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Multiple roles of ALK3 in osteoarthritis

A NARRATIVE REVIEW

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Osteoarthritis (OA) is a chronic degenerative joint disease characterized by progressive cartilage degradation, synovial membrane inflammation, osteophyte formation, and subchondral bone sclerosis. Pathological changes in cartilage and subchondral bone are the main processes in OA. In recent decades, many studies have demonstrated that activin-like kinase 3 (ALK3), a bone morphogenetic protein receptor, is essential for cartilage formation, osteogenesis, and postnatal skeletal development. Although the role of bone morphogenetic protein (BMP) signalling in articular cartilage and bone has been extensively studied, many new discoveries have been made in recent years around ALK3 targets in articular cartilage, subchondral bone, and the interaction between the two, broadening the original knowledge of the relationship between ALK3 and OA. In this review, we focus on the roles of ALK3 in OA, including cartilage and subchondral bone and related cells. It may be helpful to seek more efficient drugs or treatments for OA based on ALK3 signalling in future.

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Keywords: ALK3, Osteoarthritis, Joint

Article focus

- The important pathological changes of osteoarthritis (OA) mainly include cartilage degeneration and sclerosis of subchondral bone.
- Summarizing the mechanisms of bone morphogenetic protein (BMP) signalling in cartilage and subchondral bone-associated cells mediated by activin-like kinase 3 (ALK3) is important to gain insight into OA.
- Understanding the role of ALK3 in the pathogenesis of OA helps in the design of new treatment options.

Key messages

- OA is a highly prevalent degenerative joint disease, and its lesions in the articular cartilage and subchondral bone are worthy of attention.
- ALK3 regulates the differentiation and maturation of chondrocytes and acts as a key point of crosstalk between osteoblasts, osteoclasts, and osteocytes, thus affecting cartilage and bone development and pathological processes.
- Considering the side effects and limitations of current clinical OA treatment, there is a need to explore the clinical application of efficient drugs targeting the ALK3-mediated BMP signalling pathway.

Strengths and limitations

- This review addresses a prevalent clinical disease – OA – and summarizes the signalling mechanisms mediated by ALK3 in the pathophysiology of articular cartilage and bone and related cells.
- By reviewing new findings in recent years surrounding ALK3 signalling in articular cartilage and subchondral bone and their cellular interactions, we broaden our understanding of the impact of ALK3 involvement in the pathological process of OA and provide new strategies for future pharmacological treatment of OA based on ALK3 and its mediated BMP signalling pathway.
- Although the authors have tried to remain objective, there will be an inevitable degree of bias and subjectivity. This review discusses the association of ALK3

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with OA-related pathological changes in articular cartilage and subchondral bone, while pathological changes in other tissues (e.g. synovium) are not included.

Introduction

Osteoarthritis (OA), the most common form of arthritis, typically affects the hips, knees, hands, feet, and spine, with a high prevalence of polyarticular involvement. OA commonly results in cartilage damage accompanied by bone lesions, synovial membrane inflammation, osteophyte formation, and subchondral bone sclerosis. Activin-like kinase 3 (ALK3), as a transmembrane receptor, is widely expressed in different organs and tissues and is vitally involved in the physiology of articular cartilage[1] and subchondral bone,[2] and in the pathology of OA.[3] In recent decades, many studies have focused on the roles of ALK3 in OA from different perspectives; we believe a summary of these studies is necessary.

ALK3 belongs to the TGFβ-BMP superfamily. ALK3 is part of the receptor complexes in the transforming growth factor β (TGFβ)- bone morphogenetic protein (BMP) signalling pathway. Therefore, to study ALK3, a basic understanding of TGFβ-BMP signalling is necessary. TGFβ is a pleiotropic cytokine belonging to the TGF superfamily that exists in a variety of organisms. In the human body, there are 33 members of the TGFβ superfamily, including TGFβ itself, BMP, growth differentiation factor (GDF), and other related proteins.[4] TGFβ can control cell fate and further regulate embryonic development, angiogenesis, and other physiological processes.[4,5] It is secreted as an inactive complex, and has to be activated by a protease before binding to its receptor and playing a role in controlling cell fate.[6] Its receptors are composed of type I receptors (TGFβRI), which are divided into ALK4/5/7[7] and type II receptor (TGFβRII). TGFβ signals through the Smad-dependent or Smad-independent signalling pathway to exert biological functions (Figure 1).[8,9] The Smad-dependent pathway mainly relies on Smad 2/3 to transmit signals into the nucleus. In the Smad-independent signalling pathway, TGFβ receptors activate other signalling molecules, such as P38, and thus initiate the mitogen-activated protein kinase (MAPK) signalling pathway.[10]

BMP is an acid glycoprotein that belongs to the TGFβ superfamily. More than 40 members of the BMP have been discovered thus far. They can affect bone formation, cartilage formation, and tendon development, and are vitally involved in tissue homeostasis and embryogenesis.[11] The signal transduction caused by the interaction of BMP and BMP receptors is very important for the formation and development of cartilage. BMP receptors are serine/threonine kinase receptors belonging to the TGFβ receptor superfamily, which includes four different type I receptors: BMPR1a (ALK3), BMPR1b (ALK6), ACVR1 (ALK2), and ACVRL1 (ALK1);[7] and three different type II receptors: activin receptor 2 A (ACTR2A), activin receptor 2B (ACTR2B), and BMPR2.[12] With BMPs binding to their heterodimeric receptors, BMPR1 is activated by means of transphosphorylation of BMPR2; thus, regulated-Smad (R-Smad) 1/5/8 are phosphorylated by BMP receptor kinases.[7] Activated R-Smads form a multimeric protein complex with common-Smad (Co-Smad) 4, and then the complex translocates into the nucleus to act on the corresponding targets (DNA elements) to regulate the transcription of BMP-related genes (Figure 1). However, inhibitory Smads (I-Smads) 6/7 can interfere with the phosphorylation of Smad 1/5/8 by binding to BMPR1. Intracellular Smad-binding proteins such as Ski, Tob, or Nanog can also inhibit the BMP signalling pathway.[13] BMP antagonists such as noggin, chordin, and differential screening-selected gene aberrative in neuroblastoma (DAN) can regulate the bioavailability of BMPR by binding to BMP with high affinity.[14] Therefore, these molecules can inhibit the BMP signalling pathway by acting on BMP ligands, receptors, or downstream Smads.

The definition, distribution, and functions of ALK3. ALK3, as a BMP type I receptor on cell membrane, can be encoded by the ALK3 gene, also known as BMPR1a, 1110037I22Rik (in mice), and 10q23del (in humans), and is widely expressed in various tissues, organs, and cells. Its expression in articular cartilage and subchondral bone is of interest (Figure 2). ALK3 is a key molecule involved in cartilage formation and osteogenesis, and is essential during early embryonic development. In previous studies, the biological role of ALK3 in bone and cartilage was not known, especially in postnatal mice, since the loss of ALK3 in the early stage of mouse development can lead to embryo death.[15] However, later studies have found that ALK3 conditional knockout (cKO) in only articular regions of mice can be used to bypass embryonic death for subsequent studies.[1] For example, some studies used the Cre/lox P system induced by tamoxifen to disrupt the expression of ALK3 in a bone-specific or age-dependent manner, thereby exploring the specific role of the BMP signalling pathway in osteoblasts.[16,17] In addition to affecting the cartilage and bones of the limbs, the BMP signalling pathway also plays an important role in the development of the teeth and palate. Li et al[18] inhibited BMP signalling in the mouse palatal mesenchyme by overexpressing Noggin through Osr2-cre^{KI} and showed that the mouse exhibited complete cleft palate. In addition, structural activation of ALK3 in the palatal mesenchyme also led to similar results. The roles of ALK3 in articular cartilage and subchondral bone are the focus of the discussion in this review.

