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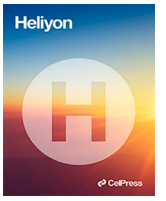

Review article

The role of NF-κB-SOX9 signalling pathway in osteoarthritis

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ABSTRAT

The nuclear factor-κB (NF-κB) signalling pathway exists in a variety of cells and is involved in the gene regulation of various physiological and pathological processes such as inflammation, immunity, cell proliferation and apoptosis. It has been shown that this signaling pathway is also involved in numerous events associated with osteoarthritis, including chondrocyte catabolism, chondrocyte survival, and synovial inflammation. SRY-related high mobility group-box 9(SOX9) is the "master regulator" of chondrocytes and one of the key transcription factors that maintain chondrocyte phenotype and cartilage homeostasis. NF-κB can positively regulate the expression of SOX9 by directly binding to its promoter region, and play a role in the formation and development of chondrocytes. This article reviews the regulatory effect of the NF-κB-SOX9 signaling axis on osteoarthritis.

1. Introduction

Osteoarthritis (OA) is a chronic progressive Osteoarthritis characterized by articular cartilage injury and reactive hyperplasia of joint margins and subchondral bone [1]. Its pathogenesis is complex, and genetic factors, environmental factors and obesity are one of the causes [2]. The incidence and disability rate of OA are higher in the middle-aged and elderly population, with an incidence of 10 percent in men and 18 percent in women over the age of 60 [3]. OA is characterized by joint pain, swelling, deformity, and is accompanied by functional impairment, which seriously affects the normal life of middle-aged and elderly patients, and brings a serious economic burden to society and families. The destruction of articular cartilage during the pathogenesis of OA is not only related to the death of chondrocytes, but also to the loss of cartilage extracellular matrix. In addition, chondrocytes are the only resident cells in articular cartilage and play an important role in maintaining the function and structure of articular cartilage by controlling the synthesis and degradation of extracellular matrix [4].

SOX9 is the "main regulatory factor" of chondrocytes, one of the key transcription factors that maintain chondrocyte phenotype and cartilage homeostasis, and is closely related to the extracellular matrix (ECM) metabolism of articular chondrocytes [5]. During limb development, inactivation of SOX9 can lead to complete loss of bone and cartilage, while upregulation of SOX9 may inhibit chondrocyte apoptosis [6,7]. Inflammatory factors are overexpressed in OA and other diseases, which significantly inhibit the biological effects of SOX9 and weaken the regeneration and repair ability of damaged cartilage tissues [7]. NF-κB is a transcription factor that plays a key role in biological processes and has been found to be involved in inflammatory and immune responses as well as in the regulation of gene expression such as cell proliferation and apoptosis [8]. Most studies have revealed that SOX9 expression is regulated

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through the NF-κB signalling pathway. The mode of action of NF-κB and SOX9 may be to positively regulate the expression of SOX9 by directly binding to its promoter region [9]. In addition, studies have also shown that NF-κB signaling is abnormally activated in OA, and is considered to be one of the most important signalling pathways, and significantly regulates OA-related inflammatory mediators [10]. The purpose of this review is to reveal the role of SOX9 and NF-κB signalling pathways in OA disease, and to provide biomarkers and therapeutic targets for the diagnosis and treatment of OA.

2. NF-kappa β family

NF-κB molecule is a protein complex, which was first discovered by RanjanSen (NIH) in the laboratory of Nobel laureate David-Baltimore through its interaction with II base pairs in immunoglobulin light chain enhancers in B cells [11]. NF-κB exists in nearly all types of animal cells and is an important nuclear transcription factor in cells. It participates in cellular responses to stimuli, such as stress, cytokines, free radicals, heavy metals, ultraviolet radiation, oxidation of Low Density Lipoprotein (LDL) and bacterial or viral antigens. NF-κB plays a key role in regulating the immune response to infection, and the abnormal activation of NF-κB signal is closely related to cancer, inflammation and autoimmune diseases [12,13]. There are mainly five members of NF-κB family in mammals, namely RelA (p65), RelB, c-Rel, NF-κB1 (p50) and NF-κB2 (p52), which form homodimers and Heterodimers to play a role. There is a 300-amino acid region at the N-terminal of these five subunits, namely REL homology domain (RHD), which mediates their dimerization into the nucleus and binding to the corresponding DNA sites, thus regulating the transcription of target genes [14,15]. In the NF-κB family, only the subunit with a transactivation domain (TADS) at the C-terminal can activate transcription, while p50 and p52 do not. Only RelA (p65), RelB and c-Rel carry a transactivation domain. Therefore, NF-κB dimers formed by p50 and p52 subunits cannot be activated for transcription, and they can only promote transcription when they form heterodimers with other NF-κB subunits containing transactivation domains [16–18]. In the absence of stimulation, the dimer formed by these subunits binds to NF-κB inhibitor protein (IκB) to form complexes and is shelved in the cytoplasm; while stimulating by inflammatory factors or other substances, IκB is phosphorylated and the NF-κB dimer is released into the nucleus to activate transcription [19].

