

Mechanistic insights into lncRNA SNHG6 during the progression of osteoarthritis

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Abstract. The present study investigated the role and preliminary regulatory mechanism of the long non-coding RNA small nucleolar RNA host gene 6 (SNHG6) in lipopolysaccharide (LPS)-induced chondrocytes. LPS-induced chondrocytes were used to mimic osteoarthritis (OA) *in vitro*. Gain- and loss-of-function experiments were performed as follows: the Cell Counting Kit-8 assay for cell viability, flow cytometry for cell cycle and apoptosis analysis, the TUNEL assay for further apoptosis detection, western blotting for apoptosis- and cell cycle-related protein levels, and ELISA for inflammatory cytokine levels. Gene expression levels were detected by reverse transcription-quantitative PCR and immunofluorescence. In LPS-induced chondrocytes and in patients with OA, reduced expression of SNHG6 and PGAP1 as well as increase expression of microRNA (miR)-944 were observed. SNHG6 targets miR-944, and PGAP1 is a target of miR-944. Overexpression of SNHG6 or PGAP1, as well as downregulation of miR-944 markedly suppressed chondrocyte apoptosis and inflammation. Furthermore, increased miR-944 expression reversed the regulatory effects of PGAP1 overexpression in LPS-induced chondrocytes. In conclusion, the role and underlying mechanism of SNHG6 in the progression of OA *in vitro* were preliminarily identified, and it was found that its overexpression remarkably inhibits chondrocyte apoptosis and inflammation. This regulatory effect may be mediated by regulating the miR-944/PGAP1 axis.

Introduction

Osteoarthritis (OA) is a chronic and disabling condition that affects the joints (1). Individuals with this condition often experience joint discomfort, swelling and restricted movement, which

negatively affect their physical capabilities and overall quality of life (2). Apoptosis of chondrocytes has been identified as a key pathological feature of OA, and mild synovial inflammation is also recognized as a contributing factor (3,4). Currently, there is no definitive cure for OA. In clinical settings, management typically involves non-pharmacological approaches such as assistive tools, aerobic activities, therapeutic exercises, lifestyle adjustments, and pharmacological interventions such as analgesics and oral or topical non-steroidal anti-inflammatory drugs (5,6). Nevertheless, conclusive evidence supporting the long-term effectiveness of these treatments for OA remains elusive. As a result, there is an ongoing need for innovative and more effective treatment options.

In total, ~2% of the human genome consists of protein-coding RNAs, whereas the remainder is composed of non-coding (nc) RNAs (7). Among these, long nc RNAs (lncRNAs) are defined as transcripts exceeding 200 nucleotides in length. Despite their lack of translation into functional polypeptides, lncRNAs play an essential role in modulating the expression of protein-coding genes (8). As novel epigenetic regulators, they influence the progression of various human diseases, including OA. Shi *et al* (9) identified a novel lncRNA, POU3F3, which modulates the microRNA (miRNA or miR)-29a-3p/FOXO3 axis to inhibit OA progression. Chen *et al* (10) reported significant upregulation of the lncRNA HOTAIR in chondrocytes affected by OA, which notably enhances inflammatory responses through the PTEN/PI3K/AKT pathway. Ni *et al* (11) demonstrated that the lncRNA DLEU1 contributes to the progression of OA by markedly promoting chondrocyte apoptosis. The lncRNA small nucleolar RNA host gene 6 (SNHG6), also known as U87HG, is located on human chromosome 8q13.1 (12). Currently, nearly all reports on SNHG6 are associated with cancer. It is overexpressed in various cancers, including colorectal cancer (13), esophageal squamous carcinoma (14) and ovarian cancer (15). Studies have demonstrated its role in enhancing cell proliferation, metastasis and epithelial-mesenchymal-transition in numerous cancer cell types (13-15). However, its role and the underlying mechanisms involved in OA progression are poorly understood.

The present study investigated the effects of SNHG6 on apoptosis and inflammation as well as its possible a mechanism of action in lipopolysaccharide (LPS)-induced chondrocytes. The miR-944/post-GPI attachment to protein 1 (PGAP1) axis may be a potential target of SNHG6 in the progression of OA.

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Materials and methods

Patients and sample collection. From January 2024 to December 2025, 30 patients (17 females and 13 males; age range, 55-75 years; mean age, 64.7 ± 6.6) with newly diagnosed with OA were recruited at our hospital. OA diagnosis was confirmed by analysis of both X-ray imaging and joint fluid. The inclusion criteria of these patients were as follows: i) Full comprehension of the study protocol and agreement to provide synovial fluid; ii) no prior medical intervention. Patients with other chronic inflammatory conditions who started treatment within three months before admission were excluded from the study. Meanwhile, 30 healthy controls (17 females and 13 males; age range, 52-73 years; mean age, 62.2 ± 6.0) underwent systemic physiological examination with no OA or rheumatoid arthritis were enrolled. Synovial fluid samples (1 ml) were collected using a syringe under local anesthesia. The ethics committee of Wenzhou People's Hospital approved the present study protocol (approval no. KY-2023-251; Wenzhou, China). Both healthy individuals and patients with OA signed written informed consent. The research complied with the principles established in the Declaration of Helsinki.

