Knockdown of long non-coding RNA AK094629 attenuates the interleukin-1β induced expression of interleukin-6 in synovium-derived mesenchymal stem cells from the temporomandibular joint

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Abstract. Interleukin (IL)-1β is a key promotor in the pathogenesis of temporomandibular joint osteoarthritis. Differentiation of stem cells to cartilage is a crucial repair mechanism of articular cartilage damage, and IL-1β has been reported to impede the differentiation by upregulating the secretion of IL-6, an important inflammatory factor. Long non-coding RNAs (lncRNAs) regulate a number of physiological and pathological processes, but whether lncRNA AK094629 contributes to the IL-1β mediated induction of inflammation remains unclear. Therefore, the aim of the present study was to investigate the effect of AK094629 on IL-1β-induced IL-6 expression in synovial-derived mesenchymal stem cells (SMSCs) of the temporomandibular joints. The results of the present study demonstrated that the expression of AK094629 in the synovial tissue of patients with osteoarthritis was positively correlated with IL-1β. In addition, IL-1β upregulated the expression of AK094629 in the SMSCs *in vitro*, and AK094629 knockdown inhibited the IL-1β mediated upregulation of IL-6. The present study also demonstrated that AK094629 knockdown downregulated the expression of the mitogen-activated protein kinase kinase kinase 4 (MAP3K4), which is upregulated by IL-1β, whereas knockdown of MAP3K4 did not affect the expression of AK094629, but reversed the upregulation of IL-6 in SMSCs. In conclusion, AK094629 knockdown attenuated the expression of IL-1β-regulated IL-6 in the SMSCs of the temporomandibular joint by inhibiting MAP3K4. Therefore, AK094629 may be a potential novel therapeutic target for the treatment of temporomandibular joint osteoarthritis.

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Introduction

Temporomandibular joint disorders (TMDs) are diseases involving pain and dysfunction in the temporomandibular joint (TMJ) and masticatory muscles (1). Osteoarthritis (OA) is a degenerative TMD characterised by progressive cartilage degeneration, subchondral bone remodelling, synovitis and chronic pain (2,3). OA of the TMJ (TMJOA) often involves all soft and hard tissues of the TMJ, resulting in pain, joint motion limitation and joint noises (4). Physiotherapy, non-steroidal anti-inflammatory drugs, arthroscopy and surgical treatment are often used in the clinical treatment of TMJOA (5). These treatments can relieve the symptoms; however, owing to the limited healing ability of avascular cartilage, they do not completely restore joint function or reverse the destruction of cartilage and other tissues (6,7). Mesenchymal stem cell (MSC) treatment is a potential new therapeutic strategy for TMJOA. Synovial-derived mesenchymal stem cells (SMSCs) have been demonstrated to have osteogenic, chondrogenic and adipogenic potential (8,9), and are recognised for their proliferation efficiency and potential to differentiate into cartilage (10).

Although TMJOA is defined as a low-grade inflammatory joint condition (5), degenerative changes in the synovium and disc of the TMJ can still be caused by persistent inflammation (11). Interleukin (IL)-1β is one of the most significant pro-inflammatory factors and has been demonstrated to cause articular cartilage inflammation (12). IL-1β is significantly upregulated in the synovial fluid, synovium and cartilage of patients with TMJOA, where it stimulates chondrocytes and rheumatoid fibroblast-like synoviocytes (RA-FLSs) to release matrix metalloproteinases (MMPs), which cause

excessive degeneration of the cartilage extracellular matrix (ECM) (13-16). IL-1β has also been demonstrated to inhibit the expression of type II collagen in MSCs, resulting in unbalanced synthesis and catabolism, which ultimately leads to cartilage destruction (17). Additionally, IL-1β can also increase the production of other inflammatory mediators such as IL-6, IL-8, and tumour necrosis factor (TNF)-α (18). Previous studies have demonstrated that IL-1β upregulates the expression of IL-6 in synovial fluid-derived and synovial-derived mesenchymal stem cells by activating the NF-κB pathway (8,9). IL-6 is also considered an important inflammatory factor associated with synovitis and OA of the TMJ and was demonstrated to be upregulated in the synovial fluid of patients with OA and correlated positively with MMPs (19-21). In addition, IL-6 also impedes MSCs in the synovial fluid from differentiating to cartilage, thus reducing the effectiveness of stem cell-based TMJOA therapy (22,23).

Long non-coding RNAs (LncRNAs) are a class of >200-nucleotide non-coding RNA molecules without an open reading frame (24,25). They are further classified into antisense lncRNAs, intergenic non-coding RNAs (lincRNAs), pseudogene lncRNAs, enhanced RNAs and intronic RNAs depending on their location in relation to protein-coding genes. The class of an lncRNA determines its functionality to a certain extent (26), with different lncRNAs being involved in chromatin modification, transcription and post-transcriptional regulation (27), making lncRNAs important regulators of a number of physiological and pathological processes, including OA (28). LncRNAs influence the progression of OA by affecting the survival of chondrocytes and synovial cells, and regulating the expression of factors associated with arthritis, such as MMPs and type II collagen alpha 1 (29). For example, LncRNA HOX transcript antisense RNA is significantly upregulated in the synovial fluid of patients with TMJOA and was demonstrated to cause an IL-1β-induced increase in the expression of MMP-1, MMP-3 and MMP-13 in the primary chondrocytes of rabbits (14). Cartilage injury-related lncRNA (lncRNA-CIR), highly expressed in the cartilage of patients with OA, has been demonstrated to degrade cartilage matrix (30). In addition, human chondrocyte inflammation-associated lincRNA (CILinc)01 and CILinc02 were significantly downregulated in the chondrocytes of patients with OA, and their knockdown promoted the IL-1β-induced expression of IL-6 and IL-8 in the chondrocyte line TC28 (31). Preliminary experiments demonstrated that the expression of AK094629 in the synovial tissue of patients with OA was positively correlated with IL-1β, and that IL-1β impedes the MSCs in the synovial fluid from differentiating to cartilage by upregulating the secretion of IL-6 (9). Therefore, the present study hypothesised that LncRNA AK094629 may be associated with the development and progression of TMJOA.