The structure of articular cartilage under physiological conditions and OA

The articular cartilage covers the articular surface and plays a role in lubrication and cushioning in joints, which is conducive to normal joint function. The articular cartilage contains four layers vertically, namely the tangential zone, transitional zone, radial zone, and calcified zone.[19] The superficial tangential zone accommodates flat-shaped chondrocytes and collagen fibres arranged along the surface; the transitional zone has a higher concentration

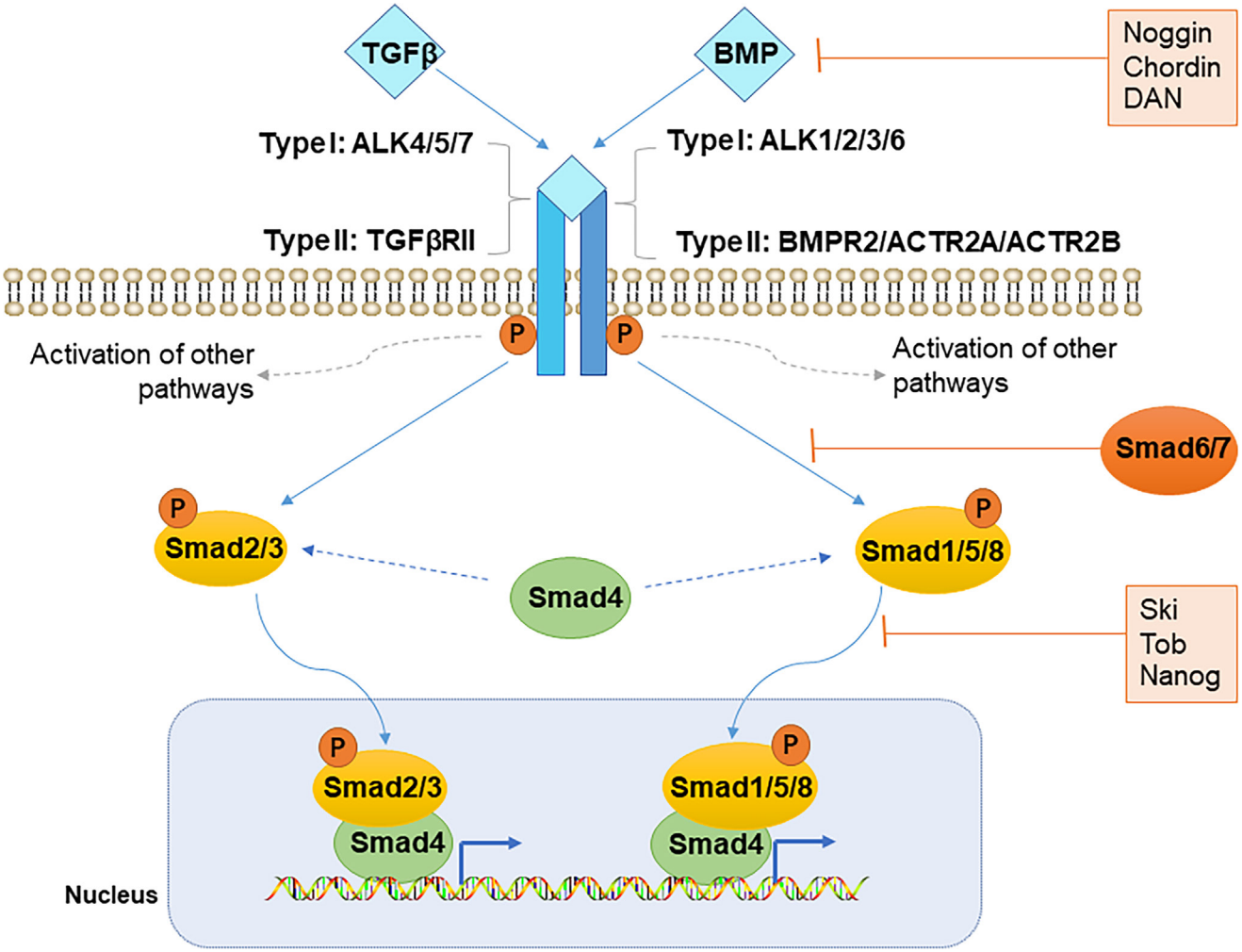

Fig. 1

Signalling by the bone morphogenetic protein (BMP) and transforming growth factor β (TGFβ) signalling. There are two Smad-based canonical pathways: Smad 2/3 for TGFβ ligands, and Smad 1/5/8 for BMPs. In the Smad-dependent signalling pathway, TGFβ/BMP binds to the receptor complex on the cell surface, which phosphorylates Smad2/3 and Smad1/5/8, respectively. Activated R-Smads form a complex with Smad4, and then transport into the nucleus to regulate gene transcription. Receptors can also activate other signalling pathways. In addition, BMP signalling pathway can be tempered by extracellular BMP antagonists (noggin, chordin, and differential screening-selected gene aberrative in neuroblastoma (DAN)), inhibitors of R-Smads phosphorylation (Smad6/7) and intracellular Smad-binding proteins (Ski, Tob, and Nanog). ACTR2A, activin receptor 2 A; ACTR2B, activin receptor 2B; ALK, activin-like kinase; TGFβRII, transforming growth factor β receptor type II; BMPR2, bone morphogenetic protein receptor type 2.

of proteoglycan aggrecan and a lower density of cells; the chondrocytes in the radial zone organize in vertical columns and secrete collagen II and aggrecan, which give the cartilage elasticity; and the chondrocytes in the calcified zone constitute the key tissue boundary between subchondral bone and articular cartilage.[20-23] Previous studies have mostly focused on collagen II/VI/IX/X/XI in articular cartilage.[23] In addition, a recent study has highlighted that collagen III may mediate the early stages of collagen II formation and the maintenance of cartilage modulus, regulating the mechanical properties of articular cartilage.[24] Articular cartilage is devoid of blood vessels, nerves, and lymphatics; therefore, the nutrients enter the cartilage by diffusing from the synovial fluid and the subchondral bone.

OA is a common chronic disease, the onset of which is related to age, strain, trauma, and other factors. The characteristic changes of OA include focal cartilage loss, subchondral bone osteosclerosis and exposure, and osteophyte formation. In articular cartilage with OA, the collagen network is disordered due to the decreased production and increased degradation of collagen II, and the number of functionally active chondrocytes decreases.[25] Although BMPs, except BMP-3, play an important role in the formation and maintenance of normal articular cartilage, overexpressed BMPs can also damage cartilage. Matrix metalloproteinase-13 (MMP-13), which is expressed in articular cartilage with OA, can degrade collagen II, IV, IX, and aggrecan.[26] The degradation of collagen II destroys the arched fibre structure of

