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LncRNA OIP5-AS1 affects the biological behaviors of chondrocytes of patients with osteoarthritis by regulating micro-30a-5p

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Abstract. – OBJECTIVE: Osteoarthritis (OA) is a prevalent chronic orthopedic disease, but its relationship with the lncRNA OIP5 Antisense RNA 1(OIP5-AS1)/micro-30a-5p axis is still under investigation. This study was designed to explore the regulatory function of this axis on chondrocytes.

PATIENTS AND METHODS: A quantitative polymerase chain reaction (qPCR) assay was carried out to quantify lncRNA OIP5-AS1 and micro-30a-5p in cartilage tissues of patients with OA, and lncRNA OIP5-AS1 siRNA, micro-30a-5p mimics, and micro-30a-5p inhibitor vectors were constructed to analyze the functions of lncRNA OIP5-AS1/micro-30a-5p on chondrocytes. In addition, the Western blot was used to determine the levels of proteins in chondrocytes, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)assay to determine the viability of chondrocytes, the flow cytometry to analyze apoptosis and cycle of them, and the Dual-Luciferase reporter gene assay to verify the targeting relationship between LncRNA OIP5-AS1 and micro-30a-5p.

RESULTS: LncRNA OIP5-AS1 in cartilage tissues of patients with OA decreased, while micro-30a-5p in them increased and increased with apoptosis. Down-regulation of lncRNA OIP5-AS1 gave rise to increased micro-30a-5p, and up-regulation of micro-30a-5p or down-regulation of lncRNA OIP5-AS1 brought about cell apoptosis and inflammatory response, and inhibited cell proliferation, while up-regulation of micro-30a-5p suppressed cell apoptosis and inflammatory response, and accelerated cell proliferation. LncRNA OIP5-AS1 affected chondrocytes by negatively regulating micro-30a-5p.

CONCLUSIONS: LncRNA OIP5-AS1 inhibits the apoptosis and inflammatory response of chondrocytes and promotes their survival by targetedly inhibiting micro-30a-5p, and both up-regulation of lncRNA OIP5-AS1 and down-regulation of micro-30a-5p is beneficial to patients with OA.

Key Words:
Osteoarthritis, LncRNA OIP5-AS1, Micro-30a-5p, Chondrocytes.

Introduction

Osteoarthritis (OA) is a prevalent chronic orthopedic disease. If it is not treated timely and effectively, it will not only compromise patients' daily life[1,2], but also increase the risk of cardiovascular disease[3]. Chondrocytes induce OA through balance disorder of phenotypes such as extracellular matrix metabolism and growth of chondrocytes themselves[4,5], effectively inhibiting the inflammatory response and apoptosis of chondrocytes is beneficial to relieving OA[6-8]. Therefore, it is possible to promote the development of OA treatment strategies by regulating the phenotype balance of chondrocytes.

The regulation of phenotypes by miRNA is crucial in the development of OA, so screening and studying miRNA with an abnormal expression are beneficial for human to understand the mechanism of OA and the formulation of subsequent treatment strategies for OA. MiR-30a is a non-coding RNA about 71bp long, and micro-30a-5p is a mature spliceosome of miR-30a. MiR-30a and its spliceosomes can regulate the development of tumors including non-small-cell lung carcinoma, colon cancer, melanoma, breast cancer and head and neck squamous cell carcinoma through different molecular networks[9-13]. For patients with OA, miR-30a can induce degradation of extracellular matrix in chondrocytes by down-regulating SRY-Box Transcription Factor 9(SOX9)[14], and our study found that micro-

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30a-5p was upregulated in cartilage tissues of patients with OA. Therefore, we suspected that micro-30a-5p, as a spliceosome of miR-30a, may also be involved in OA. LncRNA inhibits the regulation of miRNA on downstream genes by binding to miRNA through specific loci of its sequence, which is called as lncRNA sponging. Sponging belongs to the upstream regulation of miRNA, so the relationship between miRNA and diseases is regulated by it. Abnormal expression of lncRNA OIP5-AS1 is strongly linked to the pathogenesis of many diseases[15-20]. In this study, we found that lncRNA OIP5-AS1 was down-regulated in patients with OA, so we suspected that the down-regulation of it may be linked to OA.

Because of the opposite expression lncRNA OIP5-AS1 and micro-30a-5p in cartilage tissues of patients with OA, we suspected that the two may affect the development of OA together. At present, the mechanism of lncRNA OIP5-AS1 and micro-30a-5p is still under exploration, so this study would make the two express differently in cartilage tissues to study the relationship between them and OA.

Patients and Methods

Patients with OA

Cartilage tissues were sampled from 59 patients with OA in our hospital including 27 patients with femoral OA and 32 patients with knee OA. The patients with OA were selected according to the diagnostic criteria of OA formulated by American Rheumatology Society[21]. The inclusion criteria of the patients were as follows: patients diagnosed with OA according to X-ray film and clinical indexes and those who cooperated with the treatment. The exclusion criteria of them were as follows: patients with other joint diseases or mental disease, patients with other comorbid tumors, and those with a previous treatment history. The patients were informed of the information about the study during the whole study process, and the whole research was allowed by the Ethics Committee of our Hospital. The tissue samples were cut into sections, and stored in -80°C for future detection.