Cell culture, LPS treatment and transfection. Primary human normal chondrocytes (cat. no. CP-H107; <https://www.procell.com.cn/p/human-chondrocyte-cells-cp-h107-68054>) were purchased from Wuhan Pricella Biotechnology Co., Ltd. at passage 0 (P0). The use of primary human cells has obtained the approval from the Ethical Committee of Wenzhou People's Hospital (approval number: KY-2023-289; Wenzhou, China). They were cultured in DMEM/F12 medium (Hyclone; Cytiva) supplemented with 1% Penicillin-Streptomycin Solution (Beyotime Institute of Biotechnology) and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). The cells were incubated at 37°C under a humidified atmosphere containing 5% CO₂. For experimental purposes, second-passage (P2) chondrocytes with 85% confluency were selected and utilized. LPS was utilized to establish the *in vitro* OA model, since it can efficiently trigger chondrocyte inflammatory response, upregulate pro-inflammatory cytokines and matrix-degrading enzymes, and suppress the expression of cartilage-specific anabolic proteins, which closely recapitulates the pathological alterations of degenerated chondrocytes in OA. This model has been widely adopted in numerous OA-related *in vitro* mechanistic studies due to its high experimental stability, favorable repeatability, and simple operation, which is particularly suitable for the initial verification of the functional role of specific molecular axes in chondrocyte apoptosis and inflammation. To construct an *in vitro* OA cell model, normal chondrocytes were treated with LPS (5 µg/ml; Sigma Aldrich) for 12 h (16-18).

From Genomeditech (Shanghai) Co., Ltd., mimics NC (sense: 5'-AUGUAGUAUAGCAGAUGAUUAUC-3'; anti-sense: 5'-GAUAUCAUCUGCUAUACUACAU-3')/miR-944 mimics (sense: 5'-AAAUUAUUGUACAUCGGAUGAG-3'; anti-sense: 5'-CUCAUCCGAUGUACAUAUAUUU-3') and inhibitor NC (5'-GAUAUCAUCUGCUAUACUACAU-3')/miR-944 inhibitor (5'-CUCAUCCGAUGUACAUAUAUUU-3'), as well as small interfering RNA targeting SNHG6 (si-SNHG6; sense: 5'-GAUGUUGAUACAUCACAA-3'; anti-sense: 5'-UUGUGAUGUUAUCAACAUC-3')/si-PGAP1 (sense: 5'-GGU

ACGAGGUGACAGUUA-3'; anti-sense: 5'-UUAACUGUACCUCGUACC-3')/si-NC (sense: 5'-GAGACAGUUGUACGAGUAG-3'; anti-sense: 5'-CUACUCGUACAACUGUCUC-3') and overexpression of PGAP1 (OE-PGAP1), OE-SNHG6 and OE-NC were synthesized. Cell transfection was performed using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). The aforementioned oligonucleotides and plasmids (50 nM) were separately co-transfected into LPS-induced chondrocytes (1×10^5 cells/well) for 48 h. After transfection, chondrocytes were harvested, and the levels of gene expression were detected via reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

RT-qPCR. Synovial fluid samples and chondrocytes were obtained for RNA extraction using a Total RNA Extraction Kit (Foregene Co., Ltd.). Subsequently, cDNA was synthesized using the SeqHunt® First Strand cDNA Synthesis Kit (Seq-Hunt Biotechnology) according to the manufacturer's instructions. PCR analysis was performed on a Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with 2X Blue Universal SYBR qPCR Master Mix (Seq-Hunt Biotechnology) according to the manufacturer's protocol. The thermocycling conditions were as follows: 95°C for 5 min, 40 cycles of denaturation at 95°C for 10 sec, annealing at 50°C for 1 min and extension at 72°C for 30 sec. The relative expression levels were quantified by the $2^{-\Delta\Delta C_q}$ method (19), with GAPDH and U6 serving as the internal controls for data normalization. All gene-specific primers utilized in the present study are listed in Table I.

Cell viability and cell cycle measurement. Chondrocytes were seeded in 96-well plates at a concentration of 4×10^3 cells per well and cultured continuously for 48 h. After incubation, each well was supplemented with 10 µl Cell Counting Kit-8 reagent (Dojindo Molecular Technologies, Inc.) and incubated for another 2 h at 37°C. Finally, the absorbance values were then determined at a wavelength of 450 nm using a microplate reader (Hiwell Diatek).

To analyze the cell cycle, chondrocytes (1×10^5) were fixed with 75% ethanol at 4°C for 1 h. After fixation, the cells were centrifuged at 1,500 g for 5 min at 4°C, then re-suspended in phosphate-buffered saline. Subsequently, the cells were incubated with Propidium iodide/RNase A solution (Beyotime Institute of Biotechnology) in the dark for 30 min. Data were acquired on a flow cytometer (BD Biosciences). The distribution of cells in the G1, S and G2 phases was quantified by ModFit LT software (version 5.0; Verity Software House) using the Watson pragmatic model.

Cell apoptosis measurement. Based on the manufacturer's protocol of Apoptosis Detection Kit (Thermo Fisher Scientific, Inc.), 2×10^5 suspended chondrocytes were stained with Annexin V-FITC (10 µl) and PI (5 µl) at 25°C for 15 min in the dark, followed by adding 500 µl of binding buffer. Subsequently, the apoptotic chondrocytes were assessed using a flow cytometer (BD Biosciences).

For further auxiliary verification and quantitative detection of chondrocyte apoptotic levels, the One Step TUNEL Apoptosis Assay Kit from Beyotime Institute of Biotechnology was adopted for complementary testing. First, the cells were

Table I. Primer sequences.

