Increased expression of cyclooxygenase-2 in synovium tissues and synovial fluid from patients with knee osteoarthritis is associated with downregulated microRNA-758-3p expression

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Abstract. Cyclooxygenase-2 (COX-2) is a common factor in inflammation, and its specific regulatory mechanism has not been fully elucidated. The present study aimed to investigate COX-2 mRNA and protein expression levels in synovium tissues and synovial fluid from patients with knee osteoarthritis (KOA), and determine the molecular mechanism by which microRNA (miRNA/miR)-758 regulates KOA via COX-2. A total of 37 patients with KOA and 29 patients with acute knee trauma (control group) were enrolled in the present study. Reverse transcription-quantitative PCR analysis was performed to detect miR-758-3p and COX-2 mRNA expression, while western blotting and ELISA were performed to detect COX-2 protein expression in synovium and synovial fluid, respectively. The dual-luciferase reporter assay was performed to verify the interaction between miR-758-3p and the 3'-untraslated region (UTR) of COX-2 mRNA. Synovial cells were transfected with agomiR-758-3p, and the MTT assay was performed to assess cell proliferation. The results demonstrated that COX-2 expression was higher in patients with KOA than those with acute knee trauma. Conversely, miR-758-3p expression was lower in patients with KOA than those with acute knee trauma. Notably, miR-758-3p interacted with the 3'-UTR of COX-2 mRNA to regulate its expression. Overexpression of miR-758-3p inhibited the expression and release of COX-2, as well as the proliferation of human KOA synovial cells. Taken together, these results suggest that COX-2 expression is upregulated in synovium tissues and synovial fluid from patients with KOA, which is associated with downregulated miR-758-3p expression. In addition, miR-758-3p affects the proliferation of synovial cells and the expression of relevant proteins in these cells, thus promoting the occurrence and development of KOA.

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Introduction

Knee osteoarthritis (KOA) is a disease characterized by severe wear of articular cartilage and regeneration of articular margin and subchondral bone (1). Similar to hip OA, KOA can cause weight-bearing difficulty in the lower limbs, eventually resulting in limb disability (2). Inflammatory response induced by synovium tissue injury is an common cause of OA (3,4). The main symptoms of KOA include joint pain and stiffness (5). Joint pain initially appears in joint movement and gradually develops into persistent pain (5). The incidence and development of KOA are associated with age, and the incidence rate is significantly higher in women compared with men (6). According to the etiology and pathogenesis of KOA, it is divided into two subtypes, primary and secondary KOA (7). The pathological process of primary KOA is gradual, and its pathogenesis remains unclear. Secondary KOA often occurs in young adults, and common causes include accidental trauma, deformity, insufficiency of blood supply and joint inflammation (7). Notably, inflammatory mediators, proteoglycans and matrix metalloproteinases (MMPs) are all involved in the pathogenesis of KOA (8-10).

Cyclooxygenase-2 (COX-2) is a member of the cyclooxygenase isoenzyme family, which catalyzes the metabolism of arachidonic acid to form prostaglandins (PGEs). PGE2, an inflammatory mediator, can activate inositol triphosphate through its receptors (EP1R, EP2R, EP3R or EP4R) to promote the release of Ca^{2+} and inhibit Na^{+}/Ca^{2+} exchange channel to decrease Ca^{2+} outflow (11). Simultaneously, intracellular Ca^{2+} release elevates nitrogen oxides expression, which induces the production of reactive oxygen species (ROS) and oxidative stress (12,13). COX-2 expression increases with the occurrence of inflammatory reaction and oxidative stress, which in turn increases the expression levels of the inflammatory mediators, PGE2

and PGH2, as well as vascular endothelial growth factor, which may be induced by PGE2 (14). Thus, the production of oxidative stress-related substances, such as oxygen free radicals and ROS is indirectly induced, further promoting cell damages (14,15). In addition, COX-2 is closely associated with inflammation-related diseases and tumors, and it is speculated that COX-2 can promote tumor angiogenesis, tissue invasion and enhance the apoptosis resistance of tumor cells (16-18). However, the regulation of COX-2 remains unclear.

