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SOX11 promotes osteoarthritis through induction of TNF-α

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ABSTRACT

Objective: Osteoarthritis (OA) is a degenerative disease and the molecular mechanism of OA remains unclear. Transcription factor SOX11 has been proved to be involved in the development progress of OA. The present study aimed to evaluate the potential function of SOX11 during the development of OA.

Methods: SOX11 expression in patients with OA and health donator was determined with qRT-PCR. Subsequently, *in vitro* OA model was established by treating the chondrocyte cells CHON-001 with IL-1β. Next, we validated the function of SOX11 in *in vitro* OA model by using siRNAs. Finally, the relationship between SOX11 and TNF-α was explored.

Results: SOX11 was upregulated in patients with OA and in IL-1β treated cells. IL-1β significantly increased both the mRNA and protein levels of MMP13 and cleaved caspase 3, while decreased collagen II and aggrecan in CHON-001 cells. In addition, knockdown of SOX11 could significantly decrease IL-1β-induced apoptosis in CHON-001 cells. Meanwhile, IL-1β induced OA like phenomenon was significantly reversed by siRNA interference. Moreover, inhibition of SOX11 decreased the level of TNF-α in patients with OA and in IL-1β treated cell supernatant.

Conclusion: Inhibition of SOX11 could improve IL-1β-induced OA like phenomenon in CHON-001 cells, which suggesting SOX11 played an important role during the pathogenesis of OA. Thus, we hypothesized that SOX11 could be a potential target for the treatment of patients with OA.

1. Introduction

Osteoarthritis (OA) is a degenerative joint disease, also known as hypertrophic arthritis or degenerative arthritis, which is a common disease among middle-aged and elderly people [1]. According to the epidemiological survey, the incidence rate of OA in people aged 55–64 years is 40% [2]. Clinical manifestation of patients with OA includes joint pain, tenderness, stiffness, swelling, limited movement and joint deformity [3]. With the increase of aging population, the incidence of OA is on the rise each year. However, the exact etiology and pathogenesis of OA remain unclear. Therefore, doctors and research scientist are encountering to find out the causes of pathogenesis of OA so that effective prevention and treatments could be given.

The etiology of OA has been proved to be multi-factorial, including joint injury, obesity, aging, and heredity [1]. Physical therapy and proper exercise can maintain the range of motion of the joints. Meanwhile, several molecular pathways, such as transforming growth factor-β (TGF-β), Smad3, β-catenin and HIF-2α are all reported to be involved in the development of OA [4–7]. Moreover, cytokines are reported to regulate the development and progression of OA. Increased level of TNF-α, IL-1β, as well as activated NF-κB signaling pathways were observed in patients with OA [8,9]. It has been reported that some anti-inflammatory and analgesic drugs could alleviate or control the symptoms of OA with a short time [10].

SOX gene family is composed of many supergenes with conserved motifs of HMG box, which is considered to be an important transcription regulator [11]. SOX protein has important biological functions in sex determination and differentiation, early embryo development and tissue and organ formation. Meanwhile, several SOX genes have been proved to be involved in the development of OA. For instance, the reduction of SOX9 and SOX6 gene levels might be responsible for the loss of phenotypic stability of osteoarthritic chondrocytes [12]. SOX11 gene belongs to the group C of Sox family, which plays a very important role in embryonic neurogenesis and tissue remodeling [12]. Nowadays, it has also been proved that SOX11 is expressed in many different kinds of malignancies, indicating the oncogenic function of SOX11 [13,14]. On the basis of these activities of SOX11, the present study aimed to evaluate the potential function of SOX11 during the development of OA.

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2. Material and methods

2.1. Cell line and reagents

Human chondrocyte cell line CHON-001 was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), cultured in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 0.1 mg/ml G418 (Thermo Fisher Scientific, Waltham, MA, USA) and 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA). IL-1β and TNF-α were purchased from R&D (Minneapolis, MN, USA).

2.2. Knee tissue collection

Fresh OA specimens were obtained from 20 patients undergoing surgery within 4 h at the Zhangjiagang Hospital of Traditional Chinese Medicine. Control cartilage specimens were obtained from 20 donors without known history of joint disease. The study was approved by the Ethics Committee of the Zhangjiagang Hospital of Traditional Chinese Medicine and followed the guidelines of the Declaration of Helsinki. Written informed consent was obtained from all study participants.