Gene name	Primer sequence
lncRNASNHG6	Forward: 5'-ATACTTCTGCTTCGTTACCT-3' Reverse: 5'-CTCATTTTCATCATTTGCT-3'
microRNA-944	Forward: 5'-GCGGCGGAAATTATTGTACATC-3' Reverse: 5'-ATCCAGTGCAGGGTCCGAGG-3'
PGAP1	Forward: 5'-ACAGGCTCCATCTTCCACAG-3' Reverse: 5'-ACGACATAAGCAGGAAGAGC-3'
GAPDH	Forward: 5'-TGCACCACCAACTGCTTAGC-3' Reverse: 5'-GGCATGCACTGTGGTCATGAG-3'
U6	Forward: 5'-CTCGCTTCGGCAGCACA-3' Reverse: 5'-AACGCTTCACGAATTTGCGT-3'

lncRNASNHG6, long non-coding RNA small nucleolar RNA host gene 6; PGAP1, post-GPI attachment to protein 1.

stabilized in 4% paraformaldehyde for 15 min and then treated with 0.25% Triton-X 100 for 20 min to enhance permeability. Afterwards, 50 μ l of TUNEL reaction mixture was applied, and the samples were incubated at 37°C for 60 min. Following phosphate-buffered saline washes, cell nuclei were stained with DAPI (5 μ g/ml) for 5 min in the dark. Finally, the samples were analyzed using a fluorescence microscope (Olympus Corporation).

Western blot assay. To obtain chondrocyte protein extracts, RIPA lysis buffer (Biosharp Life Sciences) was utilized. Subsequently, the protein samples (30 μ g per lane) were subjected to separation via 10% SDS-PAGE, followed by electrotransfer onto a PVDF membrane. The membrane was blocked with 5% non-fat milk (Biosharp Life Sciences) for 2 h at room temperature. Thereafter, the membrane was incubated overnight at 4°C with primary antibodies directed against Caspase-3 (cat. no. ab32351; 1:5,000; Abcam), Caspase-9 (cat. no. ab32539; 1:5,000; Abcam), Cyclin A1 (cat. no. 13295-1-AP; 1:1,000; Proteintech Group, Inc.), Cyclin B1 (cat. no. 55004-1-AP; 1:2,000; Proteintech Group, Inc.), Cyclin D2 (cat. no. 10934-1-AP; 1:1,000; Proteintech Group, Inc.) and GAPDH (cat. no. 60004-1-Ig; 1:50,000; Proteintech Group, Inc.). Afterward, the membrane was further incubated with HRP-conjugated secondary antibodies (cat. no. A21010 & A21020; 1:10,000; Abbkine Scientific Co., Ltd.) for 2 h at 25°C. Protein signals were visualized using the SuperKine™ ECL Kit (Abbkine Scientific Co., Ltd.) and quantified via ImageJ software version 1.53 (National Institutes of Health).

Immunofluorescent test. Following routine dewaxing in xylene and gradient rehydration in ethanol, antigen retrieval was performed on chondrocyte specimens under standard experimental conditions. This was followed by a blocking step using goat serum (Beijing Solarbio Science & Technology Co., Ltd.). The samples were then incubated overnight at 4°C with a primary antibody specific to PGAP1 (cat. no. 55392-1-AP; 1:50; Proteintech Group, Inc.). Next, the sections were conjugated with an Alexa Fluor 488 secondary antibody diluted

at 1:200 (Invitrogen; Thermo Fisher Scientific, Inc.). Following DAPI staining (1 μ g/ml), fluorescence microscopy images of the sections were acquired using an Olympus fluorescence microscope.

ELISA. Strictly in accordance with the guidelines of commercial ELISA kits, the concentrations of IL-1 β (cat. no. KTE6013), IL-6 (cat. no. KTE6017) and TNF- α (cat. no. KTE6032) (all from Abbkine Scientific Co., Ltd.) were quantitatively detected and assessed in chondrocyte samples.

Dual luciferase reporter assay. The potential targeting relationships between SNHG6 and miR-944, as well as between miR-944 and PGAP1, were predicted using Starbase software (version 2.0; <http://starbase.sysu.edu.cn>). The partial sequences of the 3'-untranslated region (UTR) of PGAP1 and SNHG6 containing the predicted binding sites of miR-944 were synthesized by Sangon Biotech Co., Ltd. These sequences were then inserted into the pGL3 vector to construct wild-type (Wt)-PGAP1 and Wt-SNHG6 reporter vectors. Following the manufacturer's protocol, mutant type (Mut) PGAP1 and SNHG6-3'-UTR sequences, which contain the miR-944 binding sites, were generated using the Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific, Inc.). Similarly, the mutated sequences were inserted into the pGL3 vector to create Mut reporter vectors for both PGAP1 and SNHG6. Following this, chondrocytes (5x10³ cells/well) were co-transfected with mimics NC or miR-944 mimics (10 nM) as well as the constructed wild-type or mutant reporter plasmids, with the aid of Lipofectamine 3000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). At 48 h after transfection, the relative luciferase activity was measured using the Dual-Luciferase Reporter Assay System Kit (Promega Corporation). The activity of firefly luciferase was normalized to that of *Renilla* luciferase.

Statistical analysis. SPSS 20.0 software (IBM Corp.) was employed for data analysis, and all experimental data were expressed as the mean $\pm$ standard deviation. Statistical differences between multiple groups were analyzed by one-way