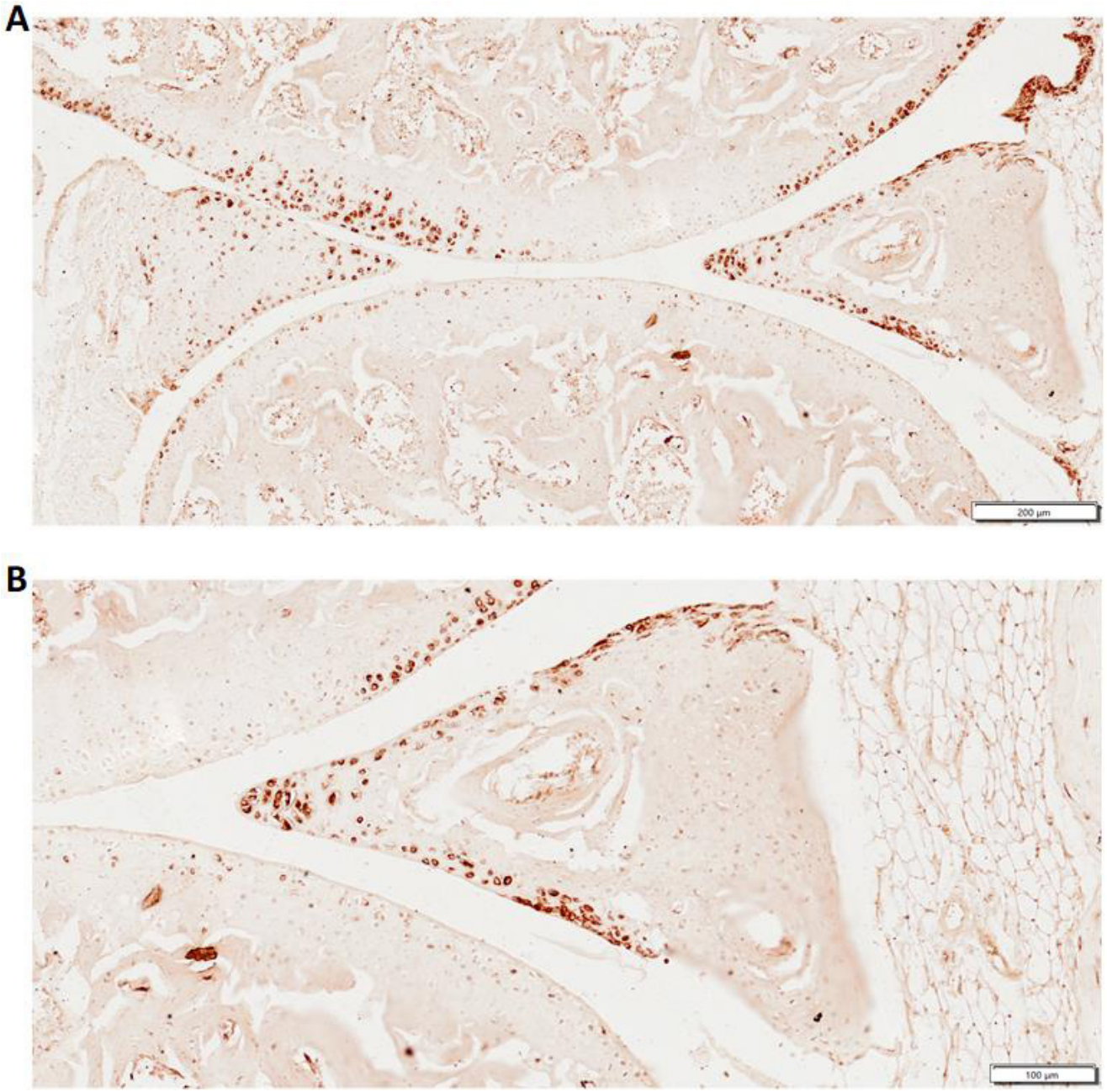

Fig. 2

Activin-like kinase 3 (ALK3) expression in a mouse knee joint. a) Whole joint. b) Meniscus. Three eight-week-old C57BL/6 J mice were provided by GemPharmatech (China). Immunohistochemistry staining on paraffin sections. Primary antibody ALK3, abcam, ab264043.

cartilage, while the degradation of aggrecan renders the chondrocytes inelastic, further leading to the destruction of cartilage.[27,28] Biver et al[13] found that an increase in BMP levels in cartilage stimulated the excessive synthesis of chondrocyte matrix and MMP-13, which can promote tissue repair and stimulate cartilage degeneration to induce OA, respectively. Mechanical damage to joints can induce articular cartilage to express terminal hypertrophy markers such as MMP-13 and lead to the damage of collagen network,[29] therefore leading to OA. The morphology and molecular expression characteristics of normal and OA knee articular cartilage are quite different (Figure 3).

The key role of subchondral bony zone in OA

The subchondral bony zone that separates articular uncalcified cartilage from the bone marrow cavity consists of calcified cartilage and subchondral bone plate.[30] It has been reported that changes at the histological level of the subchondral plate occur prior to degenerative lesions of the overlying cartilage during OA development. Therefore, the corresponding changes such as reduction in

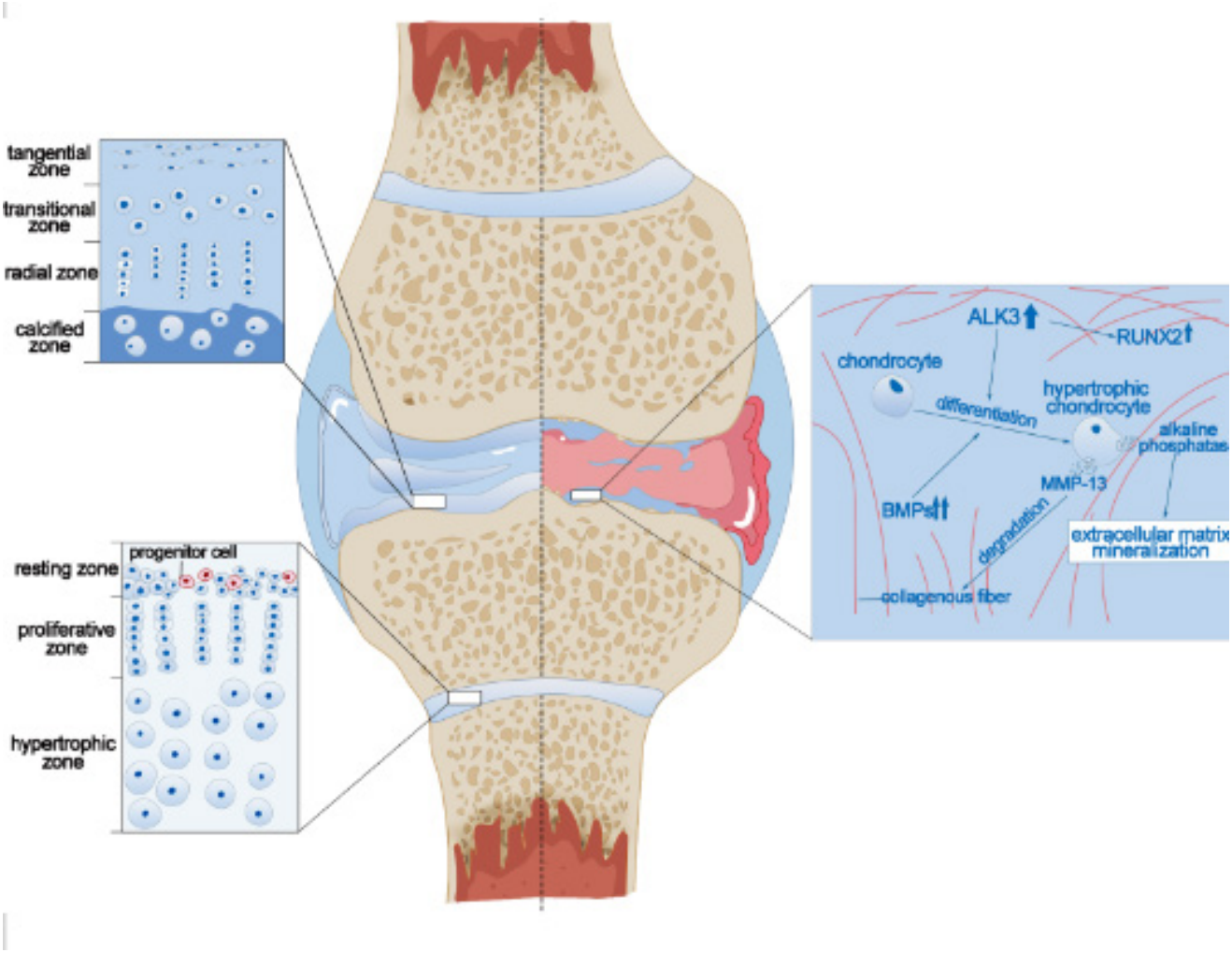

Fig. 3

Chondrocytes in growth plate and articular cartilage and activin-like kinase 3 (ALK3) regulation of osteoarthritis (OA) joint. In OA cartilage, downregulated ALK3 and overexpressed bone morphogenetic proteins (BMPs) can accelerate the differentiation from chondrocytes to hypertrophic chondrocytes. Then, the hypertrophic chondrocytes secrete matrix metalloproteinase-13 (MMP-13) and alkaline phosphatase, which can degrade collagenous fibres and promote extracellular matrix mineralization, respectively. Meanwhile, the loss or downregulation of ALK3 also upregulates runt-related transcription factor 2 (RUNX2), which can accelerate maturation of chondrocytes and contribute to the loss of appropriate anabolic function of cartilage.