2.3. Quantitative real-time PCR (qPCR) analysis

For mRNA level measurement, total RNA was isolated from cells with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, cDNA was synthesized with 2 μg total RNA using SuperScript II RNase H Reverse Transcriptase Kit (Life Technologies, Gaithersburg, MD, USA). Real-time PCR was performed with SYBR Green PCR mix (Thermo Fisher, Waltham, MA, USA). Both primers for mRNA and GAPDH were purchased from Thermo Fisher. The qPCR procedure was performed in a Bio-Rad CFX Real-time System (Bio-Rad, Hercules, CA, USA) as follows: initial denaturation at 95 °C for 10 min; 40 cycles of denaturation at 95 °C for 15 s and annealing extension at 60 °C for 30 s. The relative expression of mRNA was determined by the $2^{-\Delta\Delta}$Ct method where Ct refers to cycle threshold.

2.4. Western blot analysis

Cells were washed by PBS twice and then lysed with immunoprecipitation assay buffer containing protease inhibitors cocktail (Dalian Meilun Biotech Co., Ltd., Dalian, China). Subsequently, the lysate was centrifuged for 10 min at 12,000 rpm at 4 °C and the supernatant was moved to new tubes for further analysis. Total protein were separated in 10% SDS-PAGE gel and then transferred to polyvinylidene fluoride membranes (PVDF; Dalian Meilun Biotech Co., Ltd., Dalian, China) with wet transmembrane device. The membranes were blocked with 5% non-fat milk at room temperature for 1 h, and incubated overnight with primary antibodies (1:1000). Then membranes incubated with appropriate horseradish peroxidase-conjugated secondary antibody (1:3000) for 2 h at RT. Before developing the blots, the PVDF membranes were incubated with enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). All values were normalized to β-actin. The primary antibodies (anti-SOX11, ab170916; anti-MMP13, ab39012, anti-Cleaved caspase-3, ab49822; anti-Collagen II, ab34712; anti-Aggrecan, ab3778; anti-GAPDH, ab8245) and second antibody were provided with Abcam (Cambridge, MA, USA).

2.5. Knocking down SOX11 by siRNAs

Two siRNA were synthesized that consisted of a sequence-specific 21 nucleotide stretch designed to target SOX11 (si-SOX11-1: 5′ AGAC GGUCAAGUGCGUGUUUC; si-SOX11-2: 5′ UUUGAAGCUUGUCGGUCU UUG). Negative control was purchased from Invitrogen. SiRNAs were transfected into CHON-001 by Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Cell proliferation assay

Cell proliferation was carried out based on a colorimetric assay using cell counting kit 8 (CCK8, Dojindo, Kumamoto, Japan). In brief, cells were incubated for 24, 48 and 72 h at 37 °C. Then, cells were mixed with CCK-8 solution and incubated for another 1 h. The absorbance of cells at OD450 was measured with microplate reader (L-117, Thermo Fisher Scientific, Inc., Waltham, MA, USA).

2.7. Flow cytometry analysis

Annexin V and propidium Iodide (PI) double staining was carried out with Annexin V Apoptosis detection kit (BD, Franklin Lake, NJ, USA) according to the manufacturer's instructions. Briefly, CHON-001 cells were incubated with IL-1β, SOX11 siRNA, or TNFα for indicated time periods. After that, cells were resuspended in PBS and stained with Annexin V/PI. The apoptosis of samples were then analyzed with the flow cytometer (BD Bioscience, San Jose, CA, USA).

2.8. Statistical analysis

All data are expressed as the means ± SEM unless specified. Graphs were analyzed with GraphPad Prism 7 software. The comparison between two groups was analyzed by Student's t-test. The comparisons among multiple groups were made with one-way analysis of variance (ANOVA) followed by Dunnett's test. The level of statistical significance was set at 0.05.