3. Activation mechanism of NF-κB signaling

The main function of IκB protein is closely related to the activation of NF-κB signal, that is, shielding the nuclear localization signal and preventing NF-κB dimer from entering the nucleus to regulate gene expression. The IκB protein is composed of IκBα, IκBβ, IκBγ, IκBδ, IκBε, etc. Its family structure is characterized by multiple repeat sequences of about 33 amino acids, called multiple ankyrin repeat domains, which are mainly involved in the RHD interaction with Rel protein [20]. Only after IκB is phosphorylated and degraded by proteasome can NF-κB dimer be released into the nucleus for transcriptional activation to regulate the expression of inflammatory factors [21,22]. The enzyme responsible for the phosphorylation of IκB is IκB kinase (IKK), which is an enzyme complex. The IKK complex consists of IKKα (IKK1), IKKβ (IKK2), and a regulatory subunit, the NF-κB essential modulator (NEMO) [23]. It has been found that although IKKα and IKKβ are structurally similar, the subunit involved in the phosphorylation of IκB in the IKK complex is mainly IKKβ, and IKKα is only responsible for a small part. Among them, IKKβ is mainly involved in activating of the classical NF-κB pathway. In addition, it may also be related to the maintenance of the homeostasis of the extracellular matrix microenvironment [24]. And IKKα is involved in the activation of the non-canonical NF-κB pathway [25]. Studies have shown that mice lacking NEMO will cause severe liver function damage and death due to the apoptosis of a large number of cells, which indicates that NEMO is an essential regulatory subunit for activating NF-κB signal. On the other hand, the N-terminal of NEMO binds to the IKKs, and the C-terminal mediates its interaction with upstream signal transducers [26,27]. The activation of NF-κB signal is not only stimulated by inflammatory factors, viruses and bacteria, but also related to its post-translational modification regulation, such as phosphorylation, ubiquitin, methylation and acetylation [28].

4. Activation pathway of NF-κB signalling pathway

The NF-κB signal pathway is activated, which may regulate a various of biological functions, such as inflammatory response, immune response, stress response and tumourigenesis. However, there are two activation pathways of NF-κB signal, namely the classical activation pathway and the non-classical (alternative) activation pathway. The activation mechanism, regulatory pathway and gene expression of the two pathways are different. Below we will elaborate on these two different activation pathways.

4.1. The classical NF-κB pathway

The classical activation pathway of NF-κB is mainly involved in rapid and reversible inflammatory responses, etc, and its pathways are mostly activated by stimuli such as inflammatory factors, bacteria, viruses, Toll-like receptors, antigen receptors, and ultraviolet radiation [29]. In the absence of various stimuli, NF-κB forms a complex with the IκB protein in the cytoplasm and exists in an inactive state with no regulating functions. Only after the complex is stimulated by the previous substance, the activity can be activated to exert its function. The activation of NF-κB complex is realized by IKK kinase acting on IκB protein, phosphorylation and proteasome degradation. The key activation subunit in IKK is IKKβ, and its activity depends on the oligomerization of IKKα, IKKβ and NEMO, the three subunits of its IKK complex [30]. It has been proved that NEMO is the basic regulator of NF-κB,binding to the ubiquitin chain can cause conformational changes and assist the activation of IKK kinase complex [15,31]. The activated subunit IKKβ of the IKK kinase exerts its phosphorylation effect to phosphorylate two conserved serine residues at positions 32 and 36 of the IκB protein in the NF-κB

complex, and undergo polyubiquitination, which is finally processed by the 26S proteasome, thereby releasing NF-κB homologous or heterodimer into the nucleus. When the NF-κB signal is released into the nucleus, it can bind to the consensual binding sequence in the corresponding promoter and activator to target gene expression [32].