MicroRNA (miRNA/miR)-758 has been demonstrated to play important regulatory roles in cell metabolism, cholesterol outflow, atheromatous plaque formation and hepatitis C virus (HCV) infection (19). For example, miR-758-3p can regulate cholesterol efflux by inhibiting ABCA1 protein expression (20). Furthermore, Mandolini *et al* (21) reported that miR-758-3p regulates ABCA1 expression in human atherosclerotic plaques. Yang *et al* (19) demonstrated that miR-758-3p expression is significantly upregulated in HCV infection. In addition, abnormal miR-758-3p expression is associated with tumor progression (22). During chemotherapy for esophageal cancer, chemotherapeutic drugs can inhibit miR-758-3p expression, and the loss of control of miR-758-3p to its downstream target genes may be the reason for drug resistance of tumors (23). The bioinformatics of the present study predicted that miR-758-3p has the potential to regulate COX-2 expression. However, the underlying molecular mechanism by which miR-758-3p regulates COX-2 has not yet been investigated.

The present study aimed to investigate COX-2 mRNA and protein expression levels in synovium tissues and synovial fluid from patients with KOA, and determine the underlying molecular mechanism by which miR-758-3p regulates KOA via COX-2.

Materials and methods

Patients. A total of 37 patients with KOA who received treatment at Heze Municipal Hospital between December 2017 and July 2019 were included in the experimental group (20 men and 17 women; age range, 30-60 years; median age, 47.6 years). A total of 29 patients with acute knee trauma who received treatment at Heze Municipal Hospital during the same period were included in the control group (17 men and 12 women; age range, 28-58 years; median age, 46.9 years). All patients with KOA met the 2010 EULAR recommendations (24) for diagnosis. Patients with cancer, rheumatoid arthritis and other immune diseases were excluded from the present study. Synovium tissue samples were collected from patients with KOA during knee replacement or knee arthroscopy, while synovium tissues were collected from patients with knee trauma by resection during surgery. Part of the synovium was used to culture primary cells, while part was used for detection. Synovial fluid was respectively collected from patients with KOA and knee trauma during surgery, and blood cells were removed via centrifugation at 1,000 x g at 4˚C for 10 min. All samples were stored at -80˚C until subsequent experimentation. The present study was approved by the Ethics Committee of Heze Municipal Hospital (Heze, China; approval no. 20161226-85) and written informed consent was provided by all patients or their families prior to the study start.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from fluid and tissue samples using TRIzol® reagent (cat. no. R0016; Beyotime Institute of Biotechnology), according to the manufacturer's instructions. The purity of RNA was assessed by measuring A260/A280 on a reader. According to the manufacturer's protocol, mRNA or miRNA (2 μg) was reverse-transcribed into cDNA at 42˚C using the TIAN Script II cDNA First Strand Synthetic kit and miRcute miRNA cDNA First Strand Synthetic kit, respectively (both purchased from Tiangen Biotech Co., Ltd.). The following primer sequences were used for qPCR using SYBR-Green (cat. no. Q121-02; Vazyme Biotech Co., Ltd.): COX-2 forward, 5'- CAGCCATACAGCAAATCCTTG-3' and reverse, 5'- CAA ATGTGATCTGGATGTCAAC-3'; GAPDH forward, 5'-TGA CCTTGCCCACAGCCTTG-3' and reverse, 5'-CATCAC CATCTTCCAGGAGCG-3'; miR-758-3p forward, 5'-ACA CTCCAGCTGGGTTTGTGACCTGGTCCA-3' and reverse, 5'-TGGTGTCGTGGAGTCG-3'; and U6 forward, 5'-CTCGCT TCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATT TGCGT-3'. The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 5 min; denaturation at 95˚C for 30 sec, annealing at 55˚C for 30 sec and elongation at 72˚C for 60 sec (35 cycles); final extension at 72˚C for 2 min. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (25). If the Cq value was too high (>35), the dose was adjusted, and the experiment was repeated to increase reliable. All experiments were performed in triplicate.

Cell culture. 293T cells were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were maintained in DMEM/F-12 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), at 37˚C with 5% CO_2.

Prior to isolating KOA synovial cells, synovium tissues were washed with PBS containing 2% penicillin-streptomycin (Thermo Fisher Scientific, Inc.). Tissue samples were cut into 1-mm^3-thick sections and digested using 4 mg/ml type II collagenase (Thermo Fisher Scientific, Inc.) at 37˚C for 4 h. The solution was subsequently diluted with PBS, filtered through 200-mesh sterile sieves and centrifuged at 37˚C and 800 x g for 5 min. Cells were resuspended in DMEM supplemented with 20% FBS and 1% penicillin-streptomycin at 37˚C with 5% CO_2. Synovial cells are generally stable within 5 passages (26), thus cells at passages 3-7 were used for subsequent experimentation.