3. Results

3.1. SOX11 was upregulated in human cartilage from patients with OA and in IL-1β-treated CHON-001 cells

In order to investigate the role of SOX11 in OA, we detected the mRNA level of SOX11 in human cartilage from patients with OA or donors without joint diseases. The data indicated SOX11 mRNA level was significantly elevated in samples from patients with OA, compared with those from health donors (Fig. 1A).

Many studies suggested that IL-1β level was positively correlated with the severity of OA, and the levels of several proinflammatory cytokines, including IL-1β, were significantly increased in synovial fluid of patients with OA [15]. Therefore, in this study, IL-1β was used to induce inflammatory response in human chondrocyte cell lines CHON-001 cells, in order to mimic an *in vitro* model of OA.

The results of western blot in Fig. 1B and C displayed an increased protein level of SOX11 in cells after IL-1β (10 ng/ml) treatment. Meanwhile, the protein expression of matrix metalloproteinase (MMP) 13 was increased as well, which was consistent with a previous clinical investigation revealing that patients with articular cartilage degradation destruction had high MMP13 expression [16]. Biologically, MMP13 is a major enzyme that targets not only targets type II collagen and aggrecan but also degrades other extracellular macromolecules, such as proteoglycan, osteonectin and perlecan in cartilage [17]. Aggrecan is known as cartilage-specific proteoglycan, which is believed to be a critical component for the structure of cartilage and joint. In Fig. 1B and 1C, we found the protein levels of aggrecan and collagen II in cells were decreased by IL-1β treatment. We also noticed that the expression of cleaved-caspase-3 was upregulated, suggesting IL-1β induced apoptosis of CHON-001 cells. The mRNA levels of these OA-associated modulators were consistent with evidences of protein levels (Fig. 1D). Above data indicated that IL-1β-treated CHON-001 cell model could mimic the pathogenesis of OA, which will be used in the subsequent experiments.