analysis of variance followed by Tukey's post hoc test, whereas Student's t-test (unpaired) was used for comparisons between two independent groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of SNHG6 expression on LPS-stimulated chondrocytes. It was demonstrated that there was a notable reduction in the expression level of SNHG6 in synovial fluid of patients with OA, compared with the normal group ($P < 0.001$; Fig. 1A). SNHG6 was subsequently silenced or overexpressed in LPS-induced chondrocytes. As shown in Fig. 1B, SNHG6 expression levels were significantly reduced when transfected with si-SNHG6 ($P < 0.0001$), while was concurrently elevated upon OE-SNHG6 transfection ($P < 0.0001$). Compared with the control group, LPS treatment led to a significant decrease in cell viability (Fig. 1C, $P < 0.0001$). Meanwhile, chondrocyte viability was further inhibited following transfection of si-SNHG6 (Fig. 1C, $P < 0.0001$) but was partially restored when OE-SNHG6 was transfected ($P < 0.01$). This finding further evidenced by the results of cell frequency illustrated in Fig. 1D and E. It was observed that by contrast to the control group, G1 phase cell frequency in the LPS group were raised. Silencing of SNHG6 further extended G1 phase, whereas the overexpressed SNHG6 reduced G1 phase cell frequency. As illustrated in Fig. 1F and G, the presence of LPS significantly increased the percentage of TUNEL-positive cells ($P < 0.0001$). When transfected with si-SNHG6 in LPS-stimulated chondrocytes, the percentage of TUNEL-positive cells was further increased ($P < 0.01$); however, the positive cells were reduced when SNHG6 was overexpressed ($P < 0.01$). The results of flow cytometric analysis further validated the variations in apoptotic rates across the different groups (Fig. 1H and I; $P < 0.0001$). Subsequently, the levels of proteins associated with apoptosis (Caspase-3 and Caspase-9) and cell cycle (Cyclin A1, Cyclin B1 and Cyclin D2) were determined. Silencing of SNHG6 significantly elevated the levels of Caspase-3 and Caspase-9, and inhibited Cyclin A1, Cyclin B1 and Cyclin D2 levels (Fig. 1J; $P < 0.05$), while high levels of SNHG6 exhibited the opposite regulatory effects on these proteins ($P < 0.05$). These experimental data suggested that silencing of SNHG6 exhibited a notably inhibiting effect on chondrocyte proliferation, and a significantly promoting impact on apoptosis. Overexpression of SNHG6 exerted the opposite role in LPS-stimulated chondrocytes. Finally, the effects of SNHG6 on inflammation responses were assessed. IL-6, TNF- α and IL-1 β secretion in LPS-stimulated chondrocytes were significantly induced by the silencing of SNHG6 (Fig. 1K; $P < 0.01$) but suppressed by the overexpression of SNHG6 ($P < 0.05$).

SNHG6 binds to and negatively regulates miR-944. Starbase software was employed to predict the downstream targets of SNHG6 and identified potential binding sites between SNHG6 and miR-944 (Fig. 2A). Based on dual luciferase reporter assays, no statistical changes were observed in relative luciferase activity between the groups of SNHG6-Mut + mimics NC and SNHG6-Mut + miR-944 mimics. By contrast,

a notable reduction in luciferase activity was observed in SNHG6-Wt + miR-944 mimics group when compared with the SNHG6-Wt + mimics NC group (Fig. 2B; $P < 0.05$). Notably, in synovial fluid of patients with OA, a relatively high expression of miR-944 was identified (Fig. 2C; $P < 0.001$). Furthermore, the silencing of SNHG6 resulted in a significant increase in miR-944 expression (Fig. 2D; $P < 0.0001$), while SNHG6 overexpression was found to suppress the expression of miR-944 ($P < 0.0001$). To further verify the regulatory relationship between SNHG6 and miR-944, OE-SNHG6 or miR-944 mimics was transfected into LPS-induced chondrocytes. As illustrated in Fig. 2E and F, upregulation of miR-944 reversed the inhibitory effect of SNHG6 overexpression on miR-944 expression ($P < 0.05$), while partially attenuating its promoting effect on SNHG6 expression ($P < 0.01$). Subsequently, functional experiments demonstrated that upregulation of miR-944 reversed the regulatory effects of SNHG6 overexpression on cell proliferation, including cell viability (Fig. 2G; $P < 0.001$) and apoptosis (Fig. 2H and I; $P < 0.001$). Additionally, miR-944 upregulation was found to significantly abolish the suppressive effects of SNHG6 overexpression on the levels of IL-6, TNF- α and IL-1 β (Fig. 2J-L; $P < 0.05$).

Effects of miR-944 inhibition on LPS-stimulated chondrocytes. Given the elevated levels of miR-944 in OA, LPS-stimulated chondrocytes were transfected with a miR-944 inhibitor for functional validation (Fig. 3A; $P < 0.0001$). As illustrated in Fig. 3B and C, transfection of miR-944 inhibitor remarkably stimulated chondrocyte viability ($P < 0.01$). Both TUNEL assay and flow cytometric analysis evidenced that the transfection of miR-944 inhibitor led to a significant suppression in chondrocyte apoptosis (Fig. 3D and E; $P < 0.01$). As western blotting indicated, the levels of Caspase-3 and Caspase-9 were suppressed (Fig. 3F; $P < 0.01$), while Cyclin A1, Cyclin B1 and Cyclin D2 protein levels were elevated when miR-944 inhibitor was transfected ($P < 0.05$). Furthermore, it was observed that the suppression of miR-944 significantly reduced the levels of IL-6, TNF- α and IL-1 β in LPS-stimulated chondrocytes (Fig. 3G; $P < 0.05$).

PGAP1 is a direct downstream target of miR-944. As the predicted results of Starbase software, a potential binding site was identified between miR-944 and PGAP1 (Fig. 4A). Further experiments indicated that by contrast to the PGAP1-Wt + mimics NC group, the luciferase activity in PGAP1-Wt + miR-944 mimics group was significantly reduced (Fig. 4B; $P < 0.001$), which validated the predicted results. Compared with the normal patients, a relatively low level of PGAP1 was found in synovial fluid of patients with OA (Fig. 4C; $P < 0.001$). miR-944 inhibitor or mimics (50, 100 and 200 nM) were then transfected into chondrocytes. It was revealed that miR-944 expression was significantly downregulated upon transfection with its inhibitor (Fig. 4D; $P < 0.001$), while it was significantly upregulated after transfection with miR-944 mimics (Fig. 4D; $P < 0.01$). This suggested that miR-944 inhibitor or mimics was successfully transfected into chondrocytes. Subsequently, it was observed that transfection of miR-944 inhibitor significantly promoted PGAP1 expression (Fig. 4E; $P < 0.001$), while miR-944 mimics significantly inhibited its expression (Fig. 4E,