Cell Culture and Transfection

W1353 Chondrocytes (ATCC, Manassas, VA, USA) were transferred to a T25 cell culture flask (Thermo Fisher Company, Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, South Logan, UT, USA) containing 1% penicillin-streptomycin (100X, Solarbio , Beijing, China) and 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). The medium should be preheated to 37°C before culturing. The cells were cultured in a 5% CO_2 animal cell incubator (Binder, Shanghai, China) at 37°C until they were in a good growth condition, and then seeded into a 6-well plate for transfection. Micro-30a-5p mimics, micro-30a-5p inhibitor, NC mimics, NC inhibitor, OIP5-AS1 siRNA, and NC siRNA vectors purchased from Shanghai Sangon Biotech company (Shanghai, China) were transfected into cell lines with a Lipofectamine 2000 transfection kit (Invitrogen, Carlsbad, CA, USA) in accordance with the kit instructions. Before transfection, the medium was replaced with medium without FBS to avoid interference of serum, and after 8 hours of transfection, fresh medium was used to replace the former medium to avoid poisoning cells.

Quantitative Polymerase Chain Reaction (qPCR)

Total RNA in tissues or cells was extracted by the TRIzol method. Ultraviolet spectrophotometer was used to quantify the OD value of total RNA at 260-280. The samples with OD 260/OD 280>1.8 were used for subsequent qPCR assay. The RNA was quantified and analyzed using a reverse transcription and fluorescence quantitative kit (Tiangen Biotech company, Beijing, China) and ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA). The primers of micro-30a-5p and lncRNA OIP5-AS1 were all designed and synthesized by Shanghai Sangon Biotech Co., Ltd. For micro-30a-5p, F: 5'-TGTAAACATCCT CGACTGGAAG-3', and R: 5'-TGCGTGTCGTGGAGTC-3'; for lncRNA OIP5-AS1, F: 5'-TGCACATACACAGGTTAGAACAAG-3', and R: 5'-GAA CCT AAACTTGGGTCTCTG-3'. For U6, Upstream primer (5-3): CTCGCTTCGGCAGCACA; downstream primer (5-3): AACGCTTCACGAATTTGCGT. For GAPDH, upstream primer (5-3): AGAAGGCTGGGGCTCATTTG; downstream primer (5-3): AGGGGCCATCCACAGTCTTC. The reaction system of qPCR: 1.25 mL F primer, 1.25 mL R primer, 1.0 μL probe, 10 pg/mg RNA template, 5 μL 50×ROX Reference Dye ROX, and RNase-Free ddH_2O added to adjust 50 mL the volume. The reaction process: one cycle of reverse transcription at 50°C for 30 min, one cycle of initial

denaturation at 95°C for 3 min, followed by 40 cycles from denaturation at 95°C for 15 s to annealing at 60°C for 30 s. The data were normalized using the $2^{-DD}Ct$ method, with U6 and GAPDH as internal references.

Western Blot

The protein extract was prepared with 20 mM of Tris-HCl solution at pH 7.5 (Solarbio Company, Beijing, China) and protein inhibitor (Solarbio Company, Beijing, China). Adherent cells were digested and prepared into cell suspension, and added with 1 mL protein extract, followed by repeated pipetting until the cells were fully lysed. The suspension was centrifuged with a pre-cooling centrifuge at $1.6\times10^4\times g$ and 4°C for 20 min to take the supernatant, and the protein concentration of the supernatant was determined using the bicinchoninic acid (BCA) method. The protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated protein was transferred to a nitrocellulose (NC) membrane and let to stand at room temperature for 1 h (blocked with 5% skim milk – phosphate-buffered saline (PBS) solution). Subsequently, the protein to be detected was let to stand with β-actin primary antibody at 4°C overnight, and the NC membrane was washed with PBS solution three times, added with goat anti-rabbit secondary antibody (horseradish peroxidase (HRP) conjugant), and then let to stand for 1 h at room temperature. Finally, the NC membrane was washed with PBS solution, and visualized by enhanced chemiluminescence (ECL) reagent. The internal reference protein was β-actin, and the relative expression level of the protein to be detected was recorded as the gray value of the band to be detected/the gray value of β-actin protein band. Caspase 3, Caspase 9, Bax, Bcl-2, IL-6, IL-8, TNF-a, β-actin primary antibody, and goat anti-rabbit secondary antibody (HRP conjugant) were all purchased from Shanghai Abcam Company (Shanghai, China).

Flow Cytometry

The adherent cells were digested and prepared into cell suspension, and the concentration of the suspension was determined using a Countess TM automatic counter (Invitrogen, Carlsbad, CA, USA). The number of cells was diluted to 1×10^6. First, the cells were immobilized at 4°C for 30 min and the immobilization solution was 70% ethanol solution. Then, the immobilization solution was removed, and the cells were cultured in Annexin V-FITC/PI solution. Finally, the FACScan flow cytometer (Becton Dickinson Company, Lake Franklin, NJ, USA) was used to analyze apoptosis. The steps to detect cell cycle were similar to the above steps, but annexin V-FITC / PI should be replaced with disodium propionate iodide (50 ng / ml) / RNase (0.2 mg / ml) / 0.1% Triton X-100.

MTT Assay

Four 96-well plates were adopted for this assay. Cell suspension (100 μL) was added into one well including 6×10^3 cells, and 3 wells were chosen randomly. One plate was taken out at 24 h, 48 h, 72 h and 96 h, and was added with 5 mg/ml MTT solution at 10 μL/well each time. After 1 hour, dimethylsulfoxide (DMSO) (Solarbio Company, Beijing, China) was added and the plates were shaken lightly. Finally, enzyme mark instrument was used to determine the OD_{570} value.