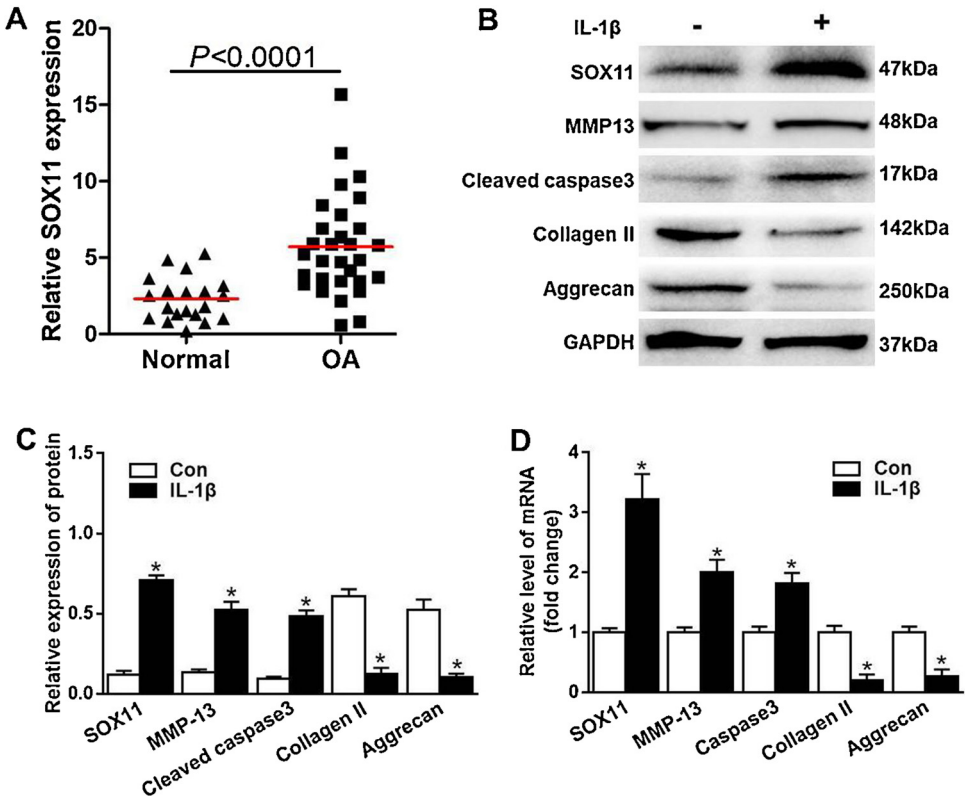

Fig. 1. Level of SOX11 in patients with OA and in IL1-treated CHON-001 cells. (A), Quantitative PCR to determine the level of SOX11 in patients with OA (n = 32) and healthy donors (n = 20). P < 0.0001, as compared to the non-OA donors. (B) CHON-001 cells were treated with 10 ng/ml IL-1β for 24 h. Then, the expression of SOX11, MMP13, cleaved caspase 3, collagen II and aggrecan in cells were detected by Western blot. (C) The quantification of protein expression of each protein was determined by the density of the band after normalization to GAPDH. Each experiment was performed in triplicate. (D) The expression of SOX11, MMP13, caspase 3, collagen II and aggrecan in IL-1β treated CHON-001 cells were detected with quantitative PCR.*P < 0.05, compared to non-treated group.

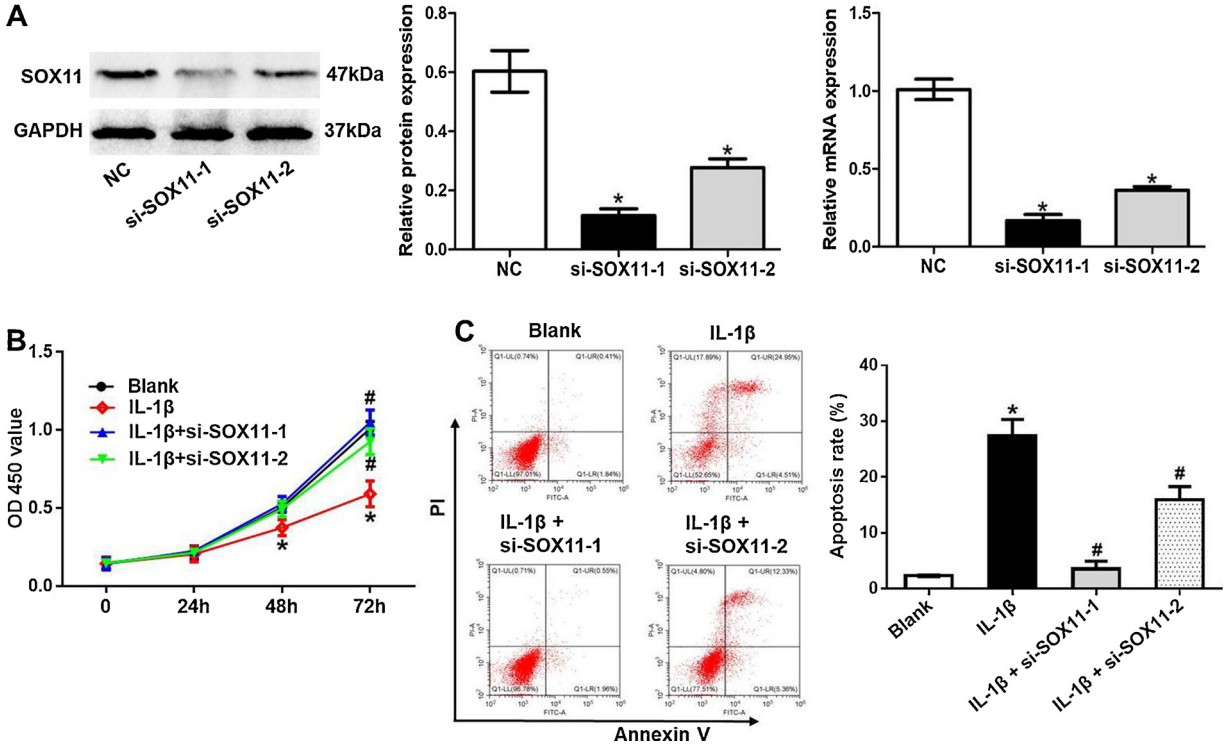

Fig. 2. SOX11 knockdown alleviated IL-1β-induced apoptosis in CHON-001 cells. (A) Western blot and quantitative PCR confirmed the knockdown efficiency of siRNAs in CHON-001 cells. (B) CHON-001 cells were transfected with siSOX11-1, siSOX11-2 for 6 h, then treated with IL-1β for 24, 48 and 72 h. Cell viability were detected with CCK8 assay. Values are expressed as means of triplicate ± SEM. *P < 0.05, compared to NC group; #P < 0.05, compared to IL-1β group. (C) CHON-001 cells were transfected with siSOX11-1, siSOX11-2 for 6 h, then treated with IL-1β for 24, 48 and 72 h. Apoptosis in cells after were detected with Annexin V/PI staining. Values are expressed as means of triplicate ± SEM. Each experiment was performed in triplicate. *P < 0.05, compared to NC group; #P < 0.05, compared to IL-1β group.