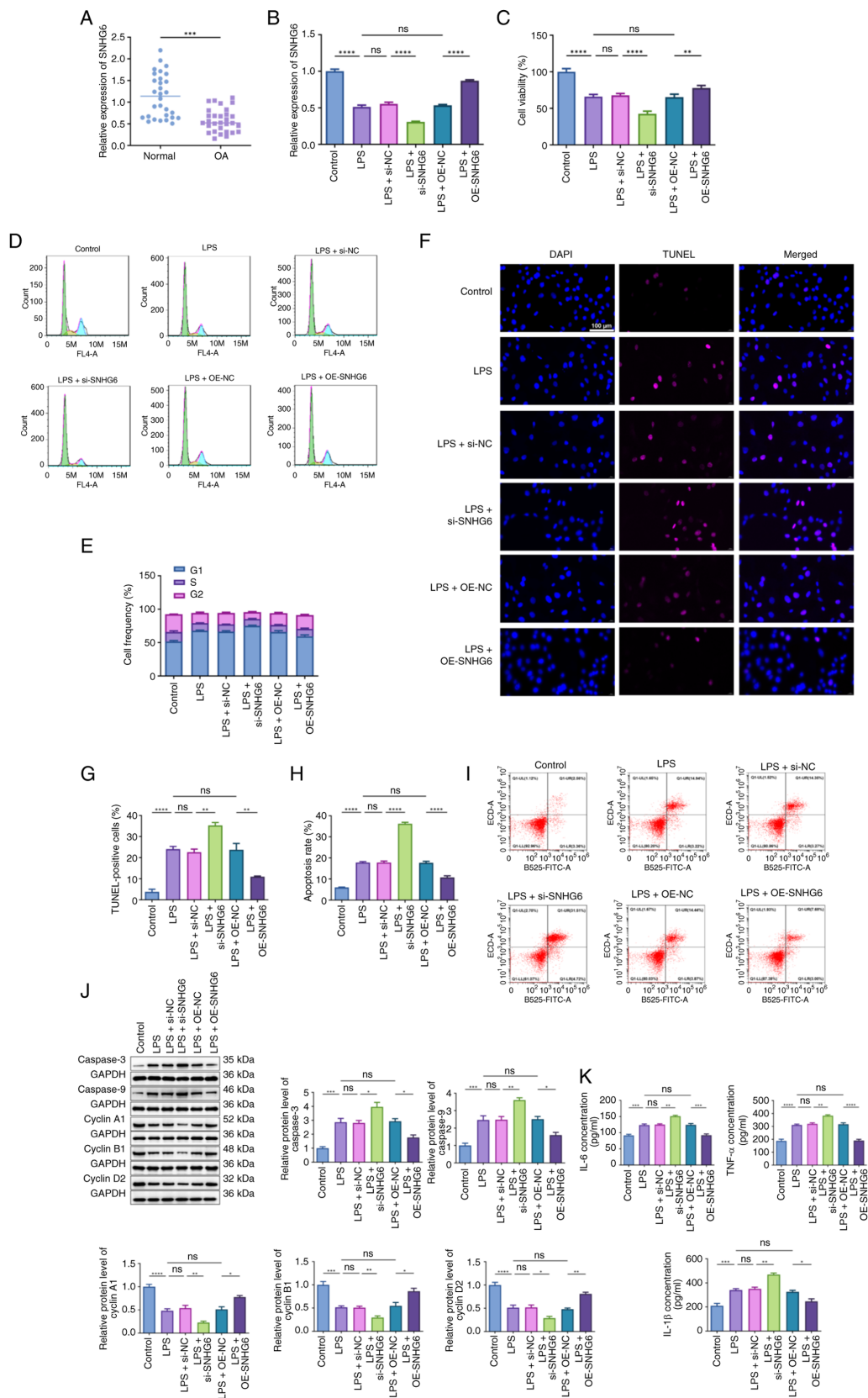


Figure 1. Effects of SNHG6 expression on LPS-stimulated chondrocytes. (A) The expression of SNHG6 in synovial fluid of normal individuals and patients with osteoarthritis was detected via RT-qPCR. (B) Following transfection of si-SNHG6 or OE-SNHG6 in LPS-induced chondrocytes, the expression of SNHG6 was detected via RT-qPCR. (C) The viability of LPS-induced chondrocytes that transfected with si-SNHG6 or OE-SNHG6 was measured by Cell Counting Kit-8 assay. (D and E) Cell frequency of LPS-induced chondrocytes was calculated via flow cytometric analysis. (F and G) The percentages of TUNEL-positive cells were assessed by TUNEL assay. Scale bar, 100 μ m. (H and I) The apoptotic rate of LPS-induced chondrocytes was calculated via flow cytometric analysis. (J) The protein levels of Caspase-3, Caspase-9, Cyclin A1, Cyclin B1 and Cyclin D2 in LPS-induced chondrocytes that transfected with si-SNHG6 or OE-SNHG6 were determined via western blotting. (K) The levels of IL-6, TNF- α and IL-1 β in LPS-induced chondrocytes that transfected with si-SNHG6 or OE-SNHG6 were determined via ELISA. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. SNHG6, small nucleolar RNA host gene 6; LPS, lipopolysaccharide; RT-qPCR, reverse transcription-quantitative PCR; si-, small interfering; OE-, overexpression; ns, no significance; NC, negative control.