subchondral plate mineralization density may indicate the early stages of OA, and we should pay attention to this in order to intervene early in the development of OA.[31] In addition, Zhen et al[32] found that increased TGFβ1 in subchondral bone can significantly increase subchondral bone volume and angiogenesis, significantly reduce proteoglycan expression, and thin the hyaline cartilage layer, thus inducing the joint to display OA phenotype, whereas inhibition of TGFβ activity in subchondral bone can inhibit OA progression. In the joint region, there are many potential instances of molecular crosstalk within and between tissues. By comparison, subchondral bone has the most intra-tissue molecular crosstalk, while ligands in articular cartilage and meniscus have the most inter-tissue molecular crosstalk with receptors in subchondral bone.[33] Those are enough to draw the attention of scholars to the role of subchondral bone in OA.

The vascular invasion into the subchondral plate observed in the articular cartilage is called the subchondral bone resorption pit, which is classified according to the degree of invasion. In grade II, the bone marrow tissue invades into articular cartilage beyond the tidemark.[34] Among them, grade II pits dominate in joints of OA, especially in the medial condyle of the knee joint.[34] The tidemark is an important line of defence against cartilage mineralization and aggressive calcification.

The role of ALK3 in the development of the joint

During embryogenesis, human bones are formed through two processes: intramembranous ossification and endochondral ossification. The latter is the main process during which the cartilage template is gradually replaced by bone. Endochondral bone formation begins

with the apoptosis of hypertrophic chondrocytes, and then it undergoes vascular invasion accompanied by the entry of osteoblast progenitor cells, which expand to the calcified cartilage to initiate angiogenesis and mineralization.[35]

Many studies have shown that ALK3 signalling is necessary for the development of postnatal articular cartilage. ALK3 can promote the differentiation of mesenchymal stem cells (MSCs) in a tissue-dependent manner. At first, the mesenchymal cells in the limb buds aggregate to form a distinct structure in a process known as mesenchymal condensation. There are active BMP signals in the mesenchyme, which are conducive to mesenchymal condensation.[36] Lim et al[37] found that either Smad4 or ALK3 cKO in the limb bud mesenchyme of mice resulted in failed mesenchymal condensation. Following mesenchymal condensation, mesenchymal cells differentiate into chondrocytes and then form the cartilage template, in which the chondrocytes sequentially undergo proliferation, maturation, hypertrophy, and finally are replaced by bone or bone marrow to complete endochondral ossification. Among these, matrix remodelling occurs via MMPs and vascularization via vascular endothelial growth factor (VEGF); VEGF receptors are necessary for the conversion of cartilage tissue into bone tissue.[38] Vascularization and MMPs also play key roles in the pathological mechanisms of OA.[39-41] These processes are intricately regulated by the BMP signalling pathway and its crosstalk signals, such as the Wnt signalling pathway.[42-45] ALK3 cKO in cartilage results in several joint defects at early developmental stages, marked synovial hypertrophy, loss of articular cartilage, and subchondral sclerosis,[1] and these consequences are the pathological manifestations of OA. These results suggest that the ALK3-mediated BMP signalling pathway is essential for the continued health and integrity of joints in the postnatal period.

The growth plate consists of a resting zone, proliferative zone, prehypertrophic zone, and hypertrophic zone. A gradient in ALK3 expression has been observed across growth plate cartilage, and the expression level of ALK3 is much higher in the resting zone and the proliferative zone.[46] In the growth plate of Col2-Cre;ALK3$^{f/f}$ (ALK3 cKO) mice, the proliferation and hypertrophy of chondrocytes were impaired, and the chondrocyte columns were disorganized, thus leading to generalized chondrodysplasia.[47,48] Jing et al[20] also observed that in ALK3 cKO mice, there was no chondrocyte column formation, but some clusters of bone and a small amount of proteoglycans were observed. The studies above suggest that ALK3 is vitally involved in regulating chondrocytes from resting to proliferation and then to terminal hypertrophy, and is also critical for cartilage development and endochondral ossification. The progenitor cells in the resting zone of the growth plate will differentiate into chondrocytes to supplement the cartilage in the growth plate under normal physiological conditions,[46] but there is a replacement of cartilage by bone-like tissues produced by progenitor cells with ALK3 deficiency for ectopic filling, while the proliferation of progenitor cells is also weakened.[49] Taken together, the loss of ALK3 can impair joint formation, especially cartilage structures, and also lead to heterotopic ossification, which is closely related to the pathological changes of OA.

Prehypertrophic chondrocytes in the growth plate secrete Indian hedgehog (Ihh) and further differentiate into hypertrophic chondrocytes.[50] Ihh and parathyroid hormone-related protein (PTHrP) are critical factors that regulate chondrogenesis and endochondral ossification. After Ihh is expressed by prehypertrophic chondrocytes, it diffuses centripetally and distally to perichondrial cells, inducing them to express PTHrP, which diffuses proximally into the chondroprogenitor zone, thereby slowing down the process of chondrocyte hypertrophy through negative feedback on Ihh[51] while promoting the proliferation of chondrocytes.[52] BMP signal can modulate Ihh expression levels and regulate chondrocyte proliferation and hypertrophy independently with Ihh signal.[53] Guo et al[54] found that Ihh signalling was downregulated in Gli1-Cre^{ERT2};ALK3$^{f/f}$ mice, and that topical application of the BMP inhibitor Noggin also resulted in reduced expression of Ihh. Therefore, ALK3, as a positive regulator of Ihh, can form a positive feedback loop with Ihh.[55] We can conclude that ALK3-mediated BMP signalling, as an interactor of the Ihh/PTHrP signalling system, can affect local prehypertrophic chondrocytes as well as distal-range PTHrP production to regulate cartilage development, and thus, a network that regulates the growth of cartilage and bone can be formed between ALK3, Ihh, and PTHrP. In the study by Mang et al,[56] it was suggested that BMP signalling is beneficial to the induction of SRY-box transcription factor 9 (SOX9). SOX9 is an essential transcription factor for cartilage formation,[57] which is necessary to maintain the normal regulation of the Ihh/PTHrP signalling system.[58] Therefore, BMP signalling can also affect the Ihh/PTHrP signalling system by regulating the expression of SOX9.

In conclusion, ALK3, which mediates BMP signalling under stable physiological conditions, is conducive to mesenchymal condensation and endochondral ossification. ALK3-mediated BMP signalling also regulates cartilage vascularization and matrix remodelling along with other signalling pathways, and may mediate OA when signalling is at pathological levels. Notably, ALK3 also interacts with the Ihh/PTHrP signalling system to form a network to jointly regulate the proliferation and differentiation of chondrocytes and the development of bone.