Mitogen-activated protein kinase kinase kinase 4 (MAP3K4) is a 180-kDa protein that phosphorylates and activates MAP2Ks, leading to the activation of MAPK pathways, including the p38 pathway (32-34). A previous study demonstrated that the p38 pathway contributed to IL-1β induced IL-6 expression (35). According to the database created by the University of California Santa Cruz (genome.ucsc.edu), AK094629 is the antisense lncRNA of the nearby MAP3K4 gene. Therefore, it was hypothesised that MAP3K4 might be related to the expression of LncRNA AK094629 in SMSCs of the TMJ.

The present study aimed to further explore the association between lncRNA AK09469 and TMJOA in order to clarify the regulatory mechanism between lncRNA AK094629 and the IL-1β-induced upregulation of IL-6 in SMSCs.

Materials and methods

Ethics statement. The present study was approved by the Institutional Ethics Board of the Hospital of Stomatology, Sun Yat-sen University (Guangdong, China). Each patient who donated synovial specimens signed an informed consent form before surgery.

Clinical samples and cell culture. Synovial specimens were obtained from ten surgically treated patients with TMJOA. The samples were placed in 1.5 ml RNase-free EP tubes containing TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was then extracted and analysed by reverse transcription-quantitative (RT-q) PCR to determine the expression levels of IL-1β and AK094629. Synovial tissues from the patients were washed three times with phosphate-buffered saline (PBS, Gibco; Thermo Fisher Scientific, Inc.) and cut into small pieces (<1 mm^3). Subsequently, 1 ml type I collagenase (Sigma-Aldrich; Merck KGaA) was added to the tube, and digestion was performed in an incubator at 37˚C for 2.5 h. After digestion, the mixture was transferred to a 15 ml tube and centrifuged at 350 x g for 5 min at room temperature, the supernatant was discarded, and the precipitate was resuspended in complete medium, composed of Dulbecco's Modified Eagle Medium (DMEM, Gibco; Thermo Fisher Scientific, Inc.) and 10% foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). The suspension was seeded into petri dishes (5,000 cells/cm^2) and cultured at 37˚C in an incubator containing 5% CO_2. The medium was refreshed every 3 days. When the cells reached ~80% confluence, they were seeded into new petri dishes after another round of digestion, centrifugation and resuspension, according to the aforementioned protocol.as Cells from passage 4-6 were selected for the experiments.

RT-qPCR. Total RNA from tissues and SMSCs was extracted using TRIzol® reagent. The mRNA was then reverse-transcribed into cDNA using the PrimeScript RT Master Mix (Perfect Real Time; Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. The following thermocycling conditions were used for RT: 37˚C for 15 min, 85˚C for 5 sec and cooled to 4˚C. qPCR was performed using the SYBR® Green RT-qPCR kit (Roche Diagnostics) and a Roche LightCycler 96 (Roche Diagnostics). The relative mRNA expression levels of AK094629, MAP3K4 and IL-6 were calculated using the $2^{-\Delta\Delta Cq}$ method (36), followed by normalisation to the GAPDH mRNA expression level. The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 10 sec; annealing at 58˚C for 20 sec; and extension at 72˚C for 30 sec, with a total of 45 cycles. The sequences of the primers used are listed in Table I. The amplification efficiencies of GAPDH,

Table I. Primer sequences for PCR.

Gene	Primer sequence (3'→5')	Amplification efficiency (%)
GAPDH		
F	GACAGTCAGCCGCATCTTCT	103
R	TTAAAAGCAGCCCTGGTGAC	
AK094629		
F	AGCGCTAAGAGTAAACGATGC	97
R	GGGGAAGAAGAAATGCTAAAG	
MAP3K4		
F	CAATCGGACTGACTTCTGGATA	105
R	TTGGGAACTTCGGACACTAAT	
IL-6		
F	ACTCACCTCTTCAGAACGAATTG	104
R	CCATCTTTGGAAGGTTCAGGTTG	

MAP3K4, mitogen-activated protein kinase kinase kinase 4; IL; interleukin; F, forward; R, reverse.

Table II. Target sequences of si-RNA and lncRNA smart silencer.

RNA	Target sequence (3'→5')
siRNA	
siMAP3K4-1	GCACTCTGTTTGTGGTTAA
siMAP3K4-2	GTGGAAGAAATACAGCTATA
siMAP3K4-3	GCAGCAGAATTCAGGCTTT
lncRNA smart silencer	
AK094629	GGACTGGAATGCTCCTACAG CAACAGACCAAGCTAACAGT GCTCAAAGTATGTTACTGCA ACTCCGGTCTCTTGACAGAA ACTTGGACTGGAATGCTGCA ATTTTCTGACCAGAAC

si, small interfering; MAP3K4, mitogen-activated protein kinase kinase kinase 4; lncRNA, long non-coding RNA.

AK094629, IL-6, and MAP3K4 primers were 1.03, 0.87, 1.14 and 1.10, respectively.

Western blot analysis. Cells were collected and lysed using RIPA Lysis Buffer (Beyotime Institute of Biotechnology) supplemented with protease and phosphatase inhibitors (Beyotime Institute of Biotechnology), which were added at a ratio of 1:99 to the RIPA Lysis Buffer. The concentration of protein was measured using a bicinchoninic assay (Beyotime Institute of Biotechnology), and the protein sample was then split between two or three 200 μl EP tubes, followed by the addition of loading buffer (sample to loading buffer ratio, 4:1) and heating to 99˚C for 10 min to denature the proteins. Proteins were separated using 6 or 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (Beyotime Institute of Biotechnology), transferred to polyvinylidene fluoride membranes (EMD Millipore), and blocked with 5% non-fat milk (BD Biosciences) for 1 h at room temperature. The blot was incubated with primary antibodies, including rabbit anti-MAP3K4 (1:1,000; cat. no. ab182165; Abcam), rabbit anti-IL-6 (1:1,000; cat. no. DF6087; Affinity Biosciences), rabbit anti-p38 (1:1,000; cat. no. AF6456; Affinity Biosciences), rabbit anti-phosphorylated (p)-p38 (1:1,000; cat. no. AF4001; Affinity Biosciences) and rabbit anti-β-actin (1:1,000; cat. no. AF7018; Affinity Biosciences) overnight at 4˚C. The blots were then washed with phosphate buffered saline supplemented with 0.05% Tween 20 three times for 10 min at room temperature and incubated with a horseradish peroxide-conjugated IgG secondary antibody (1:2,000; cat. no. 7074P2; Cell Signaling Technology) for 1 h at room temperature. The protein blots were then visualised using an ECL Kit (EMD Millipore), and ImageJ (version: 1.52t; National Institutes of Health) was used for semi-quantitative analysis.