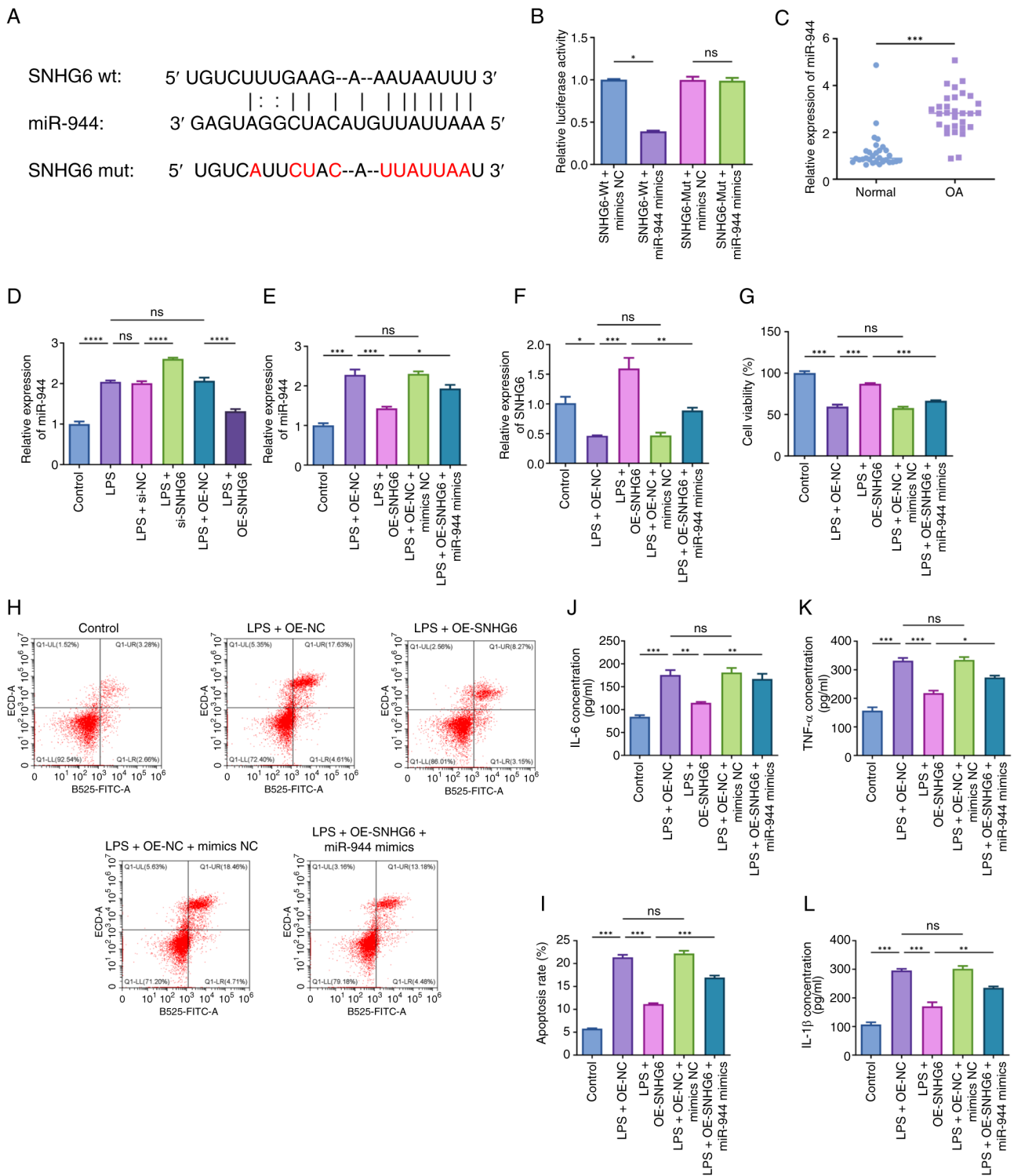


Figure 2. SNHG6 binds to and negatively regulates miR-944. (A) StarBase showed the predicted binding site of SNHG6 and miR-944. (B) The luciferase activities in chondrocytes co-transfected with pGL3-SNHG6-Wt/pGL3-SNHG6-Mut and miR-944 mimics/mimics NC were determined by dual luciferase reporter assay. (C) The expression of miR-944 in synovial fluid of normal individuals and patients with osteoarthritis was detected via RT-qPCR. (D) Following transfection of si-SNHG6 or OE-SNHG6 in LPS-induced chondrocytes, the expression of miR-944 was detected via RT-qPCR. (E and F) Following transfection of OE-SNHG6 or miR-944 mimics in LPS-induced chondrocytes, (G) cell viability, (H and I) apoptosis and (J-L) inflammatory cytokine levels were determined. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. SNHG6, small nucleolar RNA host gene 6; miR, microRNA; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; si-, small interfering; OE-, overexpression; LPS, lipopolysaccharide; ns, no significance.

$P < 0.001$); both changes occurred in a dose-dependent fashion. Further rescue experiments demonstrated that transfection of miR-944 mimics reversed the promoting effects of OE-PGAP1

transfection on PGAP1 expression (Fig. 4F and G; $P < 0.05$), whilst miR-944 inhibitor was found to partially attenuate the inhibitory effects of si-PGAP1 transfection on PGAP1 levels

