRNAs (miRNAs) have a functional impact on regulating chondrocyte differentiation and the progression of OA (Zhou *et al.*, 2019; Zhou *et al.*, 2020). miR-1 impacts endochondral ossification via the IHH pathway by modulating chondrocyte proliferation, hypertrophic differentiation, and apoptosis, resulting in diminished terminal differentiation in the hypertrophic zone of experimental mice (Cong *et al.*, 2022). Ding *et al.* (2021) employed miR-1-3p silencing techniques *in vitro* to regulate Sox9's role in abnormal ossification, discovering that silencing led to increased Sox9 expression and significantly reduced mineralized nodule formation by chondrocytes. This underscores the critical regulatory role of miRNAs in cartilage development and pathology, highlighting potential therapeutic targets for OA intervention. miRNAs regulate gene expression by binding to the 3′ untranslated regions (3′UTRs) of target mRNAs, leading to mRNA degradation or translational inhibition. This mechanism can control the expression of key genes such as Runx2, Sox9, and MMP-13, which are central to chondrocyte hypertrophy and calcification.

LncRNA serve as critical regulators across a myriad of biological processes. The upregulation of lncRNA can inhibit the expression of genes pivotal to cartilage formation, including Sox9 and Col2a1, and genes implicated in hypertrophy, like Runx2 and Col10a1 (Cao *et al.*, 2017). Utilizing bioinformatics approaches to predict the target genes of lncRNA facilitates the regulation of genes involved in chondrocyte hypertrophy. This highlights the potential of lncRNA as therapeutic targets for modulating cartilage development and addressing conditions such as osteoarthritis through precise genetic regulation. MEG3 has been found to regulate the BMP signaling pathway in chondrocytes, potentially influencing hypertrophy and calcification through this pathway. H19 regulates development in various tissues and cell types and is known to affect chondrocyte differentiation and hypertrophy by modulating the Wnt/β-catenin signaling pathway.

Circular RNAs (circRNAs) can act as "sponges" for miRNAs, sequestering and inhibiting their activity, thereby indirectly increasing the expression of miRNA targets, such as transcription factors and signaling molecules. Such as ciRS-7 known as a sponge for miR-7, it may regulate pathways affected by miR-7 in cartilaginous tissues, thereby influencing cellular behaviors including hypertrophy and calcification.

Protein

Ectonucleotide Pyrophosphatase/Phosphodiesterase Family Member 1 catalyzes the conversion of ATP to inorganic PPi. Excessive PPi reacts with Ca^{2+} to form amorphous calcium pyrophosphate precursors, culminating in CPP crystal formation. Additionally, tissue-nonspecific alkaline phosphatase hydrolyzes PPi into inorganic Pi, which is then transported into MV through sodium-dependent phosphate transporters PiT-1 and PiT-2 (Hasegawa *et al.*, 2022). Concurrent concentration of Ca^{2+} in matrix vesicles by annexin A5 results in the formation of amorphous calcium phosphate precursor. Within matrix vesicles' inner membrane, phosphatidylserine aids in stabilizing ACP. Upon binding to collagen fibrils, MV release amorphous calcium phosphate precursor, which then transforms into BCP. This sequence elucidates the complex biochemical pathways involved in cartilage mineralization and highlights potential targets for therapeutic intervention in calcification-related disorders (Ferreira *et al.*, 2023) (Fig. 4).

Collagen X serves as a primary marker for detecting chondrocyte hypertrophy (Carroll *et al.*, 2022). Typically, this collagen type is not expressed in healthy articular cartilage. Research suggests its role in the early stages of endochondral bone formation, evident from its detection in regions of hypertrophic chondrocytes and calcification sites. Additionally, Collagen X and its mRNA levels have been detected in human OA cartilage (Mangiavini *et al.*, 2022). This highlights the significant involvement of Collagen X in the pathological changes associated with OA and its potential as a diagnostic marker for cartilage degeneration (Jørgensen *et al.*, 2022).

Matrix metalloproteinases, including MMP3, MMP13, ADAMTS4, and ADAMTS5, are inducible by BCP crystals and have been linked to cartilage degradation in experimental models of OA (Chhana *et al.*, 2023). Similarly, CPP crystals can induce MMP13 in human primary chondrocytes. Mechanistically, calcium ions significantly contribute to the BCP crystal-induced production of MMP13, ADAMTS5, and IL-6 (An *et al.*, 2020). In a particular study, phosphorylated CaMK2 and the transcription factor Hes1 formed a protein complex, potentially inducing OA progression via MMP13, IL-6, and ADAMTS5. This underscores the intricate interplay between biochemical factors and molecular signaling pathways in OA pathogenesis, highlighting potential targets for therapeutic intervention (Xiao *et al.*, 2023).