Cytometric bead array (CBA) assay. SMSCs were seeded into a 6-well plate and cultured to ~80% confluence. After corresponding treatment, the supernatant was collected. The concentration of IL-6 in the culture medium was detected using the BD CBA Human IL-6 Protein Kit (BD Biosciences), according to manufacturer's instructions. Subsequently, 50 μl of culture medium from each treatment and assay diluent was added into the respective assay tubes, with the tube containing the assay diluent used as a negative control. Then, 50 μl of dilute IL-6 capture beads and 50 μl of dilute IL-6 phycoerythrin detection reagent were added to each assay tube and incubated in the dark for 3 h at room temperature, and measurements were obtained using a Cytoflex flow cytometer (Beckman Coulter, Inc.) and CytExpert software (version 2.1; Beckman Coulter, Inc.).

Transfection of small interfering (si)RNA or long non-coding (lnc)RNA smart silencer. The siRNA targeting MAP3K4 (Guangzhou RiboBio Co., Ltd.), siRNA negative control (siR NC; cat. no. siN0000001-1-5; Guangzhou RiboBio

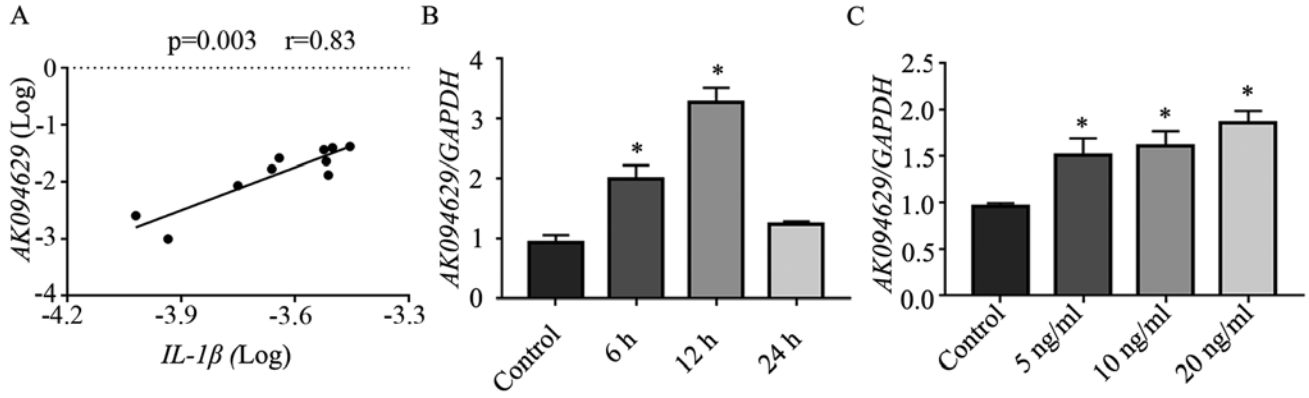

Figure 1. AK094629 in the synovial tissue of patients with TMJOA positively correlates with IL-1β and is upregulated by IL-1β in SMSCs. (A) The relationship between AK094629 expression and IL-1β in the synovium of patients with TMJOA. (B) Detection of AK094629 expression in SMSCs by RT-qPCR after stimulation with 10 ng/ml IL-1β for 6, 12 and 24 h. (C) Detection of AK094629 expression in SMSCs by RT-qPCR after stimulation with 5, 10 or 20 ng/ml IL-1β for 12 h. Patient n=10; experimental repeats n=3. *P<0.05 vs. control. TMJOA, osteoarthritis of the temporomandibular joint, RT-qPCR, reverse transcription-quantitative PCR; SMSCs, synovium-derived mesenchymal stem cells; IL, interleukin.

Co., Ltd.), lncRNA smart silencer targeting AK094629 (Guangzhou RiboBio Co., Ltd.) and lncRNA smart silencer NC (cat. no. lnc3N0000001-1-5; Guangzhou RiboBio Co., Ltd.) were diluted with 250 μl RNase-free water to a concentration of 10 mM and preserved at -20˚C. The NC used was a universal negative control that did not target any known human, mouse or rat genes. SMSCs were seeded into 6-well plates and transiently transfected with siRNA or lncRNA smart silencer after culturing to ~80% confluence. A total of 5 μl siRNA, siR NC, lncRNA smart silencer or lncRNA smart silencer NC and 5 μl of Lipofectamine® RNAiMax (Invitrogen; Thermo Fisher Scientific, Inc.) were pipetted into different non-enzyme EP tubes and diluted with 120 μl of opti-MEM (cat. no. 31985062, Gibco; Thermo Fisher Scientific, Inc.), followed by incubation at room temperature for 15 min to form the si-RNA/Lipofectamine® complexes. Then, 250 μl of the complexes were added to each well of the 6-well plates and cultured in an incubator at 37˚C. After 48 h, the culture medium was replaced and 10 ng/ml IL-1β was added to the IL-1β stimulated groups, but not the control groups. Cells were incubated for 6 h at 37˚C and then used for subequent experiments. The target sequences of siRNA and lncRNA smart silencer used in the present study are listed in Table II.

Nucleoplasm separation. When the cells reached ~80% confluence, they were digested and centrifuged at 350 x g for 5 min at room temperature, the supernatant was then discarded, and 150 μl of cell fractionation buffer (Invitrogen; Thermo Fisher Scientific, Inc.) was added and mixed gently. The mixture was left on ice for 5 min, centrifuged at 400 x g for 5 min at 4˚C and the resulting supernatant, containing the components of the cytoplasm, was transferred to a new EP tube. Subsequently, 50 μl of cell disruption buffer (Invitrogen; Thermo Fisher Scientific, Inc.) was added to the sediment, which contained the components of the nucleus, and the mixture was then placed on ice for 5 min, followed by addition of TRIzol® to extract the RNA.