3.2. SOX11 knockdown alleviated IL-1β-induced apoptosis of CHON-001

In order to further explore the function of SOX11 in OA cell model, we knocked down SOX11 by applying relevant siRNAs. As suggested in Fig. 2A, both two siRNAs successfully decreased protein level of SOX11. Consistently, mRNA level of SOX11 in cells was decreased by these two siRNAs. In addition, CCK8 assay indicated that IL-1β treatment significantly inhibited the proliferation of CHON-001 cells, which could be notably reversed by SOX11 knockdown (Fig. 2B). Meanwhile, IL-1β induced apoptosis of CHON-001 was markedly attenuated by SOX11 siRNAs (Fig. 2C). In summary, results above suggested that SOX11 knockdown alleviated IL-1β-induced apoptosis of CHON-001, indicating the potential role of SOX11 in the progression of OA.

3.3. SOX11 knockdown decreased the levels of MMP13 and cleaved caspase 3 and increased levels of collagen II and aggrecan in CHON-001 cells

Next, we further interrogated the effect of SOX11 on the expressions ofOA-associated markers. The results indicated that knockdown of SOX11 decreased both the protein level and mRNA level of MMP13 and cleaved caspase 3, while increased levels of collagen II and aggrecan in CHON-001 cells (Fig. 3A-3 F). These data suggested knockdown of SOX11 could potentially alleviate the progression of OA *in vitro*.

3.4. TNF-α was one of the downstream effectors of SOX11

Since TNF-α is a proinflammatory cytokine present in the joint during OA, we next interrogated the correlation between SOX11 and TNF-α. Firstly, the levels of TNF-α in synovial fluid and in OA cell model were detected with ELISA. The results indicated that TNF-α was obviously upregulated in patients with OA, compared with health donors (Fig. 4A). Meanwhile, in OA cell model, the level of TNF-α in culture medium was increased to around 10-folds by IL-1β treatment (Fig. 4A). However, IL-1β induced upregulation of TNF-α in medium was completely reversed by SOX11 knockdown (Fig. 4B). In addition, the anti-OA effect of SOX11 knockdown in CHON-001 cells was significantly attenuated by TNF-α (Fig. 4C, 4D). Nevertheless, cell proliferation was slightly inhibited by TNF-α (Fig. 4E). All these data implied that excess addition of TNF-α could ameliorate the protective effect of SOX11 knockdown against IL-1β in CHON-001 cells, indicating that TNF-α might function as one of the downstream effectors of SOX11. Finally, the signaling pathways associated with OA in the current study were demonstrated in Fig. 5.

4. Discussion

In this study, we revealed that SOX11 was upregulated in both OA tissues and IL-1β treated CHON-001 cells. Knockdown of SOX11 decreased molecular biomarkers of OA and apoptosis, as well as increased cell proliferation in OA cells. Thus, we hypothesized that inhibition of SOX11 probably could improve the proliferation of cartilage cells.

Expression and regulation of SOX family members in normal and osteoarthritic cartilage from adult humans was different, indicating the potential role of SOX transcription factors in OA [18]. It has been reported that SOX9 and SOX6 transcription levels were decreased in osteoarthritic chondrocytes, which might be related to the loss of structural stability in OA. SOX11 protein, the group C of Sox family, may function in the developing nervous system and play important roles in tumorigenesis and adult neurogenesis [19]. In a study conducted to compare the difference of mRNA between normal and OA cartilages, researchers found that SOX11 mRNA level was significantly upregulated in OA cartilage [20]. From our results, SOX11 was upregulated in both OA tissue and IL-1β-induced OA cellular model, which was consistent with previous results. Knockdown of SOX11 down-regulated OA related biomarkers and cell apoptosis, whereas up-regulated CHON-001 cells proliferation. All these data suggested SOX11 might be a potential therapeutic target for the treatment of OA.