Cells at the logarithmic phase ($3x10^5$) were seeded into 24-well plates and maintained in antibiotic-free DMEM/F-12 medium supplemented with 10% FBS 24 h prior to transfection, until they reached 70% confluence. In the first vial, 1 μl miR-NC (20 pmol/μl; forward, 5'-UUCUCCGAACGUGUC ACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGA ATT-3') or miR-758-3p mimic (20 pmol/μl; forward, 5'-UUU GUGACCUGGUCCACUAACC-3' and reverse, 5'-UUA GUGGACCAGGUCACAAUU-3') was added to 50 μl Opti-MEM® medium (Thermo Fisher Scientific, Inc.). In the second vial, 1 μl Lipofectamine® 2,000 reagent (Thermo Fisher Scientific, Inc.) was added to 50 μl Opti-MEM® medium. Following incubation at 37˚C for 5 min, the vials were combined and incubated for a further 20 min at

room temperature. The mixture was subsequently added to each well and incubated at 37˚C for 6 h. The medium was replaced with DMEM/F-12 medium supplemented with 10% FBS. Cells were collected for subsequent experimentation after 48 h.

Western blotting. Total protein was extracted from tissues or cells using E.Z.N.A total DNA/RNA/Protein kit (Omega Bio-Tek, Inc.). Total protein was quantified using the BCA Protein Assay kit (cat. no. ab102536; Abcam) and 50 μg protein/lane was separated by SDS-PAGE on a 10% gel electrophoresis at 100 V. The separated proteins were subsequently transferred onto polyvinylidene difluoride membranes on ice (100 V for 2 h), and blocked with 5% skimmed milk at room temperature for 1 h. The membranes were incubated with rabbit anti-human COX-2 (1:1,000; cat. no. ab15191; Abcam) and β-actin (1:5,000; cat. no. ab129348; Abcam) polyclonal primary antibodies, overnight at 4˚C. Membranes were washed three times with PBS containing Tween-20 (0.1%) for 15 min, and subsequently incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:3,000; cat. no. ab6721; Abcam) for 1 h at room temperature. Membranes were re-washed three times with PBS containing Tween-20 (0.1%) for 15 min. Protein bands were visualized using the enhanced chemiluminescence detection kit (cat. no. ab65623; Abcam) and analyzed using Image lab v3.0 software (Bio-Rad Laboratories, Inc.).

ELISA. Synovial fluid was centrifuged at 1,000 x g at 4˚C for 10 min and the supernatant was collected, which was used to detect protein contents according to the manufacturer's protocols (COX-2 ELISA kit; cat. no. ab267646; Abcam and Ca PGE2 ELISA kit; cat. no. CSB-E07965h; Cusabio Technology LLC).

Bioinformatics analysis. The miRanda database (http://www.microrna.org/microrna/home.do) was used to predict the genes that regulate COX-2 expression.

Dual-luciferase reporter assay. Based on bioinformatics analysis, wild-type (WT) and mutant regions of miR-758-3p in the 3'-UTR of COX-2 mRNA were chemically synthesized *in vitro*. The ends were joined with Spe-1 and HindIII restriction sites, and subsequently cloned into pMIR-REPORT luciferase reporter plasmids (Thermo Fisher Scientific, Inc.). Plasmids (1 μg) with WT or mutant 3'-UTR sequences were co-transfected into 293T cells with agomiR-758-3p (100 nM; forward, 5'-UUUGUGACCUGGUCCACUAACC-3' and reverse, 5'-UUAGUGGACCAGGUCACAAAUU-3') or agomiR-negative control (agomiR-NC; 100 nM; forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'; both purchased from Sangon Biotech Co., Ltd.) using Exfect Transfection Reagent (Vazyme Biotech Co., Ltd.). Following incubation at 37˚C for 24 h, cells were lysed using the dual-luciferase reporter assay kit (cat. no. E1980; Promega Corporation), according to the manufacturer's instructions, and luciferase activities were detected using the GloMax 20/20 luminometer (Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

MTT assay. Cell proliferation was assessed via the MTT assay. Following transfection, cells were seeded into 96-well plates at a density of $2x10^3$ cells/well. Each condition was assessed in triplicate wells. At 24, 48 and 72 h post-transfection, 20 μl MTT reagent (5 g/l; cat. no. C0009; Beyotime Institute of Biotechnology) was added to each well and incubated for 4 h at 37˚C. Following the MTT incubation, the purple formazan crystals were dissolved using DMSO (150 μl/well) and proliferation was subsequently analyzed at a wavelength of 490 nm, using a microplate reader (Bio-Rad Laboratories, Inc.).