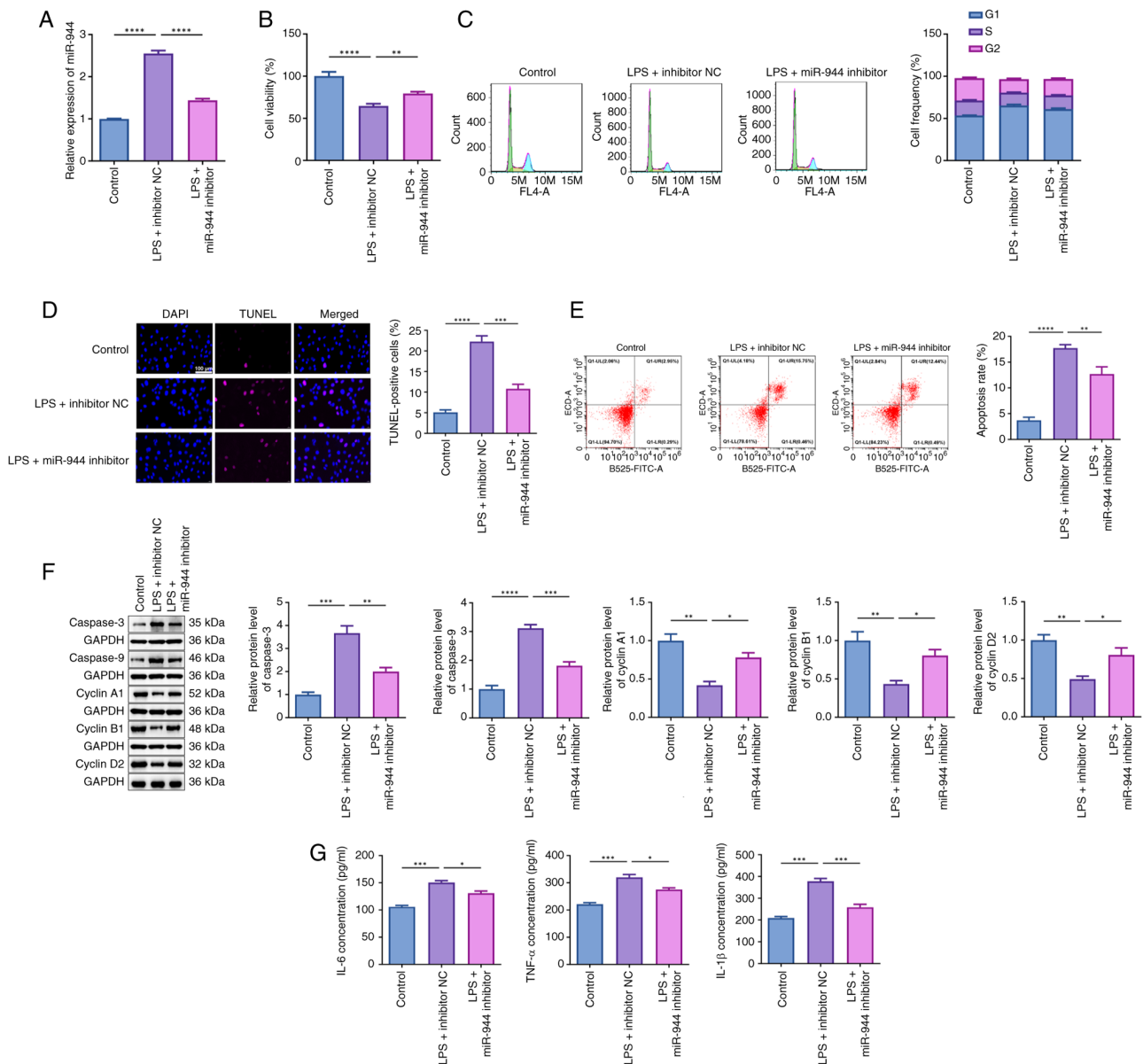


Figure 3. Effects of miR-944 inhibition on LPS-stimulated chondrocytes. (A) Following transfection of miR-944 inhibitor or inhibitor NC in LPS-induced chondrocytes, the expression of miR-944 was detected via reverse transcription-quantitative PCR. (B) The viability of LPS-induced chondrocytes that transfected with miR-944 inhibitor or inhibitor NC was measured by Cell Counting Kit-8 assay. (C) Cell frequency of LPS-induced chondrocytes was calculated via flow cytometric analysis. (D) The percentages of TUNEL-positive cells were assessed by TUNEL assay. Scale bar, 100 μ m. (E) The apoptotic rate of LPS-induced chondrocytes was calculated via flow cytometric analysis. (F) The protein levels of Caspase-3, Caspase-9, Cyclin A1, Cyclin B1 and Cyclin D2 in LPS-induced chondrocytes that transfected with miR-944 inhibitor or inhibitor NC were determined via western blotting. (G) The levels of IL-6, TNF- α and IL-1 β in LPS-induced chondrocytes that transfected with miR-944 inhibitor or inhibitor NC were determined via ELISA. * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001. miR, microRNA; LPS, lipopolysaccharide; NC, negative control.

(Fig. 4H and I; P <0.001). Moreover, as shown in Fig. 4J and K, it was found that silencing of SNHG6 significantly inhibited PGAP1 expression (P <0.0001), while the overexpressed SNHG6 promoted the expression levels of PGAP1 (P <0.01). Following further transfection of OE-SNHG6 or miR-944 mimics into LPS-stimulated chondrocytes, it was demonstrated that the transfection of miR-944 mimics reversed the promoting effects of OE-SNHG6 on PGAP1 expression (Fig. 4L; P <0.001).

miR-944 interacts with PGAP1 to modulate proliferation, apoptosis and inflammation in LPS-stimulated chondrocytes. As revealed in Fig. 5A-E, overexpression of PGAP1

significantly stimulated chondrocyte proliferation (P <0.0001), but suppressed apoptosis (P <0.001). These results were further validated by western blotting. It was demonstrated that the transfection of OE-PGAP1 in LPS-stimulated chondrocytes significantly reduced Caspase-3 and Caspase-9 levels (Fig. 5F; P <0.01), while concurrently elevating the levels of Cyclin A1, Cyclin B1 and Cyclin D2 (P <0.05). Notably, transfection of miR-944 significantly reversed the regulatory effects of OE-PGAP1 transfection in LPS-stimulated chondrocytes, including proliferation and apoptosis (Fig. 5A-F; P <0.05). Moreover, a significant reduction in inflammation responses was observed in LPS-stimulated chondrocytes when OE-PGAP1 was transfected (Fig. 5G; P <0.0001); however,