TNF-α mRNA levels were up-regulated in cartilage from patients with OA compared to normal cartilage [21]. *In vitro* and animal studies have confirmed that pro-inflammatory cytokines TNF-α can enhance cartilage degradation and induce bone resorption in OA [22–24]. It can

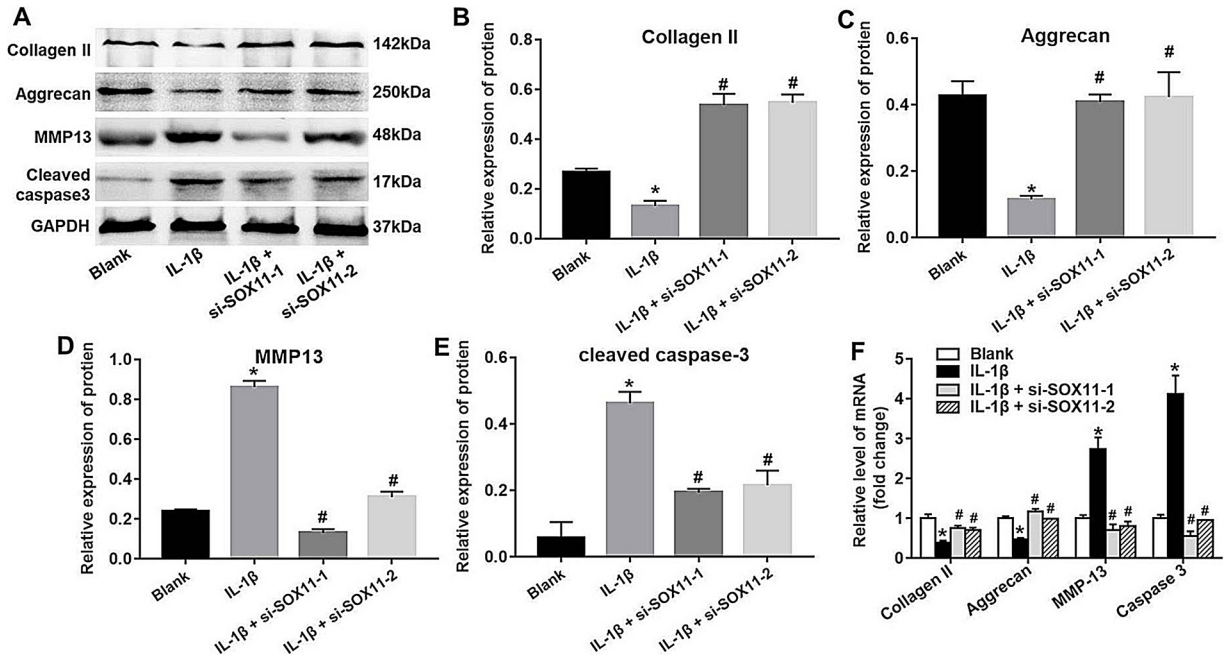

Fig. 3. SOX11 knockdown alleviated IL-1β-induced OA like phenomenon in CHON-001 cells. (A) CHON-001 cells were transfected with siSOX11-1, siSOX11-2 for 6 h, then treated with IL-1β for 72 h. The expression of collagen II, aggrecan, MMP13 and cleaved caspase 3, were detected with Western blot. (B, C, D, E) The quantification of collagen II, aggrecan, MMP13 and cleaved caspase 3 expression in cells, respectively. (F) The mRNA levels of collagen II, aggrecan MMP13 and caspase 3 were quantified using with Quantitative PCR. *P < 0.05, compared to Blank group; #P < 0.05, compared to IL-1β group. Each experiment was performed in triplicate.