The rapid activation of the classical NF-κB pathway induces inflammation and immune-related responses. Although it plays a role in protecting the body, uncontrolled activation can lead to damage or serious harm to the body, such as autoimmune diseases, chronic inflammation and tumorigenesis. Therefore, the NF-κB signalling pathway should be strictly controlled to protect the function of the organism. At present, it has been found that the classical NF-κB pathway is negatively regulated [33]. When the NF-κB complex is activated and released and enters the nucleus, the newly synthesized IκB α binds to NF-κB dimer in a concentration-dependent manner and promotes transfer to the cytoplasm, resulting in the termination of NF-κB signal transcription [34]. Studies have found that IκBβ can counteract the inhibitory effect of IκBα by binding to the nuclear dimer, resulting in continued transcriptional signaling [35,36], while IκBε can inhibit IκBα-mediated oscillations [27]. The three members of the IκB family, IκBα, IκBβ, and IκBε, cooperate with each other to ensure the normal operation of NF-κB signaling. In addition, we also found that deubiquitination plays a role in the negative regulatory mechanism of the classical NF-κB pathway, mainly in the upstream signal transduction of IKK [14]. The above results show that the negative regulation of NF-κB pathway is involved in many pathways and its standard operation is strictly regulated.

4.2. The alternative NF-κB pathway

The activation of the non-classical NF-κB pathway is a slow, long-lasting, irreversible response, and is closely related to the immune system, such as the occurrence of lymphoid organs, the development of B cells, and T cell responses [23]. Non-classical pathways are usually activated by the tumor necrosis factor receptor (TNFR) family, namely CD40 ligand (CD40L), B-cell tumor necrosis factor receptor (BAFF-R), and lymphotoxin receptor (LTβ-R) [37]. The non-classical NF-κB pathway is mediated by the transcriptional factor p100/Rel B complex, and both IKKα and NF-κB-induced kinase (NIK) are involved in its activation, but IKKβ and NEMO in the IKK complex have not been found to play a role in this pathway [38,39]. NIK, also known as mitogen-activated protein kinase kinase kinase 14 (MAP3K14), is a component of the non-classical NF-κB pathway, which is mainly responsible for the phosphorylation of IKKα and then participates in the processing of p100 [23]. P100 is an inhibitor and precursor protein of the P52 subunit. Without stimulation, P100 blocks the transcriptional activity of the P100/Rel B complex due to its N-terminal auto-inhibitory function [40]. After stimulation, the binding of TNF family receptors to ligands leads to the weakening or disappearance of ubiquitination on NIK, thereby activating NIK kinase. The restored NIK kinase can phosphorylate and activate IKKα, and then the activated IKKα can mediate the phosphorylation and ubiquitination of p100 to generate p52. Finally, p52 and Rel B form a dimer and enter the nucleus to activate target genes [20,41,42]. Therefore, NIK kinase is a key protein in activating the non-canonical NF-κB pathway [43], and the processing of p100 is an important step in this signalling pathway.

5. The functional role of NF-κB signaling in OA

Osteoarthritis is characterized by the destruction and loss of articular cartilage. Chondrocytes are resident cells in articular cartilage, embedded in the extracellular matrix. Due to the lack of blood vessels and nerve tissue on the surface of cartilage, the self-repair ability of chondrocytes is poor. As a consequence, injury, inflammation and degeneration can lead to dedifferentciation. In addition, the loss of chondrocyte phenotype will accelerate the degradation of cartilage matrix, leading to irreversible cartilage damage, resulting in OA [44]. The normal growth and development of cartilage is divided into four parts, namely, resting area, proliferative area, Prophase hypertrophic area and hypertrophic area, while the classical p65 pathway plays a role in the whole process of cartilage growth, but mainly in the resting zone and hypertrophic area [45,46]. In addition, it is also found that the non-classical NF-κB signal pathway is also involved in the development of cartilage growth plate, but in the surrounding area, this pathway is also involved in endochondral osteogenesis by regulating the proliferation and differentiation of chondrocytes [26]. However, in OA, NF-κB signalling pathway plays the opposite role due to abnormal activation. It has been found that the abnormally activated NF-κB signal pathway is closely related to chondrocyte differentiation, extracellular matrix degradation and synovitis in OA, and this pathway also plays the function of apoptosis and anti-proliferation in OA chondrocytes [47,48]. In the development of OA disease, it is primarily the NF-κB signal of the classical pathway that is abnormally activated, and the non-classical pathway plays a lesser role.