Statistical analysis. Statistical analysis was performed using SPSS 18.0 software (SPSS, Inc.). Each experiment was repeated three times. Data are presented as the mean ± standard deviation. Unpaired Student's t-test was used to compare differences between two groups, while one-way ANOVA followed by Bonferroni post hoc test were used to compare differences between multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

COX-2 expression is higher in patients with KOA than those with acute knee trauma. RT-qPCR analysis was performed to detect COX-2 mRNA expression in synovium tissues and synovial fluid. In addition, western blotting and ELISA were performed to detect COX-2 protein expression in synovium tissues and synovial fluid, respectively. The results demonstrated that COX-2 mRNA and protein expression levels were significantly higher in synovium tissues from patients with KOA compared with the control group ($P<0.05$; Fig. 1A and B). Similarly, COX-2 mRNA and protein expression levels were significantly higher in synovial fluid from patients with KOA compared with the control group ($P<0.01$; Fig. 1C and D). Taken together, these results suggest that COX-2 may play a regulatory role in the pathological process of KOA.

miR-758-3p expression is lower in patients with KOA than those with acute knee trauma. Bioinformatics analysis was performed to predict the genes that regulate COX-2 expression. The results demonstrated that miR-758-3p can potentially interact with COX-2 (Fig. 2A). RT-qPCR analysis was performed to detect miR-758-3p expression in synovium tissues and synovial fluid. The results demonstrated that miR-758-3p expression was significantly lower in synovium tissues and synovial fluid from patients with KOA compared with the control group ($P<0.05$ and $P<0.01$; Fig. 2B and C, respectively). Collectively, these results suggest that miR-758-3p expression is downregulated in patients with KOA.

miR-758-3p interacts with the 3'-UTR of COX-2 to regulate its expression. The dual-luciferase reporter assay was performed to confirm the interaction between COX-2 mRNA and miR-758-3p. As presented in Fig. 3, the luciferase activity of 293T cells co-transfected with agomiR-758-3p and pMIR-REPORT was significantly lower in the wild-type group compared with the NC group ($P<0.05$). Conversely, no significant difference was observed in the luciferase activity of cells co-transfected with agomiR-758-3p and pMIR-REPORT between the mutant and NC groups ($P>0.05$; Fig. 3). Taken