Dual-Luciferase Reporter Assay

The pmirGLO-OIP5-AS1 wt and pmirGLO-OIP5-AS1 mut vectors were constructed, and co-transfected with micro-30a-5p mimics and NC mimics into chondrocytes, respectively. The transfected cells were cultured in a 96-well plate, and their Luciferase activity was detected using the Dual-Luciferase reporter gene assay system (Promega, Madison, WI, USA) after 48 hours of culturing.

Statistical Analysis

Data in this study were analyzed statistically using SPSS 20 (IBM, Armonk, NY, USA) and visualized into required figures using GraphPad Prism 8.0. Independent-samples *t*-test was used to determine the statistical differences between patients with OA and normal individuals and between the NC siRNA group and the OIP5-AS1 group. Differences between above 2 groups were compared using the one-way ANOVA. LSD-*t* test was used to determine the post hoc pairwise comparison. The correlation between lncRNA OIP5-AS1 and micro-30a-5p was determined by Pearson's analysis. All data were analyzed using the two-tailed test, and 95% was used as the confidence interval. $p<0.05$ indicates a statistical difference. Measurement data were expressed as the mean±standard-deviation.

Results

LncRNAOIP5-AS1 Is Down Regulated in Cartilage Tissues of Patients with OA, and Micro-30a-5p Is Up Regulated in Them

Cartilage tissues were sampled from 59 patients with OA and 43 healthy individuals, and a qPCR assay was carried out to quantify micro-30a-5p and lncRNA OIP5-AS1 in them. Figure 1A showed that compared with cartilage tissues of healthy individuals, the tissues of patients with OA showed down-regulated lncRNA OIP5-AS1 and up-regulated micro-30a-5p, and Figure 1B showed compared with cartilage tissues of healthy individuals, the tissues of patients with OA showed intensified apoptosis. In view of the opposite expression trend of lncRNA OIP5-AS1 and micro-30a-5p in cartilage tissues of patients with OA, we selected human normal cartilage tissues as research objects, and constructed micro-30a-5p mimics, miR-30a inhibitor, and lncRNA OIP5-AS1 siRNA vectors to analyze the correlation between lncRNA OIP5-AS1 and micro-30a-5p with OA.

OIP5-AS1 Inhibits the Apoptosis and Inflammatory Response of Chondrocytes and Promotes Their Proliferation

To understand how OIP5-AS1 affected OA, we constructed OIP5-AS1 siRNA to regulate the level of OIP5-AS1 in cartilage tissues, and adopted the MTT assay to measure cell viability, the