The classical NF-κB signal pathway plays a key role in the catabolism and inflammation of chondrocytes [16,49]. The abnormal activation of this pathway will lead to abnormal expression of regulatory genes after binding to corresponding DNA targets due to the increase of p65 in the nucleus, such as the overexpression of interleukin-1β (IL-1β), interleukin-6 (IL-6), C-C motif chemokine ligand 5 (CCL5), cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2), which can induce chondrocyte differentiation, apoptosis and extracellular matrix degradation, and eventually lead to inflammation and degeneration of articular cartilage [50,51]. Studies have shown that tumor necrosis factor-α (TNF-α), IL-1β and IL-6 can promote the secretion of matrix metalloproteinase and reduce the production of collagen and proteoglycans. PGE2 and hypoxia inducible factor-2α can accelerate the apoptosis of chondrocytes mediated by Fas, but NF-κB can also prevent chondrocyte death induced by tumor necrosis factor-alpha, and this anti-apoptotic effect is also essential in chondrocytes [52,53]. In conclusion, NF-κB plays a bidirectional role in chondrocyte survival and apoptosis. And related researchers, using specific siRNA to inhibit the expression of NF-κB p65, found that it can block the overexpression of IL-1β, IL-6, COX-2, etc. [54]. It has also been reported that normal rat knee joint transfected with IKKβ lentivirus induces NF-κB P65 signalling pathway activity and synovial inflammation [55]. All of these demonstrate the importance of this pathway.

In addition, in chondrocyte culture of OA disease, silencing IKK α can inhibit the expression of Runx2, lead to chondrocyte hypertrophy, and increase the expression of collagen and proteoglycan [56], indicating that the non-classical NF-κB signal pathway is

also involved in the development of osteoarthritis. Related studies have shown that in hypertrophic chondrocytes, the target genes bone morphogenetic protein-2 (BMP2), C-X-C motif chemokine ligand 8 (CXCL8), C-X-C motif chemokine ligand 1 (CXCL1) regulated by this pathway, together with ELF3 and HIF-2α pathway, promote the production of the type X collagen gene (COL10A1), Matrix metalloproteinase 9 (MMP9), Matrix metalloproteinase 13 (MMP13), alkaline phosphatase and osteocalcin hypertrophy markers, resulting in chondrocyte calcification and osteophyte formation [57–59]. Therefore, targeted down-regulation of NF-κB may be considered as an effective method for the treatment of osteoarthritis.

6. Role of SOX9 in OA

SOX9 is a member of the SRY-related HMG-box (SOX) transcription factor family, located on human chromosome 17 [6], which can act on other genes to regulate various functional changes of cells [60]. Human SOX9 protein consists of 509 amino acids and contains four different domains: two trans-activation domains, HMG domain and dimerization domain [6], through which the expression of target genes can be regulated.

SOX9 is a major transcription factor during cartilage development and endochondral osteogenesis. It is critical for chondrocyte ECM gene expression and also plays a role in chondrocyte differentiation [61]. Although many transcription factors are required for cartilage regeneration, SOX9 is considered indispensable [62,63]. During the growth of normal cartilage, SOX9 can promote the gene transcription of cartilage markers type II and IX collagen and proteoglycan aggrecan, and inhibit the expression of aggrecanase and matrix metalloproteinases, thereby maintaining the phenotype of articular chondrocytes [64]. However, the expression of SOX9 gene and protein in OA is significantly decreased, which is related to the activation of the NF-κB signalling pathway by inflammatory stimulation. Several studies have shown that a large amount of activation of this pathway will reduce or inhibit the normal expression of SOX9 [65], lead to phenotypic instability and dedifferentiation of chondrocytes, and their role in matrix degradation, decreased joint function, and progression of osteoarthritis [66,67]. SRY-related high mobility group-box 5 (SOX5) and SRY-related high mobility