Overview of the multirole ALK3 in OA

One of the key lesions visible in histopathological analysis in OA is cartilage degeneration. As a protein that can promote cartilage formation, BMP can bind to ALK3, thus playing a role in OA. Therefore, the role of ALK3 in OA is worthy of further study. ALK3 is considered to be an indicator of joint changes in OA. Van et al[59] observed that the expression of ALK3 and ALK6 in bovine cartilage was downregulated with increasing age in mice, and

the cartilage inducer GDF5 was also reduced, leading to cartilage degeneration, which was related to OA. Feng and Derynck[60] observed that the articular cartilage with ALK3 deficiency in mice became thin, or even missing or fibrotic, causing the bones in the joint to rub against each other almost directly. Schmal et al[3] evaluated the cartilage defect depth using the International Cartilage Repair Society (ICRS) classification.[61] The data showed that the expression of ALK3 was negatively correlated with ICRS scores, indicating that the more severe the cartilage defect was, the lower the expression of ALK3. However, Chawla et al[62] selectively inhibited ALK3 and ALK6 using two BMP receptor inhibitors (Compound A and LDN-193189) in a 3D in vitro OA microcartilage model, and found that OA microcartilage displayed a glycosaminoglycan (GAG)- and collagen (COL)-II-rich cartilage extracellular matrix and that expression of hypertrophic genes (e.g. COL10A1, MMP13, Ihh) in chondrocytes was reduced. Thus, inhibition of ALK3 and ALK6 on chondrocytes reduced the hypertrophic properties of OA while maintaining cartilage extracellular matrix synthesis. This finding not only demonstrates that the ALK3-mediated BMP signalling pathway mediates cartilage hypertrophy and is involved in the development of OA hypertrophic properties, but also provides some guidance for the future treatment of OA. The different findings mentioned above present a contradiction between 'ALK3 knockdown causes OA development' and 'inhibition of ALK3 alleviates OA symptoms', which may be due to the different target cell types, the simultaneous inhibition of several BMP-type receptors by small molecule BMP-receptor inhibitors, and slight differences in ALK3 expression. Another key lesion in OA involves the subchondral bone, including sclerosis and osteophyte formation. Destruction of the subchondral bone layer is considered to be a sign of OA progression.[30] The physiological state of subchondral bone affects the expression of ALK3 in articular cartilage.[3] However, few studies have reported the direct effect of ALK3 in subchondral bone on OA. Therefore, summarizing the regulatory roles and mechanisms of ALK3 in the three types of bone cells will enable a better understanding of the relationship between ALK3 in subchondral bone and OA lesions.

Roles of ALK3 in cartilage, subchondral bone, and related cells

The cells in cartilage tissue are chondrocytes, which can undergo terminal hypertrophy to become hypertrophic chondrocytes. The calcified cartilage matrix can be absorbed by chondroclasts,[63] while osteoclasts can absorb both calcified and non-calcified cartilage.[64] The cells in subchondral bone tissue are osteocytes, osteoblasts, and osteoclasts. Among them, osteoblasts and osteoclasts are responsible for bone formation and resorption, respectively, to complete bone remodelling. These cells are closely involved in articular cartilage degeneration, subchondral osteosclerosis, and osteophyte formation in the development of OA. Therefore, ALK3, as a key receptor for chondrogenesis and osteogenesis, should play an important role in these cells. The change of its expression may cause OA.

The effects of ALK3 on chondrocytes. Chondrocyte maturation and hypertrophy are closely related to OA. Dysfunctional chondrocytes may lead to the degeneration of articular cartilage, and their cell-derived exosomes can promote M1 polarization of macrophages, exacerbating inflammation.[65] ALK3 has a key role in maintaining normal proliferation and hypertrophy of chondrocytes.[48] Many studies have found that the BMP signalling pathway engages in crosstalk with Ihh/PTHrP signalling and thus mediates the Ihh/PTHrP loop to regulate the physiological activities of chondrocytes. Ihh mediates cartilage hypertrophy while PTHrP can negatively affect Ihh, which means that it can inhibit the terminal differentiation of chondrocytes.[66] Zhang et al[67] demonstrated that ALK3 induced the expression of Ihh in chondrocytes and then participated in the Ihh/PTHrP loop. Ihh expression is upregulated in human OA cartilage; the upregulation in turn promotes chondrocyte hypertrophy and expression of collagen X and MMP-13, triggering OA.[68,69] Inhibition of Ihh signalling resulted in the decrease of collagen X and MMP-13, and the increase of collagen II and aggrecan, which ultimately attenuated cartilage degeneration.[70] Thus, overexpression of ALK3 may induce chondrocyte hypertrophy and cartilage destruction through significant upregulation of Ihh, leading to OA. However, this effect is partly counterbalanced by the PTHrP negative feedback loop in the presence of normal levels of ALK3 expression, which subsequently maintains the normal physiological state of chondrocytes. However, another study found that the constitutively active form of BMP type IA receptor (ca-Bmpr1a) in chondrocytes did not upregulate Ihh.[71] This discrepancy may be due to differences in the experimental model (cell, chicken, or mouse) and the different temporal and spatial stimulation and detection of BMP signals. One study suggested that the loss of ALK3 in chondrocytes led to a decrease in bone mass and hindered fracture healing, which was the opposite of the effect on osteoblasts without ALK3.[72] Therefore, the ALK3 in chondrocytes can increase bone mass, while the BMP signal in osteoblasts inhibits endogenous bone mass. Jing et al[35] observed that the deletion of ALK3 in early chondrocytes led to severe bone defects, delayed development, deformed epiphyses, and thin cortical bone, indicating that ALK3 is very important for the coupling of cartilage formation and osteogenesis in the metaphysis, and it affects the bone pattern and structure.

Not only the loss of ALK3, but also the overexpression and misexpression of ALK3, affects the normal physiological process of chondrocytes. Kobayashi et al[71] found that ca-Bmpr1a in chondrocytes of mutant mice contributed to decreasing the columnar zone of proliferating chondrocytes and increasing hypertrophic markers, suggesting that ca-Bmpr1a can accelerate terminal hypertrophy of chondrocytes. However, a previous study showed that misexpression of ca-Bmpr1a in chickens resulted in a delay

in the differentiation from chondrocytes to hypertrophic chondrocytes via PTHrP pathway,[73] which is contrary to the conclusion of a study by Kobayashi et al.[71] These results suggest that the BMP signal has different effects on the differentiation of chondrocytes in the growth plate in different cell culture systems. Taken together, the loss, overexpression, and misexpression of ALK3 will disrupt the physiological structure and function of cartilage, which may lead to OA.

Regulation of ALK3 in hypertrophic chondrocytes. Hypertrophic chondrocytes develop from chondrocytes through proliferation, maturation, and hypertrophy. Most scholars believe that hypertrophic chondrocytes are the terminal cells in the process of endochondral osteogenesis, and that they can secrete collagen X and alkaline phosphatase to promote cartilage calcification and bone formation. Others hold the opinion that hypertrophic chondrocytes can be directly transformed into osteocytes.[35] Hypertrophic chondrocytes can express VEGF, which accelerates vascular invasion and cartilage absorption during the process of endochondral ossification.[74] Accordingly, the extracellular matrix produced by hypertrophic chondrocytes provides a more beneficial environment for ossification than chondrocytes. The high expression of ALK3 in prehypertrophic chondrocytes makes prehypertrophic chondrocytes the direct target of BMPs,[73] which is crucial for their differentiation into hypertrophic chondrocytes (Figure 3). Runt-related transcription factor 2 (RUNX2) is expressed in early chondrocytes and increases progressively, reaching the highest level in hypertrophic chondrocytes. It can accelerate the maturation of chondrocytes and promotes apoptosis of hypertrophic chondrocytes, and thus it is necessary for endochondral ossification.[75] RUNX2, as a cartilage hypertrophy marker, increased in OA cartilage, resulting in cartilage matrix degradation in chondrocytes,[76,77] while RUNX2 cKO in articular chondrocytes alleviated OA lesions.[78] RUNX2 is also a key target gene of the BMP signalling pathway mediated by ALK3.[79] Overexpression of ALK3 may upregulate RUNX2, which then promotes chondrocyte hypertrophy and facilitates cartilage ossification, ultimately leading to OA.