Fluorescence in situ hybridisation (FISH) assay. When the confluence of SMSCs in the confocal laser-scanning dishes reached ~50%, the culture medium was discarded, and the SMSCs were washed with PBS three times for 5 min and fixed with 4% paraformaldehyde (PFA; Beyotime Institute of Biotechnology) at room temperature for 10 min to cross-link the protein and bind RNA to the protein. PFA was then removed, and the sample was washed with PBS three times for 5 min and incubated with 70% ethanol at 4˚C for 2 h. Next, 150 μl of hybridisation buffer containing 20 nmol/l AK094629 probe (5'-TCTGTTGCGCTTACTGCTATAT-3') was added, and the sample was incubated at room temperature overnight. After hybridisation, the solution was rinsed with 2X saline-sodium citrate (SCC) for 10 min and 1X SCC two times for 5 min at room temperature. Subsequently, the cells were incubated with anti-digoxigenin-fluorescein, fab fragments [1:200; Roche Diagnostics (Shanghai) Co., Ltd.] at room temperature for 40 min and washed with 2X SCC three times for 5 min. Then, 5 ng/ml DAPI (Beyotime Institute of Biotechnology) was added, and the mixture was incubated in the dark for 30 min. Finally, the samples were observed under a fluorescence microscope (magnification, x63). The excitation and emission wavelengths of DAPI are 359 and 461 nm, respectively, and the filter used to select these wavelengths was 410-579 nm. The excitation and emission wavelengths of FITC are 494 and 523 nm, and the filter used was 493-634 nm.

Statistical analysis. Differences between two groups were analysed using Student's t-test. Analysis of variance (ANOVA) was performed to determine significant differences between more than two groups, and the least significant difference (LSD) or Bonferroni post hoc test was used after ANOVA for additional comparisons. Correlation between the expression of IL-1β and AK094629 was analysed using Spearman's rank correlation test. P<0.05 was considered to indicate a statistically significant difference.

Results

AK094629 in the synovial tissue of patients with TMJOA positively correlates with IL-1β and is upregulated by IL-1β in SMSCs. The expression of AK094629 and IL-1β in the synovial tissues donated by ten patients with TMJOA were

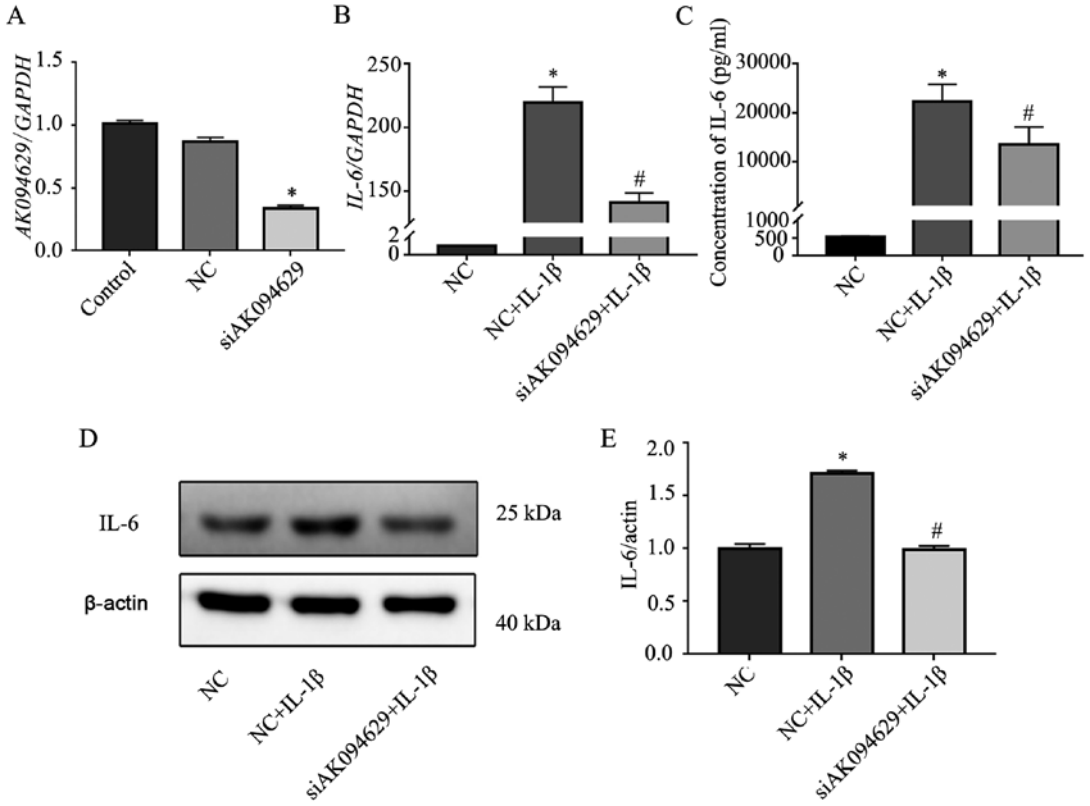

Figure 2. Knockdown of AK094629 reverses IL-1β-induced upregulation of IL-6. (A) Knockdown efficiency of lncRNA smart silencer targeting AK094629 was verified by RT-qPCR. (B) Expression level of IL-6 after knockdown of AK094629 was detected by RT-qPCR. (C) Secretion of IL-6 after knockdown of AK094629 was measured by CBA assay. (D and E) Protein levels of IL-6 after transfection with lncRNA smart silencer targeting AK094629 as detected by western blotting (D) and quantification (E) n=3. *P<0.05 vs. NC; #P<0.05 vs. NC+IL-1β. NC. IL, interleukin; lncRNA, long non-coding RNA; RT-qPCR, reverse transcription-quantitative PCR; CBA, cytometric bead array; NC, negative control; si, small interfering.