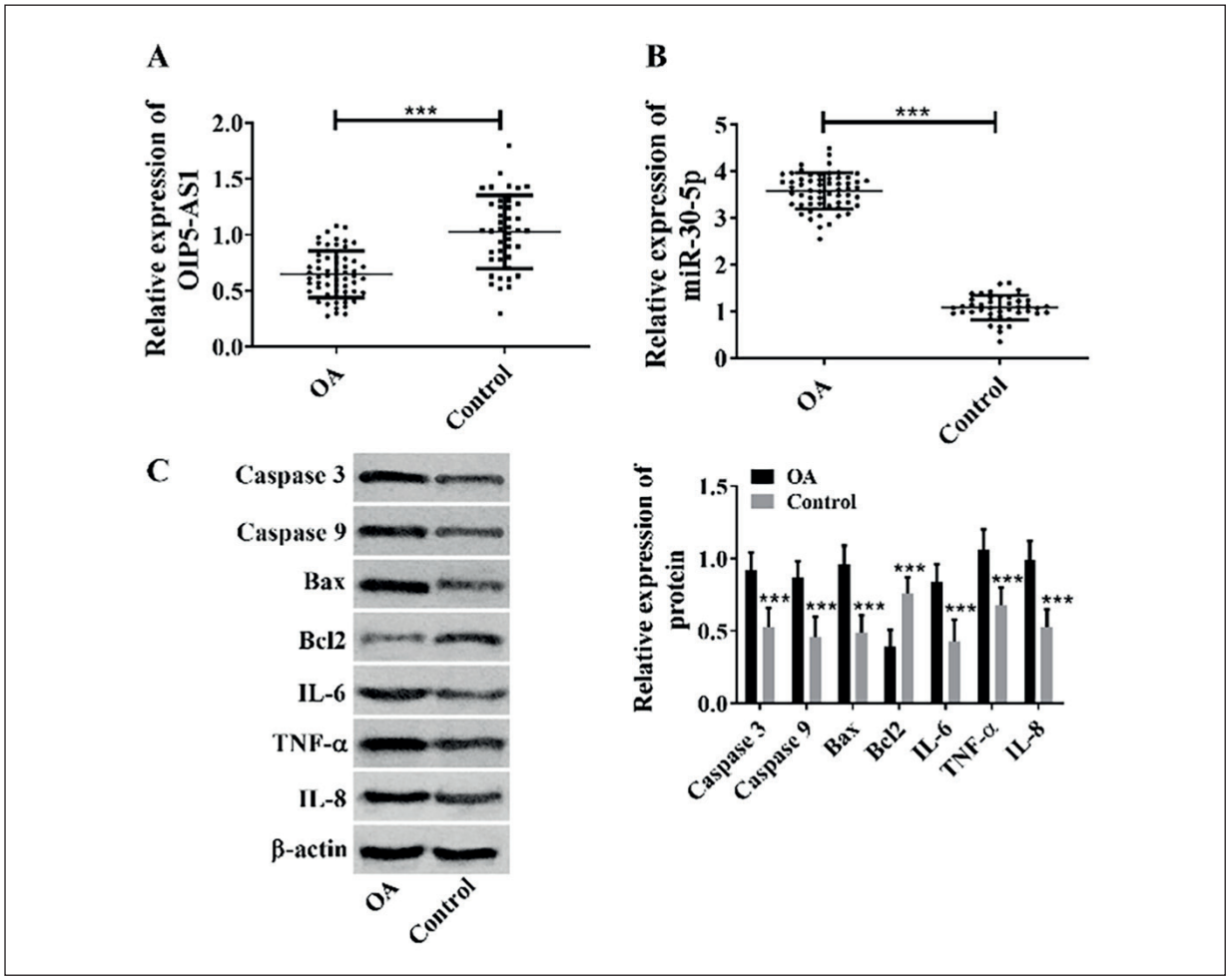

Figure 1. Abnormal expression of lncRNA OIP5-AS1 and micro-30a-5p in cartilage tissues of patients with OA. **A,** LncRNA OIP5-AS1 was reduced in cartilage tissues of patients with OA. *** indicates $p<0.001$. **B,** Micro-30a-5p was up-regulated in cartilage tissues of patients with OA. *** indicates $p<0.001$. **C,** Apoptosis in cartilage tissues of patients with OA increased. *** indicates that in comparison with the Control group, $p< 0.001$).

Western blot to determine Caspase 3, Caspase 9, Bax, Bcl2, IL-6, IL-8, and TNF-a, and qPCR to quantify micro-30a-5p. Figure 2A showed that OIP5-AS1 was downregulated in chondrocytes, while micro-30a-5p was up-regulated in them, and Figure 2B showed that down-regulation of OIP5-AS1 inhibited Bcl2 and up-regulated Caspase 3, Caspase 9, Bax, IL-6, IL-8, and TNF-a. Figure 2C showed that down-regulation of OIP5-AS1 led to a reduction in activity of chondrocytes. Considering the influence of OIP5-AS1 on apoptosis-related proteins including Caspase 3, we adopted flow cytometry to determine apoptosis and cycle of chondrocytes. Figure 2D showed that down-regulation of OIP5-AS1 promoted apoptosis of chondrocytes, causing a decrease in S-phase chondrocytes and an increase in G1-phase chondrocytes. The above results showed that OIP5-AS1 inhibited apoptosis and inflammatory response of chondrocytes and promoted proliferation of them.

Micro-30a-5p Is the Target Gene of OIP5-AS1

Because micro-30a-5p changed with changes in OIP5-AS1, we speculated that OIP5-AS1 may participate in the processions of OA by regulating micro-30a-5p, and we tried to verify this conjecture. Firstly, we predicted that there was a nucleic acid fragment binding to micro-30a-5p on the OIP5-AS1 sequence (Figure 3A). To verify the existence of the above loci, we carried out a dual luciferase reporter gene assay. Figure 3B showed that co-transfection of OIP5-AS1wt and micro-30a-5p mimics lowered the luciferase activity of chondrocytes, and Figure 3C showed that micro-