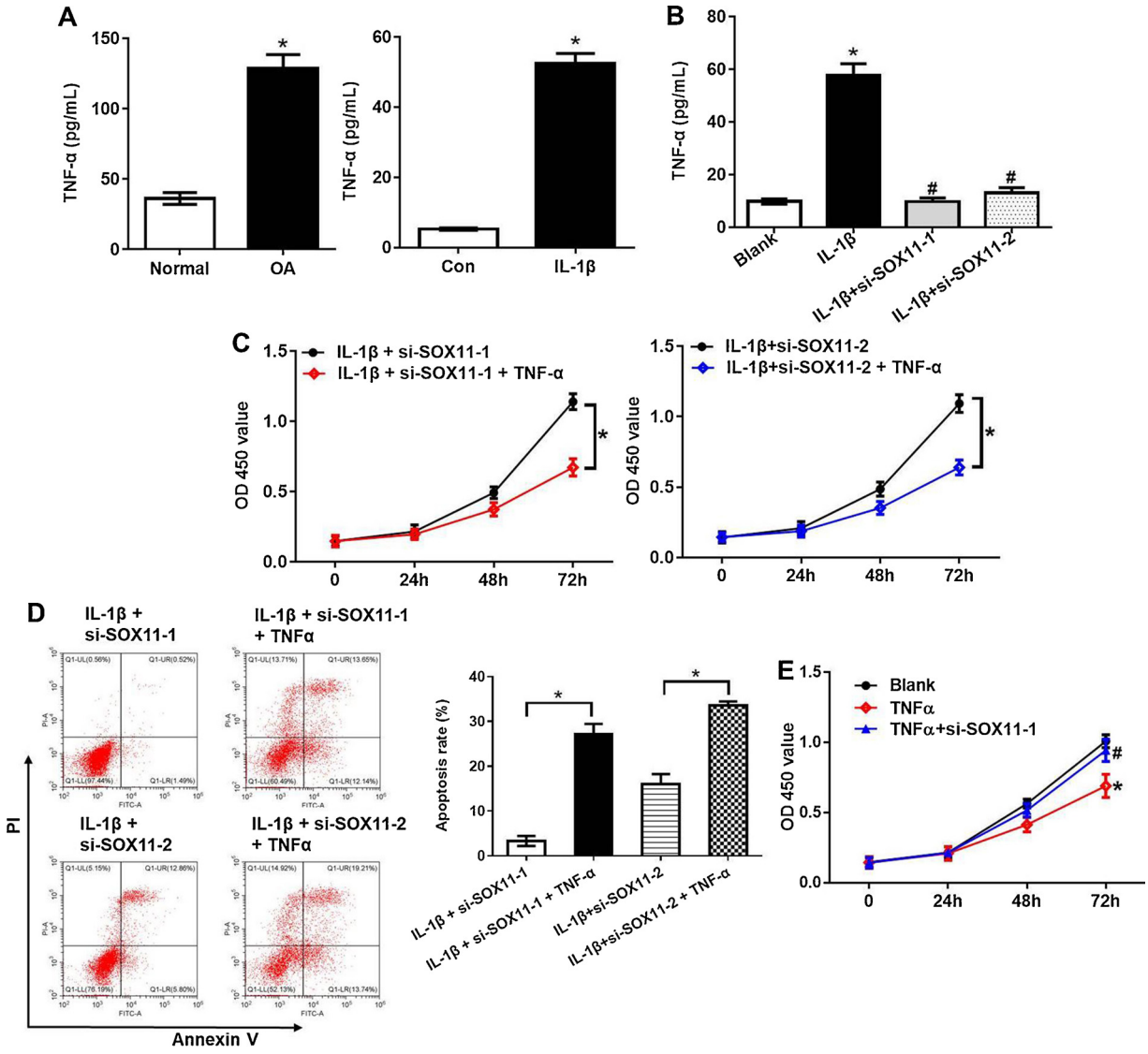

Fig. 4. TNF-α is one of the downstream effectors of SOX11. (A) TNF-α levels in synovial fluid and in supernatant of IL-1βtreated CHON-001 cells were determined by ELISA. (B) CHON-001 cells were transfected with siSOX11-1, siSOX11-2 for 6 h, then treated with IL-1β for 72 h. After that, TNF-α levels in the supernatant of cells were determined by ELISA. *P < 0.05, compared to Blank group; #P < 0.05, compared to IL-1β group. Each experiment was performed in triplicate. (C) CHON-001 cells were transfected with siSOX11-1, siSOX11-2 for 6 h, then treated with IL-1β or/and TNF-α for 72 h. After that, cell viability was detected with CCK8 assay. *P < 0.05. Each experiment was performed in triplicate. (D) Apoptosis in CHON-001 cells was determined with Annexin V/PI staining. *P < 0.05, compared to negative control. *P < 0.05. (E) The viability of CHON-001 cells was determined with CCK8 assay. *P < 0.05, compared to Blank group; #P < 0.05, compared to IL-1β group. Each experiment was performed in triplicate.