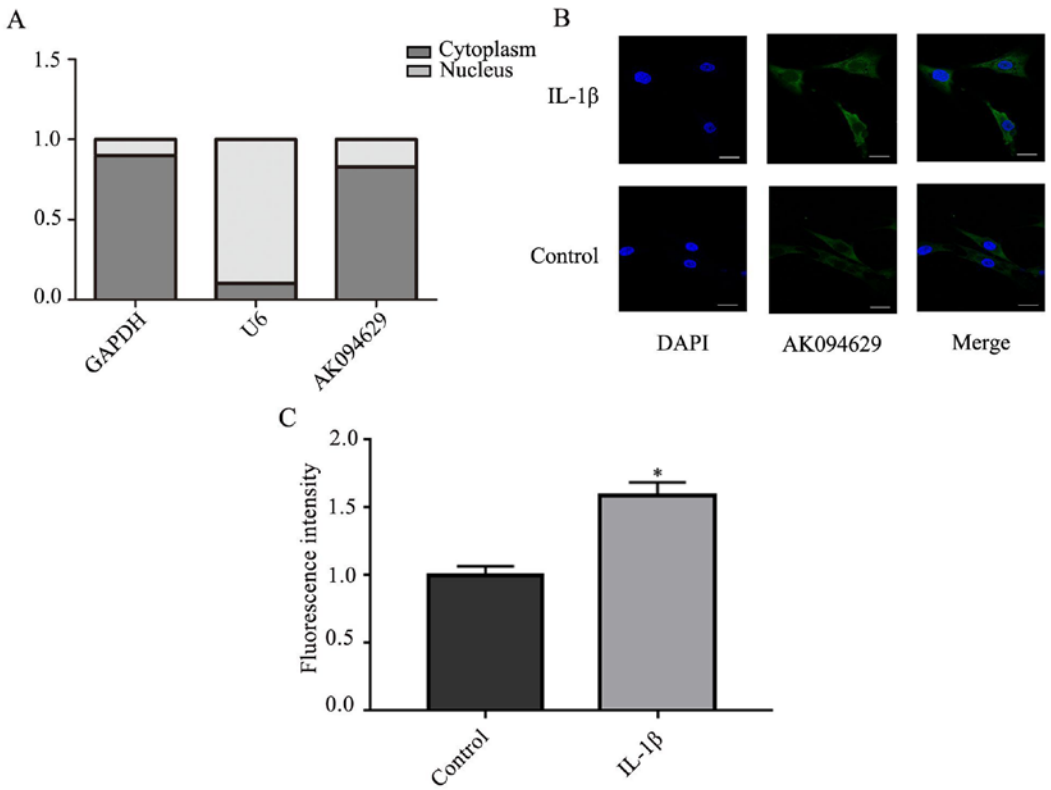

Figure 3. Subcellular localisation of AK094629 in SMSCs. (A-C) AK094629 is localised to the cytoplasm of SMSCs as demonstrated by (A) reverse transcription-quantitative PCR and (B and C) immunofluorescence. Scale bar, 20 μm, n=3. *P<0.05 vs. control. SMSCs, synovium-derived mesenchymal stem cells; IL, interleukin.