The regulation of ALK3 in osteoblasts, osteoclasts, and osteocytes. Osteoblast-mediated bone formation and osteoclast-mediated bone resorption together regulate the remodelling of OA subchondral bone. Furthermore, osteocytes can be heavily involved in the remodelling of OA subchondral bone by regulating the functions of osteoblasts and osteoclasts.[80]

ALK3 can regulate osteoblasts in terms of both quantity and activity, thus controlling osteogenesis. After the deletion of ALK3 in osteoblasts, the terminal differentiation of osteoblasts was impaired despite an increased osteoblast number, the structure of the collagen became disorganized, and mineralization was damaged.[16] Similarly, Lim et al[81] observed that by deleting ALK3 with Dmp1-Cre in osteoblasts, the number of osteoblasts in trabecular bone increased while the activity of osteoblasts decreased. Therefore, physiological ALK3 signalling has a dual role in limiting osteoblast proliferation and stimulating osteoblast activity. The latter is positively regulated by mTORC1 signalling.[81] Activation of mTORC1 signalling in preosteoblasts can induce subchondral bone remodelling and sclerosis, and accelerate post-traumatic OA development in mice.[82] Sclerostin (SOST) and dickkopf1 (DKK1) can inhibit Wnt/β-catenin signalling.[83] Kamiya et al[84] constitutively activated ALK3 in osteoblasts from Col1CreeER^{TM}:caBmpr1a mutant mice and then detected a significant increase in the expression of downstream target SOST and unchanged net bone mass, possibly due to an increase in both bone formation and bone resorption markers. In a previous study, after conditional knockout of ALK3 in osteoblasts, SOST and DKK1 were diminished, resulting in increased Wnt/β-catenin signalling and increased bone mass in mice.[85] Dorsomorphin or the BMP inhibitor Noggin can also downregulate SOST and DKK1,[85] thereby enhancing canonical Wnt signalling in osteoblasts during postnatal bone development. The Wnt signalling and its antagonists SOST and DKK1 play important mediating roles in OA. Excessive activation or low expression of Wnt signalling may lead to severe OA,[86] and the expression of DKK-1 was lower in OA osteoblasts.[87] The upregulation of DKK1 can ameliorate OA lesions such as decreased osteophytes and reduced VEGF expression.[88] In addition, some studies have reported that increasing SOST expression can reduce osteophyte volume,[89] and knockdown of SOST resulted in subchondral osteosclerosis and aggravated knee OA in mice.[90] However, Zhou et al[91] found that surgically induced OA developed more rapidly in mice overexpressing SOST compared to the wild type mice. In summary, it is important to maintain the balance of physiological level of ALK3 and the Wnt signalling to reduce the risk of OA occurrence.

BMPs can indirectly affect osteoclasts by acting on osteoblasts or directly on osteoclasts. BMP-2 enhances osteoclast-mediated bone resorption by inducing osteoclast differentiation in a concentration-dependent manner as a result of direct stimulation of osteoclasts by BMP-2 acting in concert with the RANK signalling pathway.[92] Therefore, the direct effect of BMPs on osteoclasts is to promote their resorption of bone. Li et al[93] deleted ALK3 from osteoclast progenitor monocytes using a LysM-Cre mouse line, and monocytes/osteoclasts derived from these mice showed reduced activation of Smad1/5/8, decreased osteoclastogenesis, increased early differentiation markers, and downregulated late differentiation markers, indicating that ALK3 deletion enhanced initial differentiation but hindered maturation of osteoclasts. Therefore, ALK3 mediated-BMP signalling pathway can promote osteoclastogenesis. Osteoclasts may induce sensory innervation[94] and angiogenesis[95] at the osteochondral junction, leading to the progression of OA and increased pain. In addition, osteoclasts are intimately involved in the degradation of articular cartilage. In OA joints, immature osteoclasts migrate into

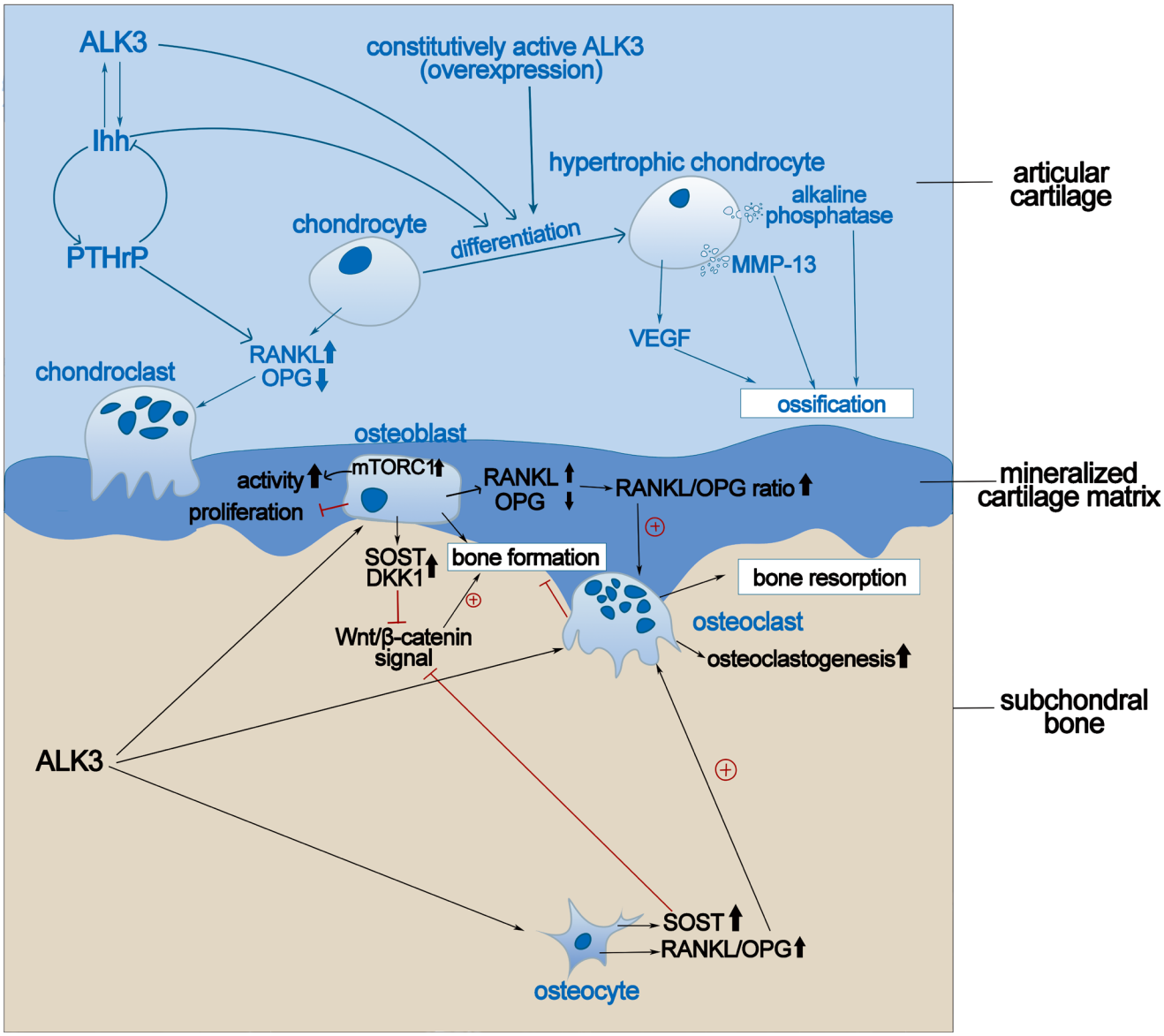

Fig. 4

Effects of activin-like kinase 3 (ALK3) on chondrocytes, hypertrophic chondrocytes, chondroclasts, osteoblasts, osteoclasts, and osteocytes, and their interactions. ALK3 plays important and multiple roles in the related cells of articular cartilage, by crosstalking with other signal pathways, such as Wnt/β-cantenin, receptor activator of nuclear factor-kappa B ligand (RANKL)/osteoprotegerin (OPG), Indian hedgehog (Ihh) Ihh/PYHrP, etc. DKK1, dickkopf1; MMP-13, matrix metallopeptidase 13; mTORC1, mammalian target of rapamycin complex 1; PTHrP, PTH-related peptide; SOST, sclerostin; VEGF, vascular endothelial growth factor.