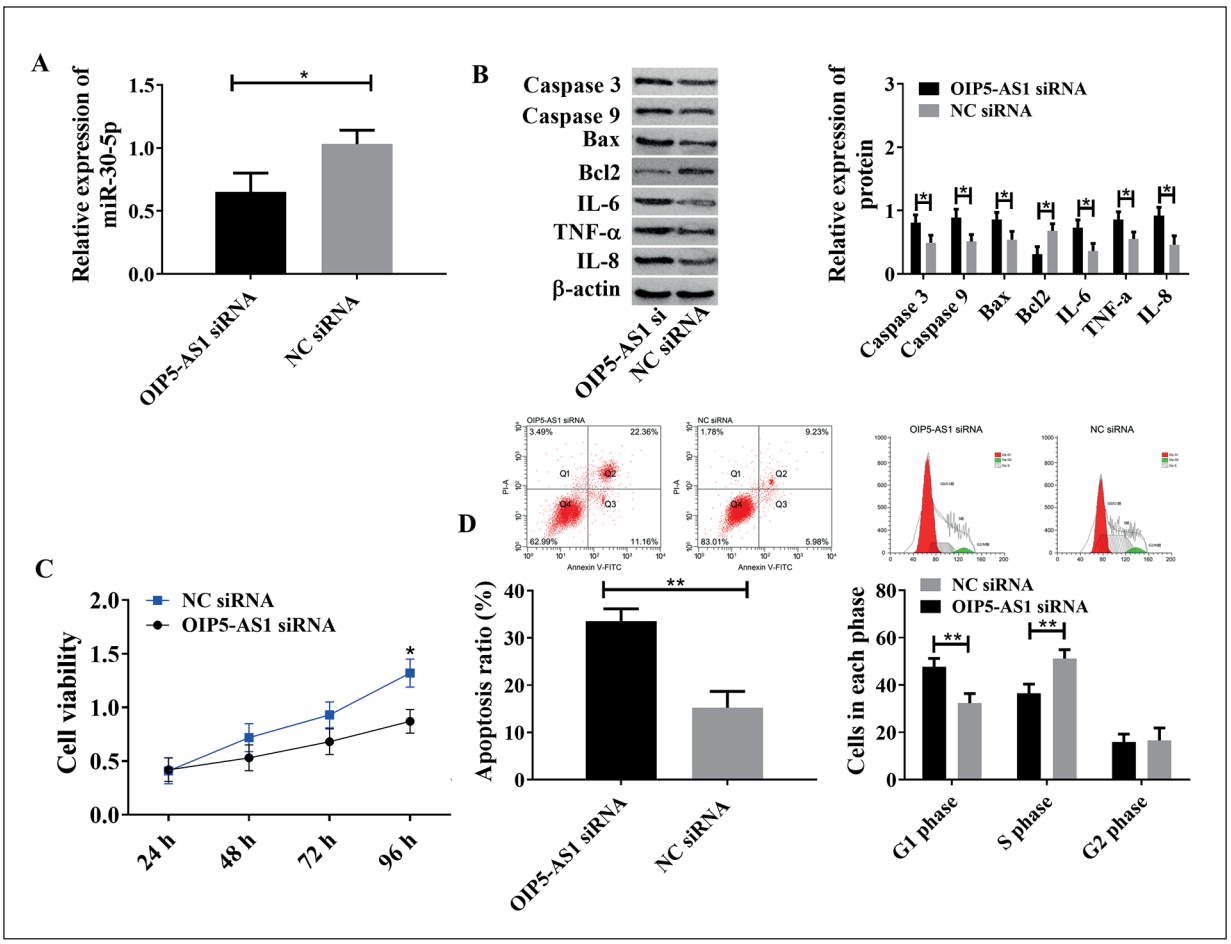

Figure 2. OIP5-AS1 inhibits inflammatory and apoptosis in chondrocytes. **A,** OIP5-AS1 was downregulated in chondrocytes and micro-30a-5p was up-regulated in them. * indicates $p<0.05$.**B,** Downregulating OIP5-AS1 promoted Caspase 3, Caspase 9, Bax, IL-6, IL-8, and TNF-a, and inhibited Bcl2. * indicates that in comparison with the NC siRNA group, $p<0.05$. **C,** Downregulation of OIP5-AS1 led to an activity decrease in chondrocytes. * indicates that in comparison with the NC siRNA group, $p<0.05$. **D,** Downregulation of OIP5-AS1 promoted apoptosis, causing a decrease in S-phase chondrocytes and an increase in G1-phase chondrocytes. ** indicates that in comparison with the NC siRNA group, $p<0.01$).

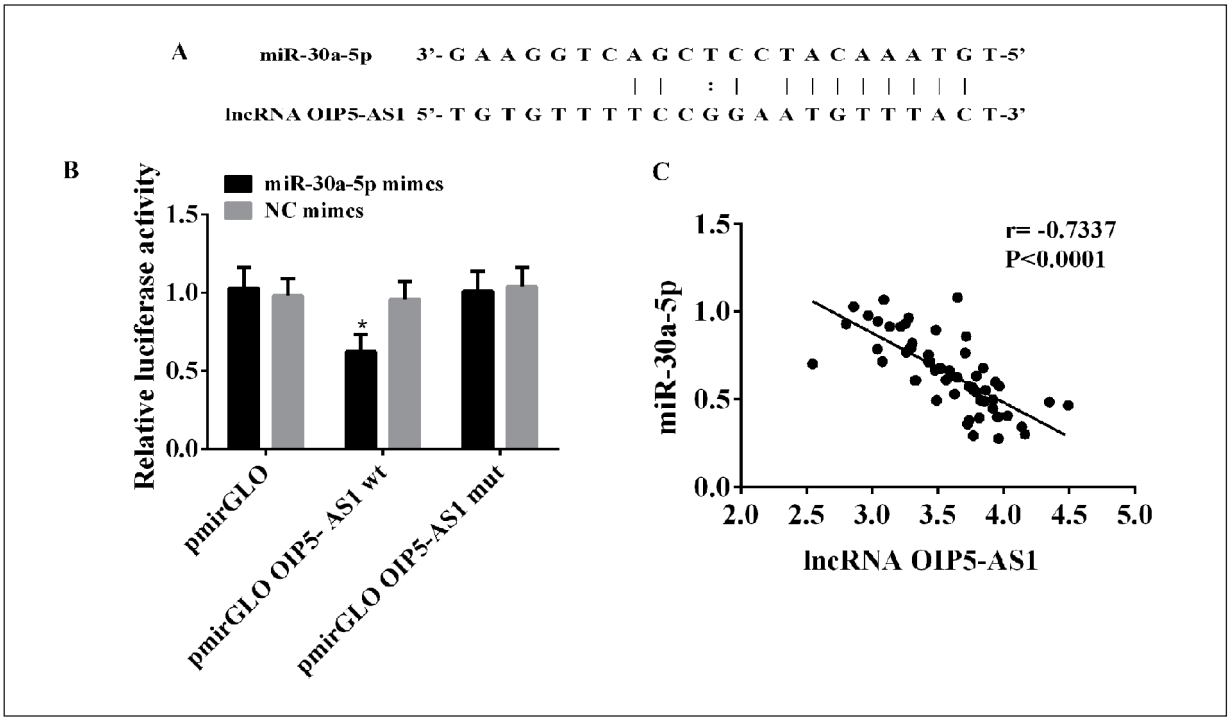

Figure 3. OIP5-AS1 negatively regulates micro-30a-5p through locus binding. **A,** It was predicted that there were binding loci between OIP5-AS1 and micro-30a-5p according to TargetScan database; **B,** Co-transfection of OIP5-AS1 wt and micro-30a-5p mimics lowered the Luciferase activity of chondrocytes. * indicates that in comparison with the NC mimics group, $p<0.05$. **C,** Micro-30a-5p was negatively correlated with OIP5-AS1.