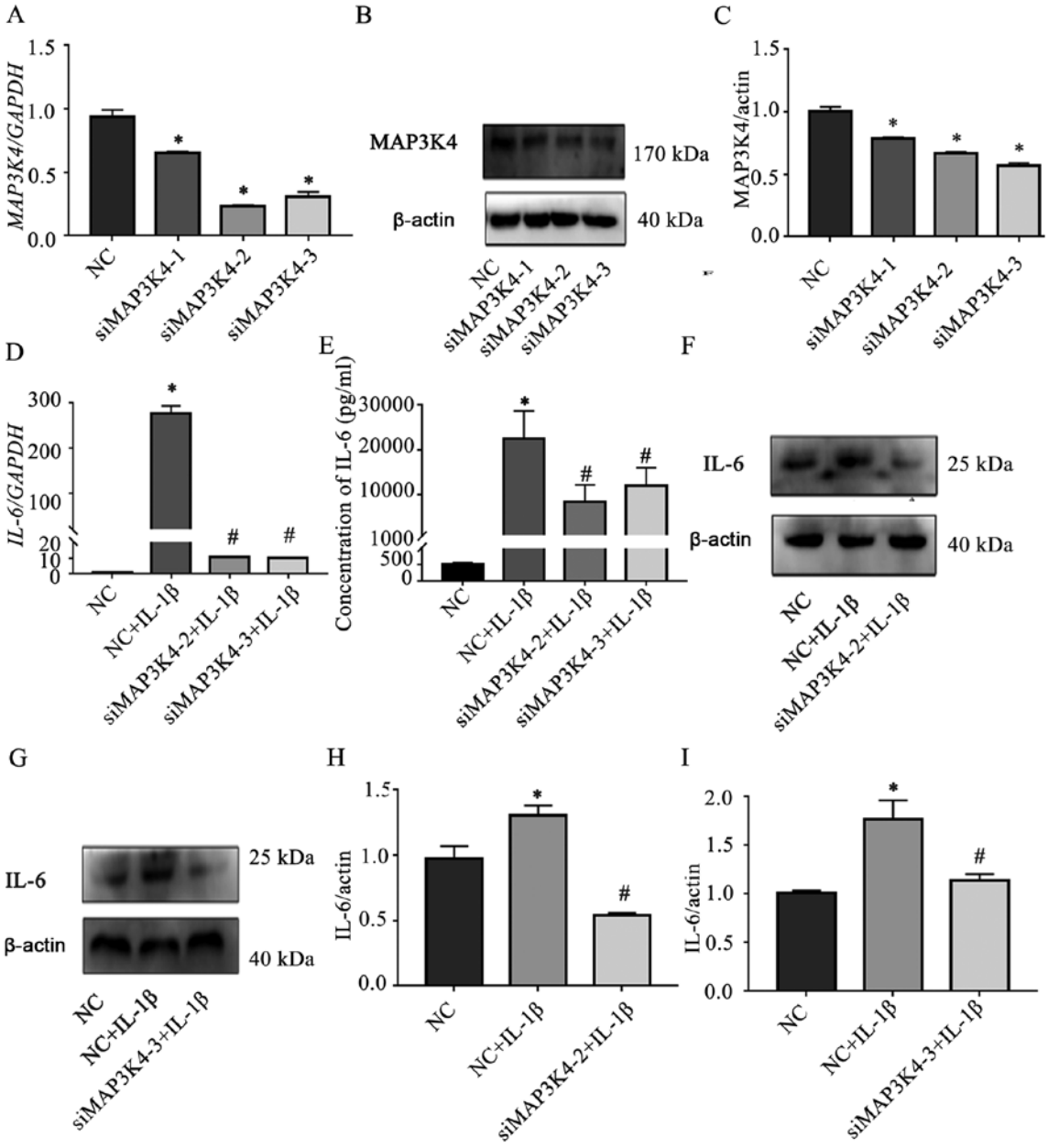

Figure 4. Knockdown of MAP3K4 attenuates the IL-1β-induced upregulation of IL-6 in SMSCs. (A and B) The knockdown efficiency post-transfection with three MAP3K4 targeting siRNAs was verified by (A) RT-qPCR and (B) western blotting. (C) Quantification of the representative blots demonstrated in panel B. (D) The expression level of IL-6 after knockdown of MAP3K4 was detected by RT-qPCR. (E) Secretion of IL-6 post-transfection with siMAP3K4-2 and siMAP3K4-3 was measured by CBA assay. (F and G) Representative western blots demonstrating the expression of IL-6 in SMSCs after knockdown of MAP3K4 using (F) siMAP3K4-2 and (G) siMAP3K4-3. (H and I) Quantification representative blots demonstrated in panels (H) F and (I) G. n=3. *P<0.05 vs. NC group; #P<0.05 vs. NC+IL-1β group. MAP3K4, mitogen-activated protein kinase kinase kinase 4; IL, interleukin; si, small interfering; RT-qPCR, reverse transcription-quantitative PCR; CBA, cytometric bead array; NC, negative control.

determined by RT-qPCR. AK094629 positively correlated with IL-1β according to the Spearman's rank correlation test (Fig. 1A). AK094629 was significantly upregulated in SMSCs stimulated with 10 ng/ml IL-1β to for 6, 12 and 24 h compared with that in unstimulated SMSCs (Fig. 1B). In addition, SMSCs stimulated with 5, 10 and 20 ng/ml IL-1β for 12 h demonstrated upregulated expression of AK094629 in a concentration-dependent manner (Fig. 1C; Table SI).

AK094629 knockdown reverses IL-1β-induced upregulation of IL-6. To determine the effects of AK094629 on IL-1β-induced expression of IL-6 in SMSCs, the cells were transfected with the AK094629 targeting lncRNA smart silencer. The knockdown efficiency was ~60% as verified by RT-qPCR (Fig. 2A; Table SII). In addition, RT-qPCR, CBA assay and western blotting analysis demonstrated that AK094629 knockdown attenuated the IL-1β induced upregulation of IL-6 (Fig. 2B-E; Tables SIII-V).

Subcellular localisation of AK094629. To determine the subcellular localisation of AK094629, the expression of AK094629 in the cytoplasm and nucleus was investigated. The results demonstrated that AK094629 was predominantly located in the cytoplasm (Fig. 3A-C; Table SVI). The