stimulate the production of other cytokines such as IL-6, matrix metalloproteinases and prostaglandins [22], and suppress the level of proteoglycans and type II collagen [25]. Our data suggested that inhibition of SOX11 could decrease the level of TNF-α, which improved current knowledge of OA by demonstrating the potential correlation between SOX11 and TNF-α. TNF-α is synthesized as inactive precursor and must be activated by an enzyme before being released extracellularly in their active forms. Therefore, the inhibition of TNF-α maturation by specific convertase inhibitors appears to be an attractive therapeutic target. Güler-Yüksel et al reported that treatment with TNF-α inhibitor infliximab might reduce hand osteoarthritis, suggesting that anti-TNF-α therapy might be effective against OA [26]. In addition, two pilot studies and one case report showed positive results of anti-TNF- α treatment in OA [27–29]. A large amount of knowledge about the pathophysiology of OA and potential targets for therapeutic strategies were generated from experimental studies. Caution should be exerted in extrapolating *in vitro* and *in vivo* results to the clinical settings. Therefore, the evidences of anti-TNF-α in treatment of patients with OA still need further research.

In conclusion, we demonstrated that SOX11 was involved in cell apoptosis in the pathogenesis of OA. Knockdown of SOX11 improved the molecular biomarkers of OA in cellular models and increased the proliferation of cartilage cells. Mechanistically, SOX11 inhibition decreased the level of another pro-inflammatory factor, TNF-α. This study allowed for a better understanding of the OA disease, which may have potential therapeutic value by specifically and effectively retarding the progression of disease.

Conflicts of interest

The authors declare no competing financial interests.

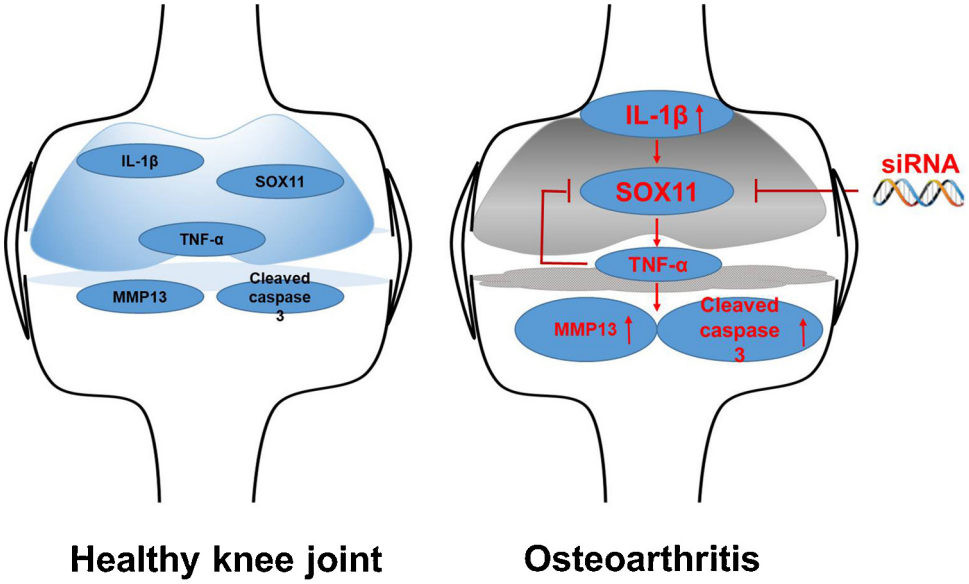

Fig. 5. The color signal cartoon illustrated the signaling pathways associated with OA in the current study.

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