the hypertrophic cartilage zone and form ossification centres,[96] while mature osteoclasts can destroy the osteochondral junction and resorb cartilage and calcified cartilage.[64] Therefore, overexpression of ALK3 can increase osteoclastogenesis and activity, which may lead to OA. However, there are few studies on the direct causal relationship between osteoclast ALK3 and OA.

In recent years, many studies have proven that there is communication between osteoblasts and osteoclasts. Osteoblasts can affect the formation and proliferation of osteoclasts through direct cell-to-cell contact or secreted molecules, while secreted molecules are probably much more important, functionally coupling osteoblasts with osteoclasts by influencing receptor activator of nuclear factor-kappa B ligand (RANKL)/osteoprotegerin (OPG).[13,97] In addition, Zhang et al[98] cocultured osteoblasts/osteoclasts with primary cells of Sirtuin 6 (SIRT6)-deficient mice and found that SIRT6-deficient osteoblasts play a significant role in promoting osteoclastogenesis, which was more effective than the depletion of SIRT6 in osteoclasts, indicating that the increased osteoclast formation is mainly due to the paracrine mode of osteoblasts rather than SIRT6-null osteoclasts. ALK3-induced BMP signalling in osteoblasts can affect RANKL/OPG through

Table I. Characteristics of bone morphogenetic protein signalling-related treatments for osteoarthritis.

Treatment classification	Advantages	Disadvantages	References
BMP2/BMP7	Cartilage repair	Production of autoantibodies; induction of inflammation, swelling, and heterotopic ossification; increased osteoclast activity	84,114 - 116
CK2.1 (an ALK3 mimetic peptide)	Increased collagen II/IX; cartilage repair without inducing chondrocyte hypertrophy	Not available	117
LDN193189 (a BMP pathway inhibitor)	Reduced cartilage hypertrophy and MMP-13; cartilage repair	Not available	42
Thermosensitive gel delivery system (encapsulates BMP-2 and GAG molecules)	Anti-inflammatory activity; inhibition of cartilage degrading enzymes; cartilage repair	Not available	118,119
GDF5	Induction of aggrecan and SOX9; reduced collagen X; cartilage repair	Formation of osteophytes	120,121
R399E	Induction of aggrecan and SOX9; reduced collagen X; lower cartilage hypertrophy and osteogenic activity; cartilage repair	Not available	56
PTH	Preserved glycosaminoglycans; reduced chondrocyte apoptosis; cartilage repair	Not available	122
ACI (chondrocytes-based)	Cartilage repair	Poor recovery; invasiveness of the process; loss of differentiation phenotype	123,124
BM-MSCs-based tissue engineering (combined with BMP-2 and TGFβ1)	Chondrogenic induction; induction of minimal hypertrophy; downregulation of collagen I and HtrA1; cartilage repair	Not available	125
Synovial-derived MSCs-based tissue engineering (combined with BMP-2)	Mimicry of the communication between synovial membrane and cartilage through synovial fluid; subchondral bone repair; cartilage repair	Not available	126

ACI, autologous chondrocyte implantation; ALK3, activin-like kinase 3; BM-MSCs, mesenchymal stem cells from bone marrow; BMP, bone morphogenetic protein; GDF, growth differentiation factor; MSC, mesenchymal stem cell; PTH, parathyroid hormone.

crosstalk with the Wnt pathway, thereby regulating the differentiation and activity of osteoclasts to change bone resorption.[99] The loss or inactivation of ALK3 in osteoblasts reduced RANKL but increased OPG, leading to the inhibition of osteoclast function.[72,100] Not only can osteoblasts affect osteoclasts, but osteoclasts can also regulate some physiological activities of osteoblasts through ALK3. Okamoto et al[101] deleted ALK3 in differentiated osteoclasts using $Ctsk^{Cre/+}$;$ALK3^{f/f}$ mice, and found that deletion of ALK3 in differentiated osteoclasts increased osteoblastic bone formation, which proved that ALK3 in differentiated osteoclasts negatively regulated osteoblast-mediated bone formation. Therefore, the conditional knockout of ALK3 in osteoclasts leads to a decrease in bone resorption and an increase in bone formation activity, which regulates coupling with osteoblasts. ALK3 affects bone remodelling by regulating the functions of osteoblasts and osteoclasts. A previous study showed that reduced bone resorption was more significant than reduced bone formation in the vertebrae and ribs of ALK3 cKO mice, resulting in increased bone mass.[72] In contrast, studies in long bones attributed the increased bone mass to an increase in the number of osteoblasts.[16,100] Thus, there may be differences in the cellular mechanism of increased bone mass in different types of bones, and this deserves further study. Taken together, ALK3 mediates osteoblast-osteoclast communication with the involvement of RANKL/OPG. In OA osteoblasts, OPG was significantly upregulated, resulting in a decrease in RANKL/OPG, which may be related to subchondral osteosclerosis and osteophyte formation.[87] Another recent study showed that increased OPG may be a sign of early OA, suggesting increased osteogenic activity and cartilage destruction, while increased RANKL level correlated with the severity of OA may indicate increased osteoclast activity and subchondral bone resorption in advanced OA.[102] Thus, ALK3 may play an important role in the assessment of OA progression and severity by regulating the RANKL/OPG ratio in osteoblasts and osteoclasts.

Osteocytes are osteoblasts in the terminal differentiation stage and also play a unique role in bone remodelling. Wang et al[103] suggested that osteocytes, not osteoblasts, are indispensable cells for bone mineralization. He et al[104] found in Dmp1-Cre; $ALK3^{f/f}$ mice a significant reduction in SOST expression in osteocytes, which in turn suppressed preosteoblast proliferation in

the trabecular region of the bone and increased cancellous bone phenotype. Kamiya et al[105] used the Dmp1-Cre system to delete ALK3 in an osteocyte-specific manner in mice and consequently observed that the number of osteocytes increased while the messenger RNA levels of SOST and RANKL/OPG all decreased, and the bone mass significantly increased compared to the control group due to the lack of osteoclast numbers. Thus, ALK3 in osteocytes can promote preosteoblast proliferation by maintaining SOST expression, and inhibit Wnt signalling to control bone mass; it can also affect osteoclast formation by regulating RANKL/OPG. In addition, osteocytes can also independently regulate bone remodelling. Liu et al[106] found that Notch signalling in osteocytes stimulates bone mineralization in a cellular and time-dependent manner, resulting in increased bone mass.

Taken together, ALK3 in bone has dual effects on limiting the proliferation and stimulating the activity of osteoblasts, and it regulates the coupling of osteoblasts and osteoclasts by affecting RANKL/OPG. Furthermore, BMP signals in osteoblasts also regulate bone mass through crosstalk with Wnt/β-catenin signals. In addition to the interaction between osteoblasts and osteoclasts, osteocytes also affect Wnt/β-catenin signalling and RANKL/OPG through ALK3, thereby affecting osteoblasts and osteoclasts. Therefore, osteoblasts, osteoclasts, and osteocytes interact with each other and jointly regulate the biological characteristics of bones, thus affecting the occurrence of OA. Meanwhile, their interaction also regulates the growth and development of articular cartilage, which is closely related to the process of OA.