30a-5p was negatively correlated with OIP5-AS1 in patients with OA. The above results uncovered that OIP5-AS1 negatively regulated micro-30a-5p in chondrocytes by binding to micro-30a-5p.

Micro-30a-5p Inhibits Proliferation of Chondrocytes and Promotes Apoptosis and Their Inflammatory Response

Since OIP5-AS1 may participate in the development of OA through targeted inhibition of micro-30a-5p, we constructed micro-30a-5p mimics and micro-30a-5p inhibitor vectors to study the function of micro-30a-5p. The results are shown in Figure 4. Figure 4A showed that up-regulation of micro-30a-5p up-regulated Caspase 3, Caspase 9, Bax, IL-6, IL-8, and TNF-a, and inhibited Bcl2, while down-regulation of it exerted opposite effects. Figure 4B showed that up-regulation of micro-30a-5p lowered viability of chondrocytes, while down-regulation of it enhanced the viability of them. Figure 4C showed that upregulation of micro-30a-5p suppressed the cycle of chondrocytes and promoted their apoptosis, while down-regulation of it exerted opposite effects. The above results uncovered that micro-30a-5p inhibited the proliferation of chondrocytes and promoted the apoptosis and their inflammatory response.

Rescue Experiment

We transfected OIP5-AS1 siRNA and OIP5-AS1 siRNA+micro-30a-5p inhibitor into chondrocytes, respectively, and analyzed corresponding changes in chondrocytes. Figure 5 showed that compared with the OIP5-AS1 siRNA group, the OIP5-AS1 siRNA+micro-30a-5p inhibitor group showed decreased micro-30a-5p expression, weakened apoptosis, and strengthened proliferation, and also showed down-regulated Caspase 3, Caspase 9, Bax, IL-6, IL-8, and TNF-a, and up-regulated Bcl2. The above results uncovered that inhibiting micro-30a-5p could offset the down-regulation of micro-30a-5p caused by OIP5-AS1 siRNA, and could promote cell proliferation and inhibit cell apoptosis.

Discussion

In this study, we found down-regulation of lncRNA OIP5-AS1 in cartilage tissues of pa-