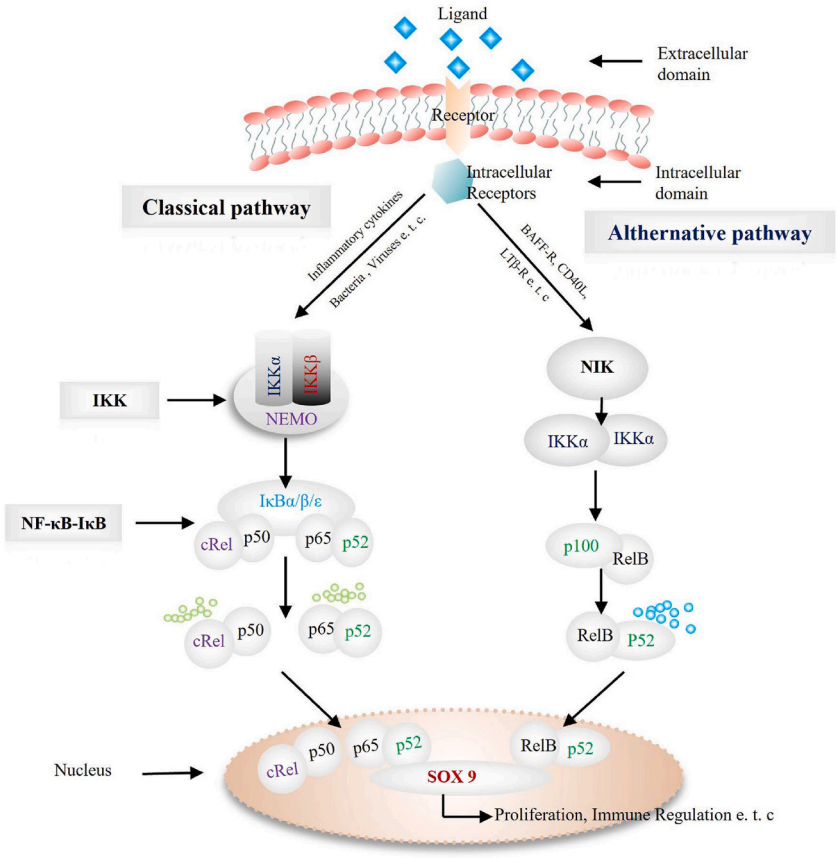

Fig. 1. The classical NF-κB signalling pathway and alternative NF-κB signalling pathway. The classical NF-κB pathway is activated by a large number of agonists, such as the inflammatory cytokines, bacteria and viruses e.t.c. Activation of this pathway depends on the IKK complex, which phosphorylates the inhibitor of IκBα to induce rapid degradation. The alternative NF-κB pathway is activated by a limited number of agonists, such as the CD40L, BAFF-R and LTβ-R e.t.c. This pathway requires NIK and IKKα to promote the processing of the p100 precursor into p52, which results in dimerization and activation of the p52/RelB heterodimer. When the NF-κB homologous or heterodimer enters the nucleus, it binds to the promoter site of SOX9 gene to regulate the phenotype of chondrocytes.

group-box 6 (SOX6) also belong to the transcription factor family. Studies have found that SOX5 and SOX6 can bind to form homologous dimer and heterodimer, but due to the lack of trans-activated domain, they often bind with SOX9 to form SOX Trio, which plays a role in cartilage formation. In the gene experiments of SOX5 and SOX6 in mice, knocking out any of these genes resulted in mild skeletal abnormalities at birth. When all knocked out, although SOX9 was expressed normally, mice die in the fetal abdomen because of incomplete bones [68], which indicates that SOX5 and SOX6 are also indispensable in cartilage formation, and their functions are closely related to SOX9. In addition, it was also found that the transactivation activity of SOX triplet was significantly higher than that of SOX9 alone, but the molecular interaction between them was not clear [69,70]. During the early stages of chondrocyte hypertrophy, Sox9 regulates the transcription of target genes by interacting with activator protein-1 (AP-1) family members such as Jun and Fosl2, thereby promoting chondrocyte hypertrophy [71]. However, in the late stage of hypertrophy, SOX9 can inhibit chondrocyte hypertrophy, thereby playing an important role in chondrocyte differentiation and inhibiting chondrocyte apoptosis [72]. SOX9 is a key transcription factor for the differentiation of cartilage mesenchymal cells into chondrocytes [73,74]. It is also involved in regulating various stages of chondrocyte differentiation. Therefore, targeting SOX9 would be a good option for OA treatment.

7. Functional role of NF-κB-SOX9 signalling pathway in OA

NF-κB transcription factor signaling and SOX9 gene are simultaneously expressed in the growth and development of cartilage (including pathological changes), and the regulated target gene expression is also significantly different under different stimulation environments. Whether it is physiological changes in cartilage growth or pathological changes of noxious stimuli, the specific binding mode between NF-κB signaling and SOX9 is not very clear, but several studies have found possible binding modes between them and mode of action. In OA, both the classical NF-κB signaling pathway and alternative NF-κB signaling pathway are involved in the gene regulation of SOX9, and the activation and regulation of their signaling pathways are shown in the structural diagram below Fig. 1.