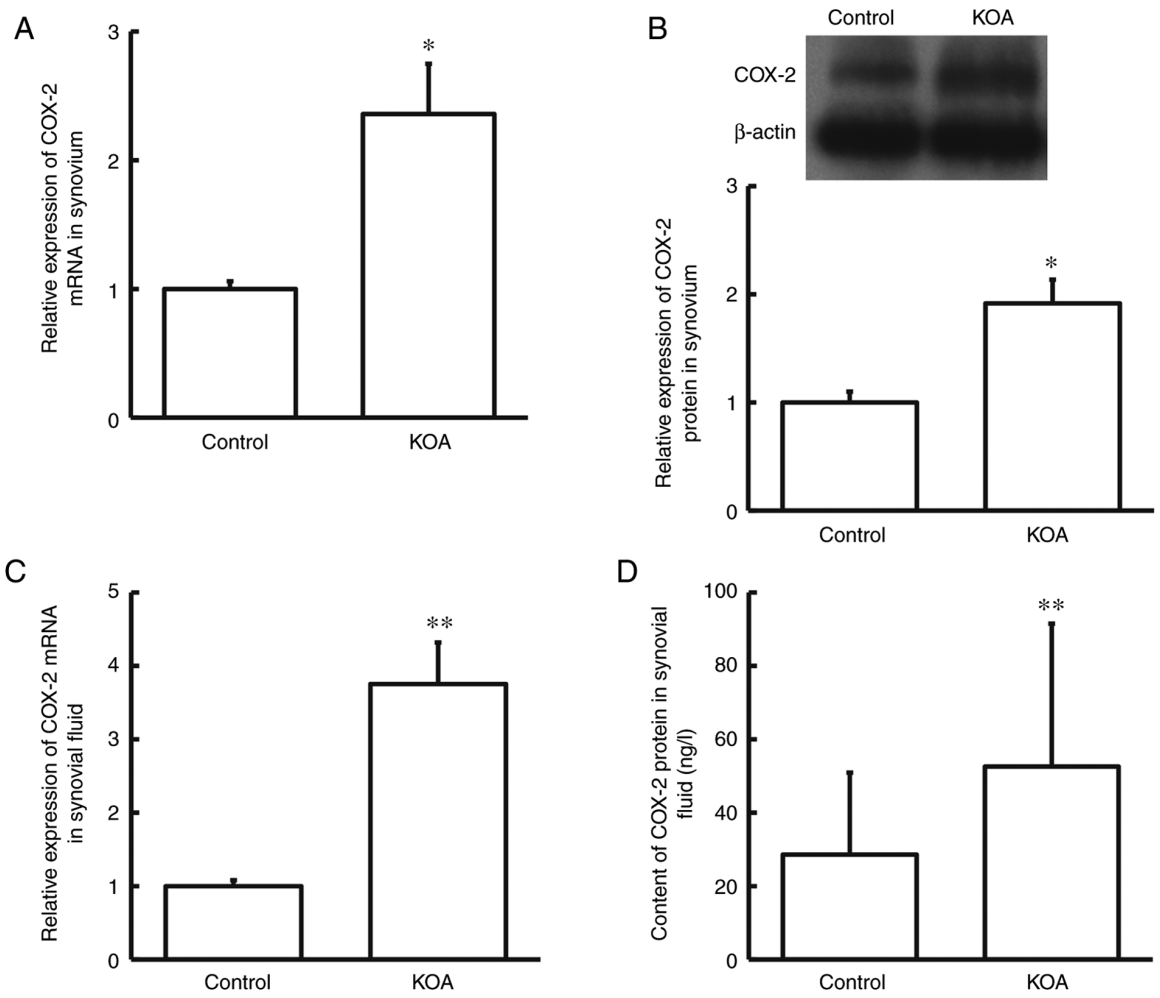

Figure 1. COX-2 expression in synovium tissues and synovial fluid. RT-qPCR and western blot analyses were performed to detect COX-2 (A) mRNA and (B) protein expression levels in synovium tissues from patients with acute knee trauma and KOA, respectively. RT-qPCR analysis and ELISA were performed to detect COX-2 (C) mRNA and (D) protein expression levels in synovial fluid from patients with acute knee trauma and KOA, respectively. *P<0.05, **P<0.01 vs. control group. COX-2, cyclooxygenase-2; RT-qPCR, reverse transcription-quantitative PCR; KOA, knee osteoarthritis.

together, these results suggest that miR-758-3p interacts with the 3'-UTR of COX-2 to regulate its expression.

Overexpression of miR-758-3p inhibits the expression and release of COX-2, as well as the proliferation of human KOA synovial cells. To investigate how miR-758-3p regulates COX-2 expression, KOA synovial cells were transfected with agomiR-758-3p. The results demonstrated that miR-758-3p expression was significantly higher in cells transfected with agomiR-758-3p compared with the agomiR-NC group (P<0.01; Fig. 4A). Conversely, COX-2 mRNA and protein expression levels were significantly lower in the agomiR-758-3p group compared with the agomiR-NC group (P<0.01 and P<0.05; Fig. 4B and C, respectively). ELISA indicated that COX-2 protein expression was significantly lower in the supernatant of cells transfected with agomiR-758-3p compared with the agomiR-NC group (P<0.01; Fig. 4D). COX-2 is also known as prostaglandin-endoperoxide synthase-2 (PTGS-2). PGE2, which is biosynthesized from arachidonic acid by COXs (PTGSs), is a physiologically active lipid (27). Notably, PGE2 protein expression was significantly lower in the supernatant of cells transfected with agomiR-758-3p compared with the agomiR-NC group (P<0.01; Fig. 4E). The results of the MTT assay demonstrated that cells transfected with agomiR-758-3p proliferated at a slower rate at 48 and 72 h compared with the agomiR-NC group (P<0.05; Fig. 4F). Collectively, these results suggest that overexpression of miR-758-3p inhibits the expression and release of COX-2, as well as the proliferation of human KOA synovial cells.