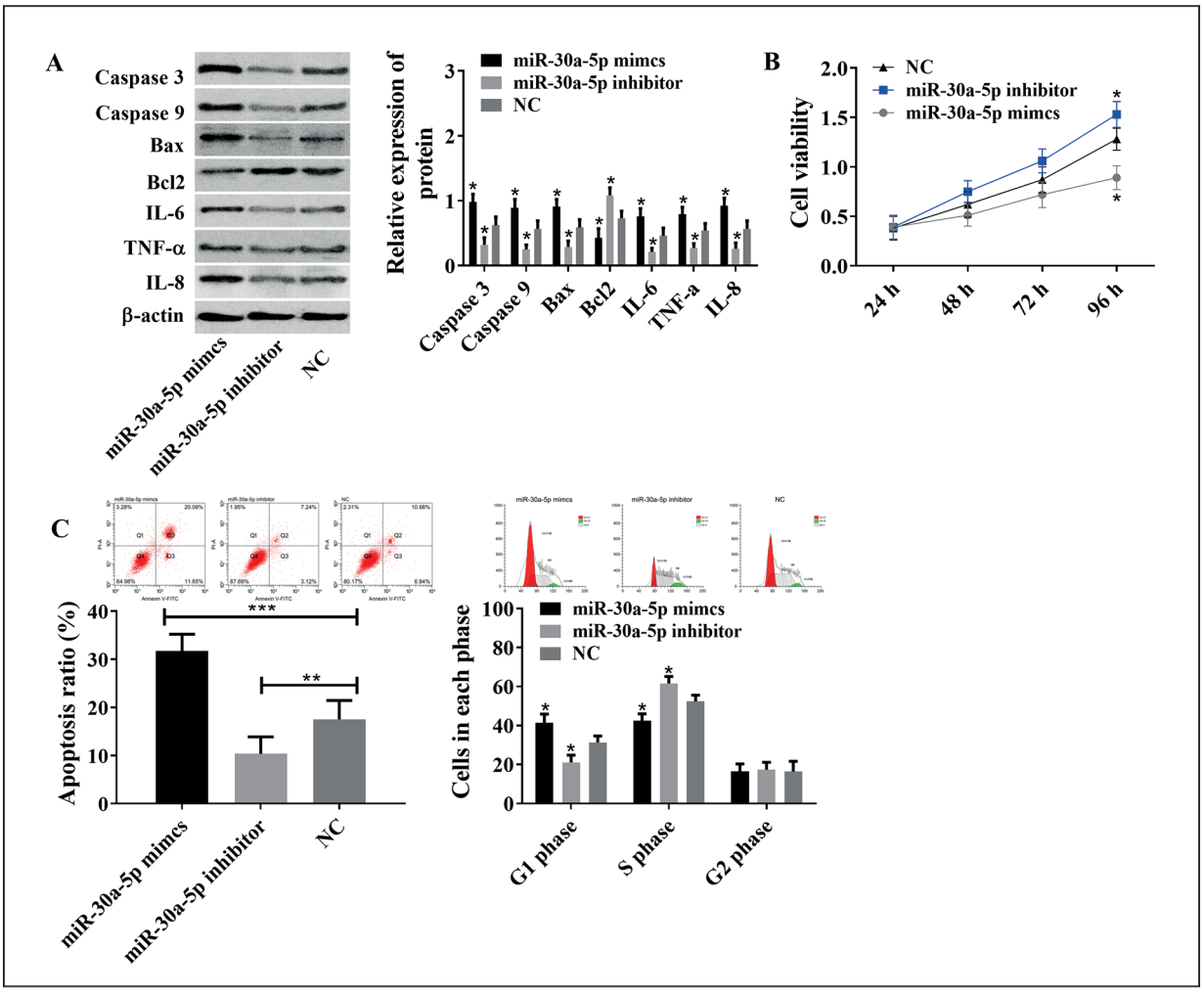

Figure 4. Micro-30a-5p inhibited proliferation of chondrocytes, and promoted apoptosis and inflammatory response. **A,** Upregulation of micro-30a-5p promoted the expression of Caspase 3, Caspase 9, Bax, IL-6, IL-8, and TNF-a, and inhibited the expression of Bcl2, while downregulation of it exerted opposite effects. * indicates that in comparison with the NC group, $p<0.05$. **B,** Upregulation of micro-30a-5p lowered the viability of chondrocytes, and down-regulation of it improved the viability of them. * indicates that in comparison with the NC group, $p<0.05$. **C,** Up-regulation of micro-30a-5p suppressed the cycle and apoptosis of chondrocytes, and downregulation of micro-30a-5p exerted opposite effects. * indicates that in comparison with the NC group, $p<0.05$; ** indicates that in comparison with the NC group, $p<0.01$, and *** indicates that in comparison with the NC group, $p<0.001$).

tients with OA, up-regulation of micro-30a-5p in them, and strong apoptosis of chondrocytes in the patients, so we suspected that the abnormal expression of lncRNA OIP5-AS1 and micro-30a-5p might be related to apoptosis of chondrocytes of patients with OA. We also found that down-regulation of lncRNA OIP5-AS1 caused up-regulation of micro-30a-5p. We predicted that there were binding loci between them based on the Starbase database. For the purpose of verifying it, we constructed OIP5-AS1 wt and OIP5-AS1 mut vectors and co-transfected them with micro-30a-5p mimics into chondrocytes, respectively. It came out that co-transfection of micro-30a-5p mimics and OIP5-AS1 wt decreased the luciferase activity of chondrocytes, which implied that miR-30a+-5p could bind to lncRNA OIP5-AS1. The above results suggested that lncRNA OIP5-AS1 may participate in the development of OA by inhibiting micro-30a-5p.

Afterwards, we regulated the expression of lncRNA OIP5-AS1 and micro-30a-5p in chondrocytes using lncRNA OIP5-AS1 siRNA, micro-30a-5p mimics, and micro-30a-5p inhibitor vectors to verify their effects on chondrocytes. It came out that down-regulation of lncRNA OIP5-