Growth factors, including BMP and various cytokines, significantly influence chondrocyte hypertrophy (Zhong *et al.*, 2015). While various BMPs enhance cartilage formation, dysregulated BMP levels may precipitate cartilage calcification and accelerate degeneration (Wu *et al.*, 2024). BMP stimulates DNA synthesis and cell replication, promoting mesenchymal cell differentiation into osteoblasts. Research exploring the BMP-chondrocyte hypertrophy relationship has analyzed novel BMP-2 binding proteins and antagonists within the chondrocyte extracellular matrix, suggesting that inhibiting BMP-2/Smad1 activity could suppress premature chondrocyte hypertrophy, as evidenced by quantified Col10a1 gene expression and

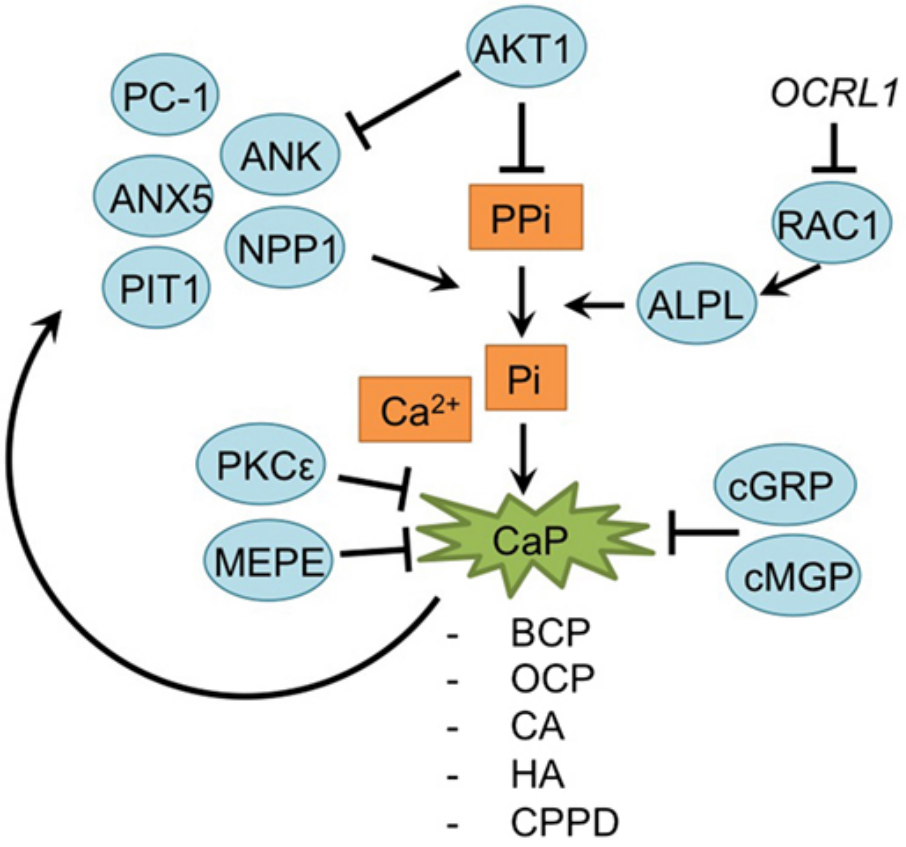

Fig. 4. Overview of the mineralization process and newly acquired insights into its relation to the development of the osteoarthritic (OA) chondrocyte hypertrophic phenotype. The mineralization process was updated with recent insights in the role of mineralization in the hypertrophic switch occuring during OA development. © 2018 Ripmeester, Timur, Caron and Welting. Reprinted with permission from ref (Ripmeester *et al.*, 2018) Front. Bioeng. Biotechnol.

Smad1 activity. In human cartilage, BMP proteins localize to hypertrophic chondrocytes within calcified areas (Yang *et al.*, 2014). BMP2 signaling induces the transdifferentiation of mesenchymal stem cells into proliferative chondrocytes and calcification. Furthermore, BMP proteins may induce chondrocyte hypertrophy and cartilage crystal nucleation through mechanisms like mitochondrial autophagy and apoptosis, highlighting their complex role in cartilage biology and pathology (Anderson *et al.*, 2000).

Bone sialoprotein (BSP), an anionic extracellular matrix protein, can induce basic calcium phosphate BCP. The glutamic acid region of BSP is involved in nucleation, and BSP further induces chondrocyte hypertrophy (Sadowska and Ginebra, 2020). In human OA, BSP expression is limited to proliferative calcifying chondrocytes. Additionally, BSP acts as an active regulator of crystal growth; it binds to collagen, and its phosphorylation amplifies crystal growth tenfold relative to its unphosphorylated form (Zhang *et al.*, 2016a). This highlights BSP's multifaceted role in OA pathogenesis, from promoting chondrocyte hypertrophy to facilitating crystal growth, underscoring its potential as a target for therapeutic intervention in OA.