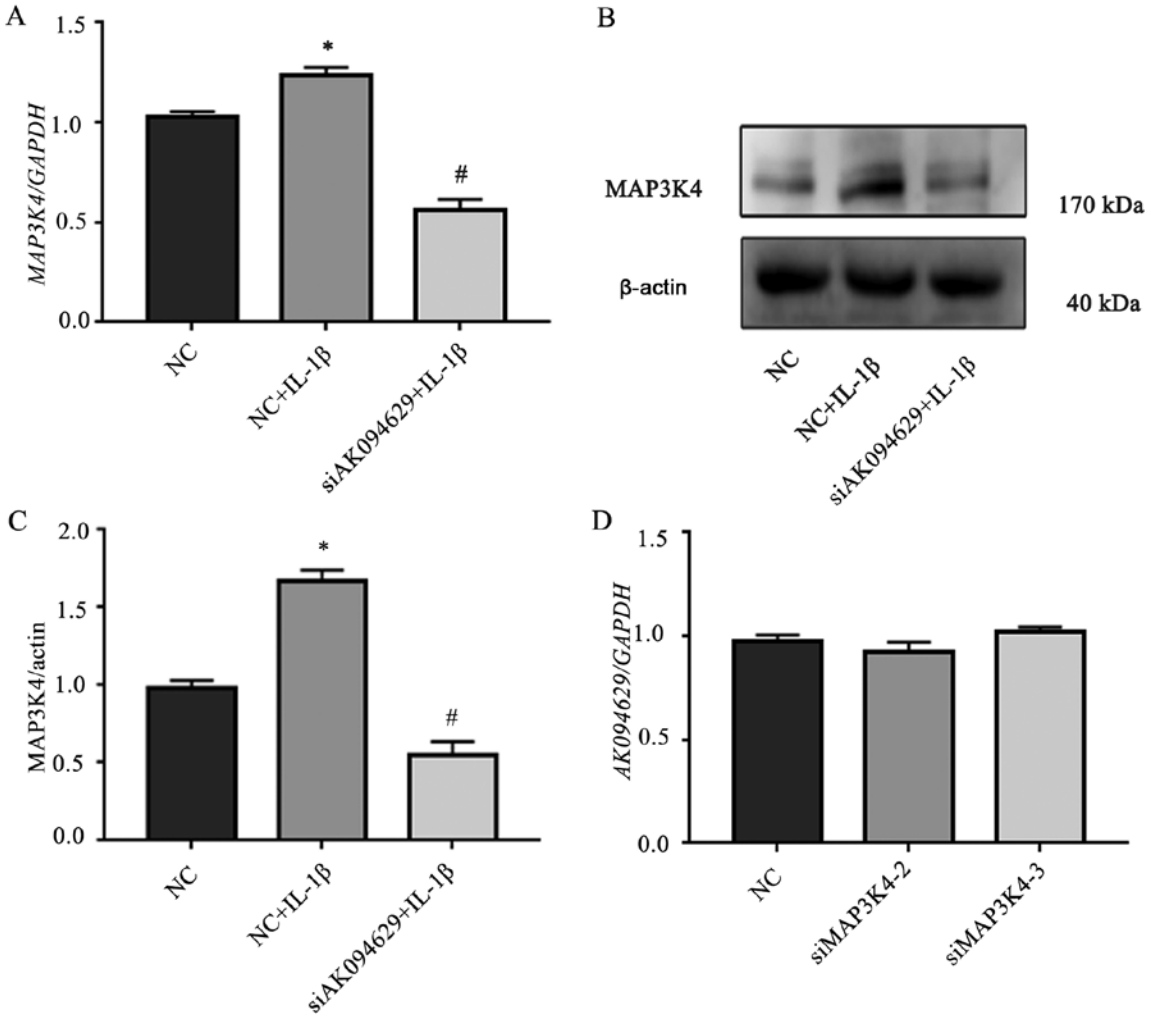

Figure 5. AK094629 effects the expression of MAP3K4, but MAP3K4 has no effect on the expression of AK094629. (A) Expression of the MAP3K4 gene after knockdown of AK094629 was detected by RT-qPCR. (B and C) Expression of the MAP3K4 protein after knockdown of AK094629 was determined by (B) western blotting and (C) quantification. (D) Detection of AK094629 expression by RT-qPCR after knockdown of MAP3K4 using siMAP3K4-2 and siMAP3K4-3. *P<0.05 vs. NC group; #P<0.05 vs. NC+IL-1β group. MAP3K4, mitogen-activated protein kinase kinase kinase 4; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering; NC, negative control; IL, interleukin.

specificity of the AK094629 probe in binding to AK094629 was verified using the FISH assay (Fig. S1).

Knockdown of MAP3K4 attenuated the IL-1β-induced upregulation of IL-6. The role of MAP3K4 on the IL-1β-induced IL-6 expression in SMSCs was explored by transfecting three MAP3K4-targeting siRNAs into the cells; the knockdown efficiencies were verified by RT-qPCR and western blotting (Fig. 4A-C, Tables SVII-VIII), and two of the siRNAs with greater knockdown efficiency were selected for subsequent experiments. RT-qPCR, CBA and western blotting demonstrated that knockdown of MAP3K4 inhibited the IL-1β-induced upregulation of IL-6 in SMSCs (Fig. 4D-I, Tables SIX-XII). In addition, the expression levels of MAP3K4 were significantly upregulated in SMSCs stimulated with IL-1β compared with those in unstimulated cells (Fig. 5A-C; Tables SXIII-XVI).

AK094629 effects the expression of MAP3K4, but MAP3K4 has no effect on the expression of AK094629. The association between AK094629 and MAP3K4 was analysed by knocking down AK094629 and MAP3K4 with lncRNA smart silencer and two siRNAs (siMAP3K4-2 and siMAP3K4-3), respectively. Knockdown of AK094629 with the lncRNA smart silencer downregulated the expression of MAP3K4 based on RT-qPCR and western blotting results (Fig. 5A-C, Tables SV and XIII). However, no significant difference was observed in the expression of AK094629 when MAP3K4 was knocked down compared with the negative control (Fig. 5D; Table SXIV).

MAP3K4 or AK094629 knockdown inhibits p38 expression. The effects on the expression levels of p38, a molecule downstream of MAP3K4, following MAP3K4 and AK094629 knockdown were also explored. Western blotting demonstrated that knockdown of MAP3K4 or AK094629 inhibited the expression of p38 and p-p38 in SMSCs compared with the negative control group. (Fig. 6; Tables SXV and XVI).

Discussion

Although the causes of TMJOA are various and complex, inflammation has been demonstrated to serve an important