At present, it has been confirmed that there is a transactivation domain in the C-terminal of SOX9, and there is also a transactivation domain in the signal molecule of NF-κB and NF-κB signal in chondrocytes was also demonstrated to be responsible for the transactivation of SOX9 in Yoshida et al. [75,76]. Moreover,NF-κB enters the nucleus to play a role after activation, and SOX9 itself is a nuclear expression gene, so it is possible for them to bind in the nucleus and regulate the expression of target genes. In the latest study, Buhrmann et al. speculated that after activation of NF-κB signal into the nucleus, SOX9 gene expression was regulated by acting on the promoter region of SOX9. They first determined the genomic sequences of several highly conserved regions located in the proximal promoter region of the human and mouse SOX9 gene, and then found that NF-κB family member p65 had the strongest activation of human SOX9 promoter activity by luciferase reporter gene detection, and used immunohistochemical localization to verify that there was indeed an intermolecular interaction between them in the process of chondrocyte differentiation. Chromatin immunoprecipitation (ChIP) assay showed that the SOX9 promoter containing NF-κB motif was bound to p65 in vivo [77]. Colter et al. found that p65 binds to SOX9 through two CCAAT mods in its promoter, which are also located in the proximal element of SOX9 [78]. In addition, Hamadou et al. found in inflammatory bowel disease (IBD) that SOX9 may regulate NF-κB activity by allele-specific binding to its promoter at the SNP locus through experiments such as the dual luciferase reporter gene assay [79]. In summary, we can speculate that NF-κB in chondrocytes enters the nucleus and exerts its regulatory role by binding to the promoter of SOX9 to form the SOX9-p65-NF-κB complex. In a study of prostate cancer, changes in SOX9 gene and protein expression were found to be associated with stimulation of NF-κB signaling. Namely, the induction of SOX9 during development reprogramming may be the result of NF-κB signaling elevation [80]. This is the same result as our study of NF-κB signaling pathway and SOX9 gene in OA, and also further affirms their functions [81,82]. Under the stimulation of inflammatory factors such as IL-1 and TNF-α, the expression of SOX9 is restricted, at least regulated by NF-κB signaling; while in normal cartilage growth or cancer cells, the regulation of SOX9 by NF-κB is promoted, The two contradict each other. In fact, the negative regulation of SOX9 by NF-κB in OA and other contexts occurs at the post-transcriptional level through an RNA-sequence-dependent mechanism, rather than at the transcriptional level, which explains the dual outcomes of NF-κB regulation of SOX9 [83]. As a result, NF-κB signaling and SOX9 can interact with each other and regulate gene expression, which must be closely related to the transactivation domain that exists between the two. The specific mechanism for regulating chondrocyte differentiation and skeletal development needs further study.

8. Conclusions

SOX9 is one of the key genes regulating chondrocytes, and abnormal activation of NF-κB signaling in OA leads to decreased SOX9 gene expression, which affects the repair of chondrocytes and extracellular matrix, thereby aggravating the symptoms and progression of OA patients. Although biological agents targeting specific genes in osteoarthritis have been used clinically to reduce inflammation and delay disease progression, the therapeutic effect is not apparent. Recently, a study found that the significant reduction of chondrocyte extracellular matrix and chondrocyte production transcription factor SOX9 in OA was closely related to the abnormal activation of NF-κB signalling pathway, and demonstrated that both SOX9 and NF-κB act by forming a complex in chondrocytes [8]. The interaction between NF-κB and SOX9 genes may reveal many potential drug therapy targets; therefore, we can target the NF-κB-SOX9 signalling pathway, study and design inhibitors targeting this pathway, etc., to upregulate SOX9 genes expression, thereby improving the symptoms and progression of OA disease.

Data availability statement

Question: Has data associated with your study been deposited into a publicly available repository?

Response: No, data availability is not applicable to this article as no new data were created or analyzed in this study.

Ethics statement

Review and/or approval by an ethics committee was not needed for this study because it's a review article.

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CRediT authorship contribution statement

Bin Tian: Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Liang Zhang:** Writing – original draft, Project administration. **Jiang Zheng:** Writing – original draft, Software. **Xin Kang:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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