Discussion

KOA is a chronic degenerative disease of the bone and joints. The main pathological changes of KOA are articular cartilage degeneration and hyperosteogeny (28). The main clinical symptoms of KOA include pain, swelling, limited movement, friction sound and deformity of knee joints (5). The incidence of KOA continues to increase with age and obesity, and thus has attracted great interest in clinical practice (29).

Currently, the treatment strategies for KOA lack efficiency. Western medicine is classified into drug treatment and surgical treatment (30). COX-2 is a membrane binding protein, and its expression in cartilages is elevated following impacts by physical and chemical factors, such as trauma, inflammatory

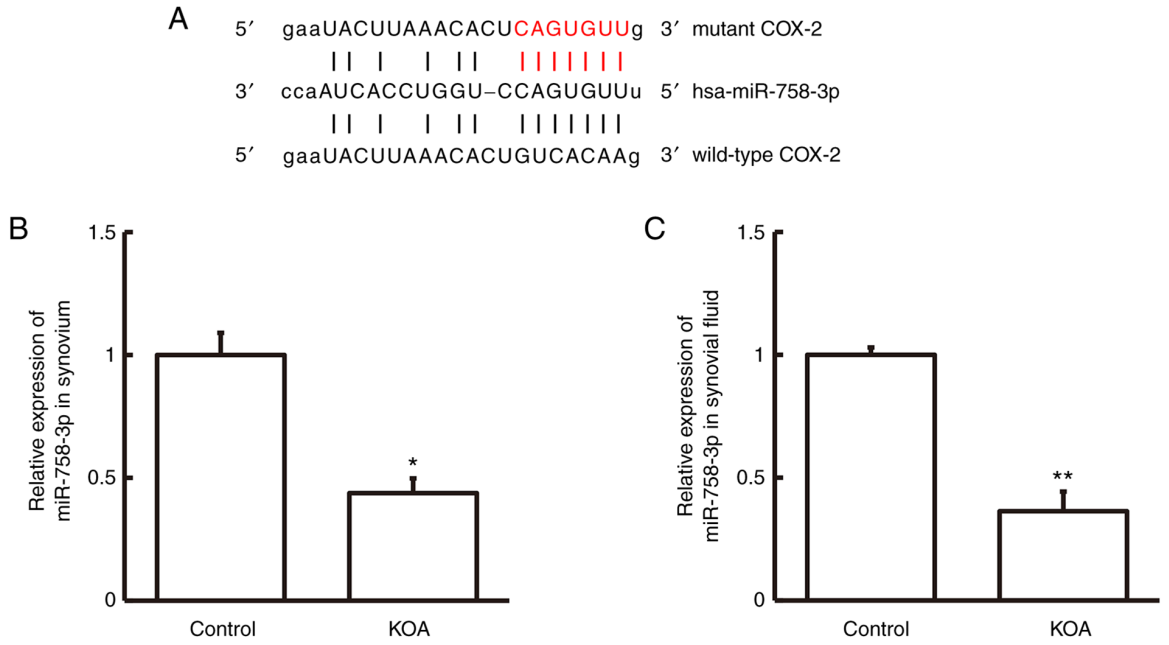

Figure 2. Bioinformatics prediction of upstream miRNA of COX-2, and miR-758 expression in synovium tissues and synovial fluid. (A) miR-758-3p can potentially interact with COX-2. Reverse transcription-quantitative PCR analysis was performed to detect miR-758-3p expression in (B) synovium tissues and (C) synovial fluid from patients with acute knee trauma and KOA, respectively. *P<0.05, **P<0.01 vs. control group. miRNA/miR, microRNA; COX-2, cyclooxygenase-2; KOA, knee osteoarthritis.