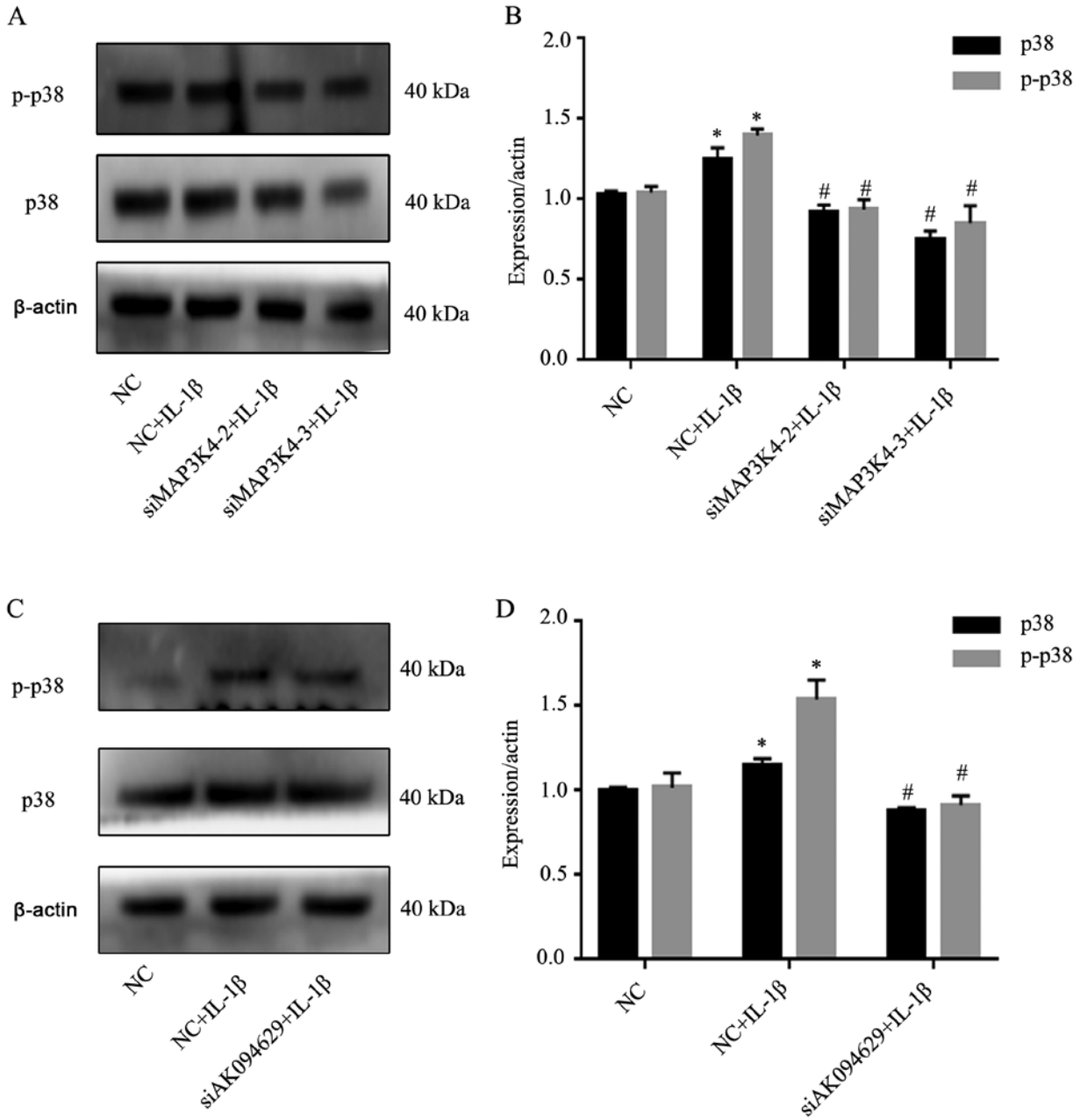

Figure 6. Knockdown MAP3K4 or AK094629 inhibits the expression of p38. (A and B) The expression of p38 and p-p38 protein after transfection with siMAP3K4-2 and siMAP3K4-3 was determined by (A) western blotting and (B) quantification. (C) Representative western blots of p38 and p-p38 in SMSCs after knockdown of AK094629. (D) Quantitation of the representative blots demonstrated in panel C. n=3. *P<0.05 vs. NC; #P<0.05 vs. NC+IL-1β. MAP3K4, mitogen-activated protein kinase kinase kinase 4; SMSCs, synovium-derived mesenchymal stem cells; IL, interleukin; si, small interfering; NC, negative control.

role in its development (13). IL-1β is one of the most important pro-inflammatory factors and a mediator of cartilage degeneration and joint inflammation (37,38). A previous study demonstrated that the expression of IL-1β was significantly increased in the synovial fluid, synovial membrane and cartilage of patients with OA (13). IL-1β can stimulate the production of other inflammatory factors such as IL-6 (8,9), which can subsequently promote the destruction of TMJ tissues by stimulating osteoclast formation, bone resorption, chondrocyte MMP production and inhibiting the differentiation of SMSCs to cartilage (39,40). Therefore, blocking the effects induced by IL-1β may be beneficial to the prognosis and outcome of patients with TMJOA.

IL-6 is an exocrine protein, and for an exocrine protein, the characterisation of extracellular expression levels is more important than intracellular expression. In the present study, this was performed using a CBA assay, which reflected the expression level of IL-6 in the culture medium, thus displaying the continuous accumulation of IL-6. However, the real-time intracellular protein and gene expression levels provided more detailed information about this process; thus, the intracellular and extracellular expression levels of IL-6 in SMSCs were characterised in the present study.

The abnormal expression of certain lncRNAs has been reported to be associated with various pathological processes of OA, including the degradation of ECM, inflammatory

reaction, apoptosis and angiogenesis (24,31). The results of the present study demonstrated the expression of AK094629 in the synovium of patients with TMJOA to be positively correlated with IL-1β. This suggested that AK094629 may be involved in the IL-1β-mediated pathophysiological processes and may be linked to the occurrence and development of TMJOA.

Stem cell chemotaxis to the lesion and differentiation to cartilage is an important repair mechanism of articular cartilage damage (41). A previous study demonstrated that IL-1β, which was significantly upregulated in dysfunctional TMJs, impeded the mesenchymal stem cells in the synovial fluid of the TMJ from differentiating to cartilage through upregulating the secretion of IL-6 (9). In the present study, the expression of AK094629 was also significantly upregulated in SMSCs of the TMJ when stimulated with IL-1β. Knockdown of AK094629 reversed the increase of IL-1β-induced IL-6 expression to some extent. These results suggested that AK094629 contributes to the IL-1β-induced upregulation of IL-6 in the SMSCs of the TMJ. Therefore, lncRNA AK094629 may be a novel therapeutic target of TMJOA.

MAP3K4, also termed MEKK4, is a 180-kDa protein, which is activated by growth factors, inflammatory factors and environmental stress (42). MAP3K4 phosphorylates and activates MAP2Ks, leading to the activation of MAPK pathways, including the pathways of p38 and JNK (32-34). A previous study demonstrated that MAP3K4 serves an important role in the development of skeletal muscles and the neural tube (43), although its role in OA remains unclear. The results of the present study demonstrated that the expression of MAP3K4 was also significantly upregulated in SMSCs after stimulation with IL-1β and that MAP3K4 was downregulated at the gene and protein levels when AK094629 was knocked down. In addition, no significant difference was observed in the expression levels of AK094629 following the knockdown of MAP3K4, whereas the IL-1β-induced upregulation of IL-6 was inhibited in SMSCs. Therefore, MAP3K4 may be one of the downstream targets of AK094629. A limitation of the present study was that only siRNA for MAP3K4 knockdown was used; thus in order to explore the relationship between AK094629 and MAP3K4, an MAP3K4 inhibitor should have been used, and this result should also be confirmed using clinical samples.

Based on the structural characteristics and cellular localisation of lncRNAs, they are divided into several types and influence gene expression through a number of mechanisms, such as chromatin remodelling, competitive endogenous RNA, stability of RNA and recruitment of scaffold proteins at the transcriptional and post-transcriptional levels (44,45). In the present study, the results of RT-qPCR and FISH demonstrated that AK094629 was predominantly located in the cytoplasm. AK094629 is an antisense lncRNA of the nearby MAP3K4, and changes in their levels were correlated; however, the mechanism through which lncRNA AK094629 affects the expression of MAP3K4 is still unclear and needs to be elucidated in future studies.

In summary, the present study demonstrated that in the SMSCs of the TMJ, knockdown of the lncRNA AK094629 reversed the IL-1β-induced upregulation of IL-6 by inhibiting MAP3K4 expression. These findings indicated that lncRNA AK094629 may be a potential novel therapeutic target in the treatment of TMJOA.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

ZZ and YS conceived the experiments. JJ, YS and JS conducted the experiments and drafted the manuscript. WL and LQ analysed the data. KS and YH collected the synovial membrane tissue specimens from the patients and participated in the cell culture experiments. JZ and RY participated in the cell culture experiments. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were approved by the Institutional Ethics Board of the Hospital of Stomatology, Sun Yat-sen University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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