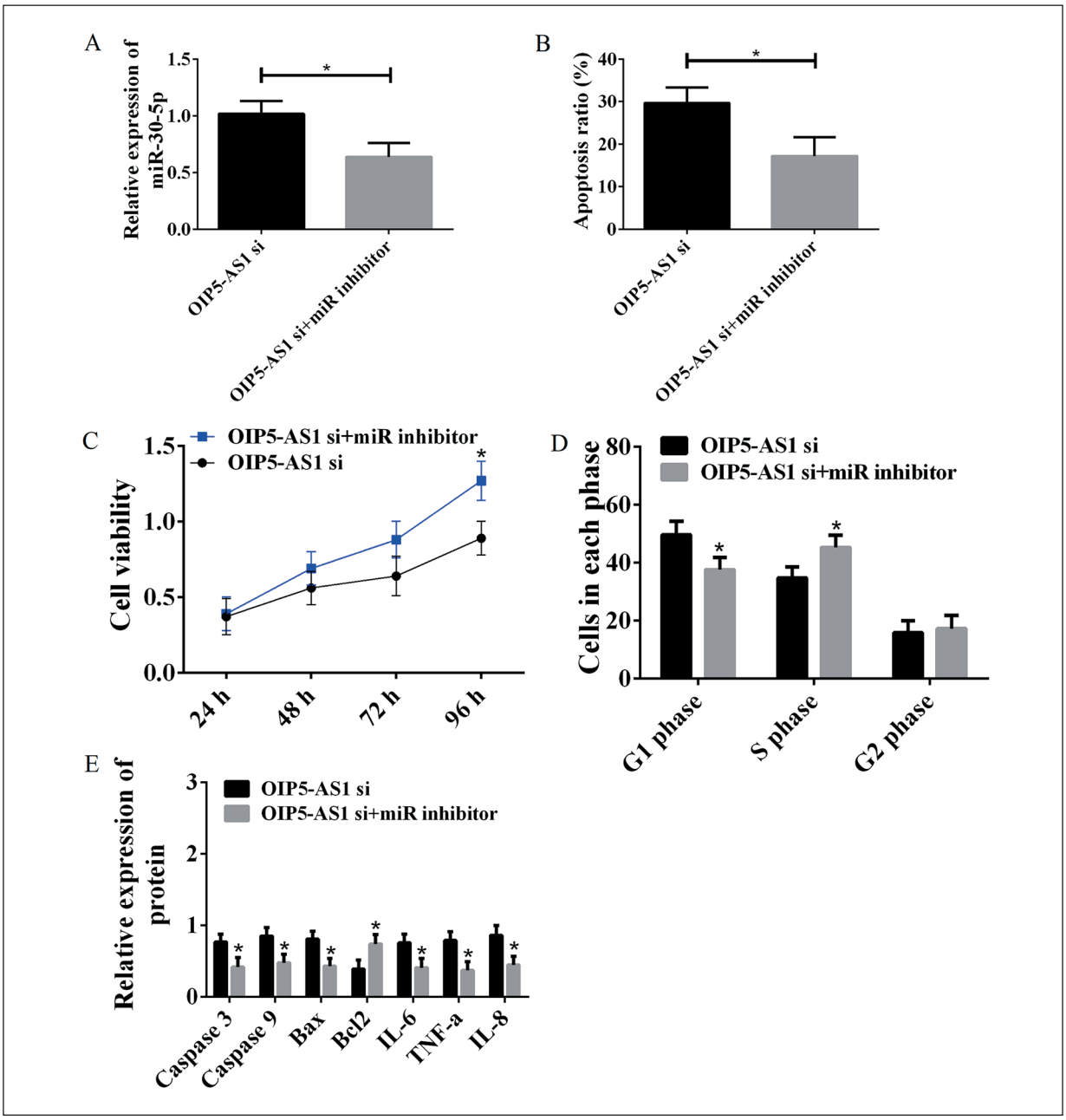

Figure 5. Rescue experiment. **A,** Compared with the OIP5-AS1 siRNA group, the OIP5-AS1 siRNA+micro-30a-5p inhibitor group showed down-regulated micro-30a-5p expression. * indicates $p<0.05$. **B,** Compared with the OIP5-AS1 siRNA group, the OIP5-AS1 siRNA+micro-30a-5p inhibitor group showed down-regulated Caspase 3, Caspase 9, Bax, IL-6, IL-8, and TNF-a, and up-regulated Bcl2. * indicates $p<0.05$. **C,** Compared with the OIP5-AS1 siRNA group, the OIP5-AS1 siRNA+micro-30a-5p inhibitor group showed intensified cell viability. * indicates $p<0.05$. **D,** Compared with the OIP5-AS1 siRNA group, the OIP5-AS1 siRNA+micro-30a-5p inhibitor group showed weakened cell apoptosis and intensified cell cycle. * indicates $p<0.05$).

AS1 or up-regulation of micro-30a-5p strengthened apoptosis and secretion of pro-inflammatory factor, while down-regulation of micro-30a-5p brought about opposite effects. The above results verified that lncRNA OIP5-AS1 inhibited apoptosis and inflammatory response of chondrocytes through micro-30a-5p.

Micro-30a-5p is an important cell regulator, which can regulate cell biological behaviors by directly or indirectly regulating protein expres-

sion. Apoptosis is an endogenous process controlled by proteins. Caspase 3 and Caspase 7 can promote the apoptosis process, and Bcl2 can improve the viability of cells and inhibits apoptosis of them. Therefore, regulating the expression of the three will directly cause the start or stop of apoptosis. Micro-30a-5p induces apoptosis by activating Caspase 3 and Caspase 7 and inhibiting Bcl2[22,23], and it also promotes cell apoptosis by targeting ZEB2[24]. In addition, it regulates the secretion of cytokines through the MAPK/ERK pathway and STAT1[25,26]. According to the results of our study, lncRNA OIP5-AS1 gave rise to programmed changes in downstream pathways or target genes by inhibiting micro-30a-5p, which led to the reduction of pro-apoptotic proteins (Caspase 3, Caspase 9, and Bax) and pro-inflammatory factors (IL-6, IL-8, and TNF-a) and finally inhibited apoptosis of chondrocytes and their inflammatory response.

In our study, we explored how lncRNA OIP5-AS1 regulated the proliferation and apoptosis of chondrocytes through micro-30a-5p, finding that down-regulation of lncRNA OIP5-AS1 led to excessive up-regulation of micro-30a-5p in chondrocytes, and up-regulation of micro-30a-5p accelerated apoptosis and inflammatory response of the cells. Micro-30a-5p, as an intermediate transmitter, not only mediates the regulation of lncRNA OIP5-AS1 on chondrocytes, but also participates in the regulation of downstream target genes. Therefore, in the future, we will continue to explore the regulation of lncRNA OIP5-AS1/micro-30a-5p axis on downstream target genes and effects of the regulation on programmed behaviors of chondrocytes.

Conclusions

LncRNA OIP5-AS1 inhibits the apoptosis and inflammatory response of chondrocytes and promotes survival of them by targeting micro-30a-5p, and both up-regulation of lncRNA OIP5-AS1 and downregulation of micro-30a-5p are helpful to alleviate inflammatory response and apoptosis of chondrocytes in patients with OA. Therefore, lncRNA OIP5-AS1/micro-30a-5p axis provides a novel idea for the treatment of OA.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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