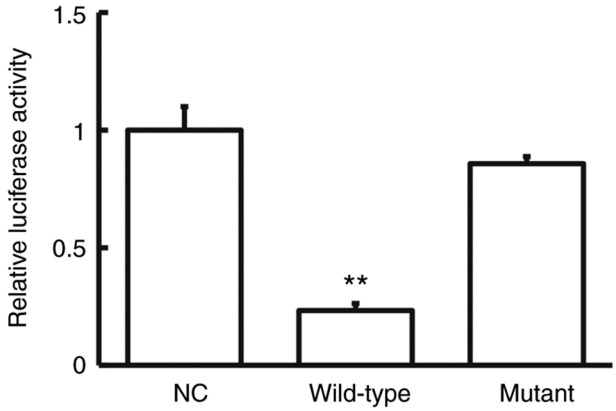

Figure 3. Relative luciferase activity of 293T cells co-transfected with agomiR-758-3p and wild-type or mutant 3'-untranslated region of the COX-2 gene. The dual-luciferase reporter assay was performed to confirm the interaction between miR-758-3p and COX-2 mRNA. **P<0.01 vs. NC group. miR, microRNA; COX-2, cyclooxygenase-2; NC, negative control.

mediators and cytokines (31). COX-2 can promote the synthesis of PGE2, which induces the production of nitric oxide (32). PGE2 is a multifunctional inflammatory mediator that increases the production of MMPs and catabolic substances, affects the structure and function of the joint, and accelerates the destruction of joint structure (33). Thus, it can be used as a target for KOA therapy.

The results of the present study demonstrated that COX-2 expression was abnormally high in synovium tissues and synovial fluid from patients with KOA, suggesting that COX-2 plays an important biological role in KOA. However, the regulation of COX-2 in KOA remains unclear. Bioinformatics analysis revealed that miR-758-3p regulates COX-2 expression. miRNAs regulate mRNAs by inhibiting translation (34). It has been reported that miR-758-3p can target HMGB3 and inhibit the proliferation and metastasis of cervical cancer cells via the Wnt/β-catenin signaling pathway (35). Similarly, miR-758-3p targets HMGA1 to inhibit the malignant phenotype of osteosarcoma via the Wnt/β-catenin signaling pathway (36). miR-758-3p also inhibits the malignant progression of retinoblastoma by directly targeting PAX6 (37). In addition, miR-758-3p also inhibits lung cancer cells (38,39). Based on these findings, it was speculated that miR-758-3p is closely associated with inflammation.

The results of the present study demonstrated that miR-758-3p expression was significantly lower in synovium tissues and synovial fluid from patients with KOA compared with the control group. Considering the abnormally high COX-2 expression, it was speculated that downregulated miR-758-3p expression induces upregulated COX-2 expression, which was confirmed via the dual-luciferase reporter assay. Synovial cells were subsequently transfected with agomiR-758-3p and the MTT assay was performed to assess cell proliferation. The results demonstrated that overexpression of miR-758-3p decreased COX-2 mRNA and protein expression levels, as well as the secretion of COX-2. In addition, overexpression of miR-758-3p decreased the proliferative ability of the cells.

The present study has a limitation in the small number of patients, which will be overcome in the future by performing studies with a larger number of patients. COX-2 is an inducible enzyme of inflammatory cytokines, including interleukin (IL)-1 and tumor necrosis factor-α (TNF-α). Notably, IL-1 and TNF-α have been detected in synovial fluid from patients with KOA (40). However, the present study failed to investigate whether COX-2 expression was affected by IL-1 or TNF-α, in addition to miR-758-3p.