The regulation of ALK3 in chondroclasts. Chondroclasts are multinucleated cells that have ultrastructural characteristics similar to osteoclasts, and they can absorb mineralized cartilage and degrade cartilage matrix.[63] At present, there are no studies directly linking chondroclasts with ALK3, but some studies have focused on molecules closely related to the expression of ALK3 in chondroclasts, such as RANKL, OPG, and PTHrP. It was found that chondroclasts localized at the osteochondral junction of the OA joint, and may mediate cartilage destruction and subchondral osteosclerosis together with mesenchymal stem cells.[107] The increase of chondroclasts is associated with the development of OA.[108] Therefore, it is worthwhile to understand the regulatory roles of ALK3-related molecules on chondroclasts. Koide et al[109] found that in the presence of a certain dose of IL-1a, BMP-2 can efficiently promote the production of osteoclast-like multinucleated cells (OCLs), such as chondroclasts. Sipola et al[110] found that VEGF-A can promote chondroclast-mediated cartilage resorption, and VEGF and BMP-4 act synergistically to affect both bone formation and bone healing. Chondrocytes can produce OPG. When RANKL is increased and OPG is decreased in chondrocytes, chondroclast production can be stimulated, which consequently accelerates cartilage resorption.[111] RANKL in chondrocytes can be increased by PTHrP, tumour necrosis factor alpha (TNF-α), and interleukin 1 (IL-1), and the decrease in OPG can accelerate the absorption of cartilage calli by chondroclasts, thereby accelerating fracture healing.[112] The loss of RUNX2 in chondrocytes changed the ratio of RANKL/OPG and therefore impaired the production of chondroclasts.[113] Since ALK3 in chondrocytes can increase the expression of PTHrP by facilitating the expression of Ihh, and thus increase RANKL/OPG in turn, the overexpression of ALK3 in chondrocytes may indirectly upregulate the production and differentiation of chondroclasts and promote cartilage absorption, thus leading to OA. This needs to be verified by more experimental studies in the future.

Clinical applications and outlook

At present, the potential application of molecules related to BMP signaling pathway in OA treatment strategies has attracted many researchers to explore (Table I). Previous studies have found that local application of BMP-2 in a short time can stimulate cartilage repair. When recombinant BMP-7 was used to treat knee OA by intra-articular injection, BMP-7 was well tolerated, and there was no tendency for ectopic bone formation.[114] However, a large amount of exogenous BMPs can cause side effects such as the production of autoantibodies. These side effects are presumably due to a broader population of cell types exposed to the high-dose BMPs.[84] In addition, some studies have found that mature recombinant BMP is insoluble at neutral pH, and therefore it forms large molecular weight (MW) agglomerates that induce noteworthy inflammation, swelling, and heterotopic ossification,[115] and a high dose of BMP-2 can increase osteoclast activity.[116] Due to the high dose, high cost, and accompanying side effects of this treatment, an alternative, more efficient BMP signalling-based treatment is needed.

CK2.1, an ALK3 mimetic peptide, binds to hyaluronic acid-based hydrogel particles to form a controlled release system. When injected locally into the intra-articular capsule of OA mice, this controlled release system increased collagen II and collagen IX, and significantly repaired cartilage without inducing chondrocyte hypertrophy.[117] Therefore, CK2.1 is a promising drug that may be used for OA treatment in the future. Liu et al[42] found that LDN193189, a BMP pathway inhibitor, inhibited cartilage hypertrophy and MMP-13 secretion, and thus cartilage degeneration, by specifically targeting ALK2/ALK3 in OA chondrocytes and MSCs. Thus, LDN193189 could be a candidate for the treatment of OA, but this requires further clinical trials. Li et al[118] developed an intra-articular injection thermosensitive gel delivery system (based on Pluronic F127) that encapsulates BMP-2 and GAG molecules (hyaluronic acid or chondroitin sulfate) for the repair of cartilage damage in OA. Hyaluronic acid can provide cells with a good cartilage-forming microenvironment that helps to maintain the chondrocyte phenotype.[119] Chondroitin sulfate is sulfated GAG, which has anti-inflammatory activity and inhibits the production of cartilage degrading enzymes. As a cartilage inducer, GDF5 can induce aggrecan and SOX9 expression

(both markers associated with chondrogenesis and ECM production), while inhibiting collagen X expression,[120] indicating that GDF5 is a potential therapeutic agent to treat OA. However, a study observed an unwanted formation of osteophytes in GDF5-treated animals.[121] Although R399E, a mutant form of GDF5, exerted the same effects on chondrocytes as GDF5, the R399E-treated cartilage showed lower cartilage hypertrophy and osteogenic activity, so it can be used as a potential cartilage repair factor.[56] In addition, intra-articular injections of parathyroid hormone (PTH) have been shown to improve OA by preserving glycosaminoglycans in articular cartilage and reducing chondrocyte apoptosis.[122]

In addition to the intra-articular application of molecular drugs, there are several MSC-based tissue engineering approaches for the treatment of OA. At present, cartilage engineering based on autologous chondrocyte implantation (ACI) offers hope for the treatment of cartilage lesions of knee OA.[123,124] However, the use of chondrocytes may bring some complications such as poor recovery, the invasiveness of the process, and loss of differentiation phenotype during expansion.[123] Therefore, other sources of cells have been considered for alternatives to chondrocytes, the most promising of which are MSCs. Legendre et al[125] obtained the best chondrogenic induction, as well as minimal hypertrophy induction, by combined action of BMP-2 and TGFβ1 under hypoxic conditions using readily available MSCs from bone marrow (BM-MSCs), and then targeted downregulation of type I collagen and HtrA1 messenger RNA and protein levels in OA chondrocytes with short interfering RNAs (siRNAs), leading to a more typical chondrocyte phenotype. Reisbig et al[126] implanted synovial-derived MSCs in a synovial-based decellular extracellular matrix scaffold expressing green fluorescent protein (GFP) or BMP-2, and found that the BMP-2 group had higher bioactivity in promoting articular cartilage growth and subchondral bone repair. Thus, this co-culture model mimics the communication between synovial membrane and cartilage through synovial fluid, demonstrating that bioactive implantation adjacent to cartilage injury can significantly improve cartilage growth and subchondral bone repair, and prevent the progression of OA.

In summary, ALK3 can regulate the differentiation and maturation of chondrocytes by participating in the Ihh/PTHrP loop and it can couple osteoblasts, osteocytes, and osteoclasts by regulating RANKL/OPG, which forms a cell network to jointly regulate the development of cartilage and bone, or even pathological processes, and can also regulate bone mass, bone strength, and other skeletal biological characteristics through crosstalk with Wnt signalling (Figure 4). The precise communication of signals within or between cells maintains the normal development and function of joints. Both overexpression and deletion of ALK3 may cause OA, so it is crucial to maintain physiological levels of ALK3. However, the conclusions of some studies are contradictory, which may be related to the use of different experimental animals, anatomical locations, and culture systems (in vitro or vivo). The detailed reasons for these discrepancies need to be further clarified. In addition, it is worth exploring in greater depth how to minimize the side effects caused by the application of BMPs in the clinical treatment of OA. The above treatments are more or less related to the BMP signalling pathway and have their own characteristics. However, no studies have reported direct ALK3-based treatments for OA. Therefore, clarifying the role of ALK3 can help to explore efficient ALK3-based drugs and advance the development of clinical treatment for OA in the future.

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