Dentin matrix protein 1 (DMP1), expressed in cartilage, exhibits opposing roles in crystal nucleation and growth (Lin *et al.*, 2014). It suppresses crystal nucleation and OA development in mice by inhibiting chondrocyte transdifferentiation and hypertrophy (Bernabei *et al.*, 2023). Conversely, basic calcium phosphate BCP crystals persist in growth on collagen fibers *in vitro*, with Dmp1$^{-/-}$ mice displaying delayed calcification in calcifying cartilage and subchondral bone. Overall, phosphorylation is crucial for DMP1's function as a calcification inhibitor, whereas dephosphorylation transforms it into a calcification activator. This elucidates the significant impact of DMP1 and its post-translational modifications on cartilage health and disease, highlighting a potential therapeutic target for managing OA and related calcification disorders.

Organelle

Mitochondria play a role in a wide array of cellular processes, such as apoptosis, aging, and pathological states, including calcification in OA. Notable mitochondrial alterations include mitochondrial respiratory chain (MRC) dysfunction and ROS production (Geurts *et al.*, 2020). Considering ATP as the primary anionic source for CPPD, its formation or that of other microcrystals can be modulated by the balance of ATP production and consumption, as mediated by mitochondrial MRC activity (Franklin *et al.*, 2016).

Mitochondria contain enzymes that regulate their activity and function, providing ATP through the oxidation of organic Pi. Research assessing OA progression in mice with homozygous mitochondrial DNA mutations reported an increase in hypertrophic chondrocytes within calcifying cartilage of the joints (Geurts *et al.*, 2020). Observations included lower-level cartilage degeneration, primarily characterized by the loss of proteoglycans. Somatic mitochondrial DNA mutations may result in elevated subchondral bone turnover and hypertrophy in calcifying cartilage, underscoring the critical role of mitochondrial function and genetic integrity in the pathogenesis of OA (He *et al.*, 2020).

Conclusion

The development and progression of OA are intrinsically linked to chondrocyte hypertrophy, apoptosis, and calcification. While not all instances of cartilage calcification are associated with chondrocyte hypertrophy, hypertrophic chondrocytes serve as critical intermediaries in the calcification of OA lesions, subchondral bone sclerosis, and the eventual progression to the terminal phase of the growth plate. Furthermore, markers indicative of chondrocyte hypertrophy positively correlates with OA severity. Despite extensive research, a comprehensive understanding of the interactions among chondrocyte behavior, growth, death, matrix remodeling, and signaling pathways remains elusive. Chondrocyte hypertrophy's impact on cartilage calcification involves complex regulatory mechanisms, highlighting the need to identify key genes regulating chondrocyte hypertrophy. Recent studies have increasingly concentrated on structural changes in joint cartilage due to reduced collagen production or the induction of degrading enzymes. This direction will guide future research into the mechanisms underlying OA development. Targeting specific signaling pathways and molecular mechanisms is crucial for developing effective therapeutic strategies. Key pathways such as Wnt/β-catenin, TGF-β/SMAD, NF-κB, and Notch play significant roles in chondrocyte hypertrophy and calcification. Interventions like small molecule inhibitors, gene therapy, biologics, and cell therapy hold promise in modulating these pathways. Current clinical trials are exploring the efficacy of Wnt signaling inhibitors (e.g., Lorecivivint), TGF-β pathway modulators, and γ-secretase inhibitors for Notch signaling. Future research directions should focus on multi-targeted approaches, personalized medicine, and innovative drug delivery systems to enhance treatment efficacy and joint health. These studies will significantly propel the understanding of the pathogenesis of degenerative joint diseases, laying the foundation for developing targeted therapeutic strategies.

List of Abbreviations

3′UTRs, 3′ untranslated regions; ALP, alkaline phosphatase; ATF4, activating transcription factor 4; BCP, Basic calcium phosphate; BMP, Bone morphogenetic protein; BSP, Bone saloprotein; C/EBPβ, CCAAT/enhancer-binding protein β; CPPD, Calcium pyrophosphate dihydrate deposition disease; CircRNAs, Circular RNAs; DMP, Dentin matrix protein; ECM, Extracellular matrix; GH, Growth hormone; IHH, Indian hedgehog; IL, interleukin; LncRNA, Long noncoding RNA; MV, Matrix vesicle; MirR, MicroRNA; MRC, mitochondrial respiratory chain; OA, Osteoarthritis; PTHrP, Parathyroid hormone-related peptide; ROS, Reactive oxygen species; RNS, Reactive nitrogen species; scRNA-seq, single-cell RNA sequencing; SOC, Secondary ossification center; TGF, Transforming growth factor; TNF, tumor necrosis factor; VEGF, Vascular endothelial growth factor.

Author Contributions

Conceptualization: JJS, DMX; Design: ZNH, YPY; Funding acquisition: YPW, XDZ; Supervision: ZNH, YPY, XFW; Materials: YH, JJS; Interpretation: YPW, ZNH; Literature Review: XDZ, DMX, JPY, JX, XFW; Writing – original draft: JPY, JX; Writing – review & editing: JPY, YH. All authors contributed to editorial changes in the manuscript, read and approved the final manuscript, and have participated sufficiently in the work to take public responsibility for appropriate portions of the content. All authors have 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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