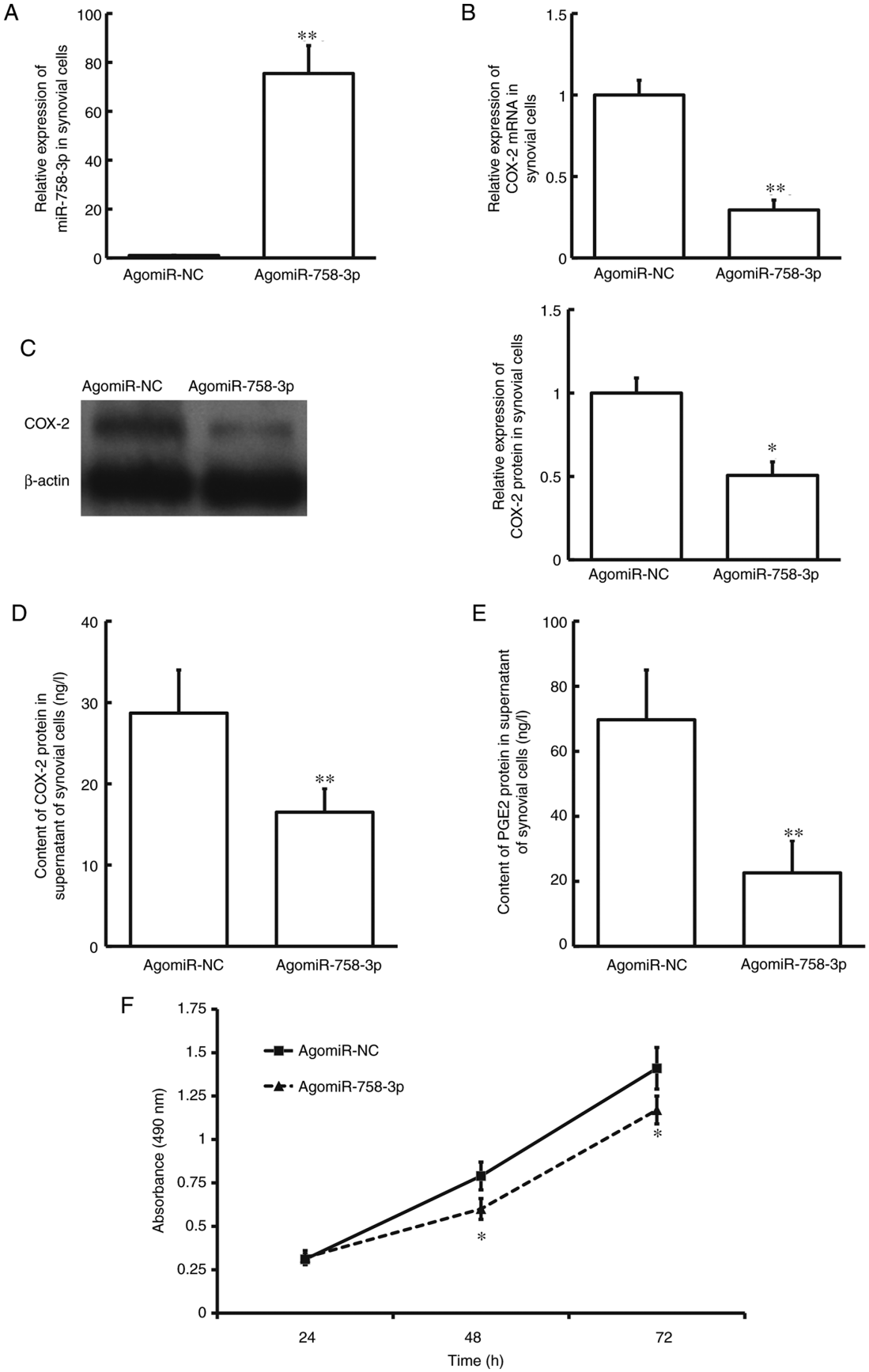

Figure 4. Effect of miR-758-3p on COX-2 expression and the proliferation of synovial cells. (A) miR-758-3p expression in synovial cells transfected with agomiR-NC or agomiR-758-3p. Reverse transcription-quantitative PCR and western blot analyses were performed to detect COX-2 (B) mRNA and (C) protein expression levels in synovial cells transfected with agomiR-NC or agomiR-758-3p, respectively. ELISA was performed to detect (D) COX-2 and (E) PGE2 protein expression levels in the supernatant of synovial cells transfected with agomiR-NC or agomiR-758-3p, respectively. (F) The MTT assay was performed to assess the proliferation of synovial cells transfected with agomiR-NC or agomiR-758-3p. *P<0.05, **P<0.01 vs. agomiR-NC group. miR, microRNA; COX-2, cyclooxygenase-2; NC, negative control; PGE2, prostaglandin E2.

In conclusion, the results of the present study demonstrated that miR-758-3p regulates COX-2 transcription factor by directly targeting and changing COX-2 protein expression, which in turn affects the proliferation of synovial cells. Taken together, these results suggest that miR-758-3p, in joint fluid, may be used as a diagnostic biomarker for patients with KOA.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZL and XH designed the present study. ZL, JS and TL performed the experiments. ZL, JS and XH analyzed the data. ZL and XH interpreted the results and drafted the initial manuscript. ZL, JS and XH confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Heze Municipal Hospital (approval no. 20161226-85; Heze, China) and written informed consent was provided by all patients or their families prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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