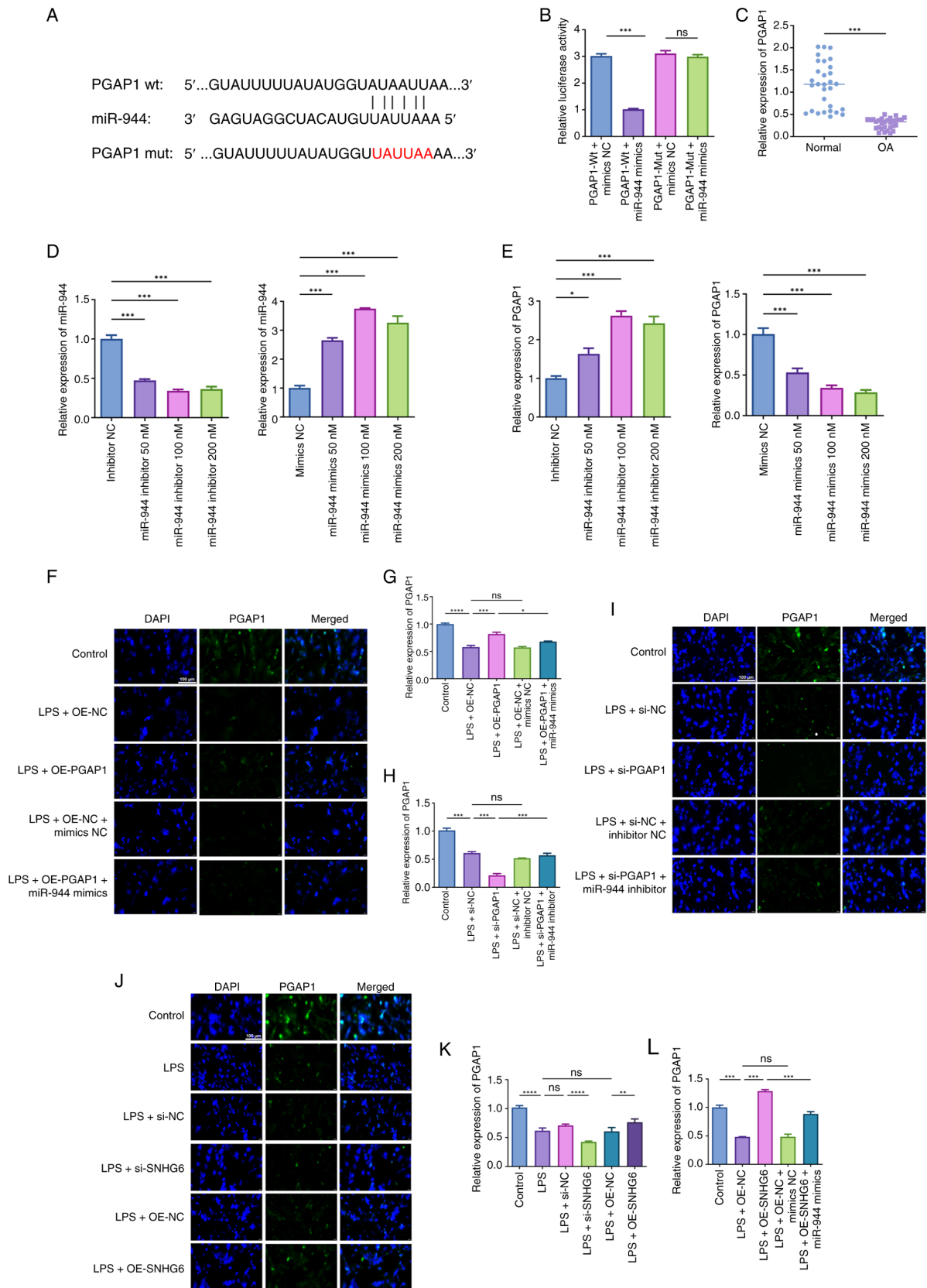


Figure 4. PGAP1 is a direct downstream target of miR-944. (A) StarBase showed the predicted binding site of miR-944 and PGAP1. (B) The target relationship between miR-944 and PGAP1 was verified by dual luciferase reporter assay in chondrocytes. (C) The expression of PGAP1 in synovial fluid of normal individuals and patients with osteoarthritis was detected via RT-qPCR. (D and E) Following transfection of miR-944 inhibitor or miR-944 mimics, the expression of (D) miR-944 and (E) PGAP1 in chondrocytes was detected by RT-qPCR. (F-I) Following transfection of (F and G) OE-PGAP1/miR-944 mimics or (H and I) si-PGAP1/miR-944 inhibitor, the expression of PGAP1 in LPS-induced chondrocytes was detected by immunofluorescence tests. Scale bar, 100 μ m. (J and K) Following transfection of si-SNHG6 or OE-SNHG6, the expression of PGAP1 in LPS-induced chondrocytes was detected by immunofluorescence tests. Scale bar, 100 μ m. (L) Following transfection of OE-SNHG6 or miR-944 mimics, the expression of PGAP1 in LPS-induced chondrocytes was detected by RT-qPCR. ns: no significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. PGAP1, post-GPI attachment to protein 1; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; si-, small interfering; OE-, overexpression; LPS, lipopolysaccharide; NC, negative control; ns, no significance.

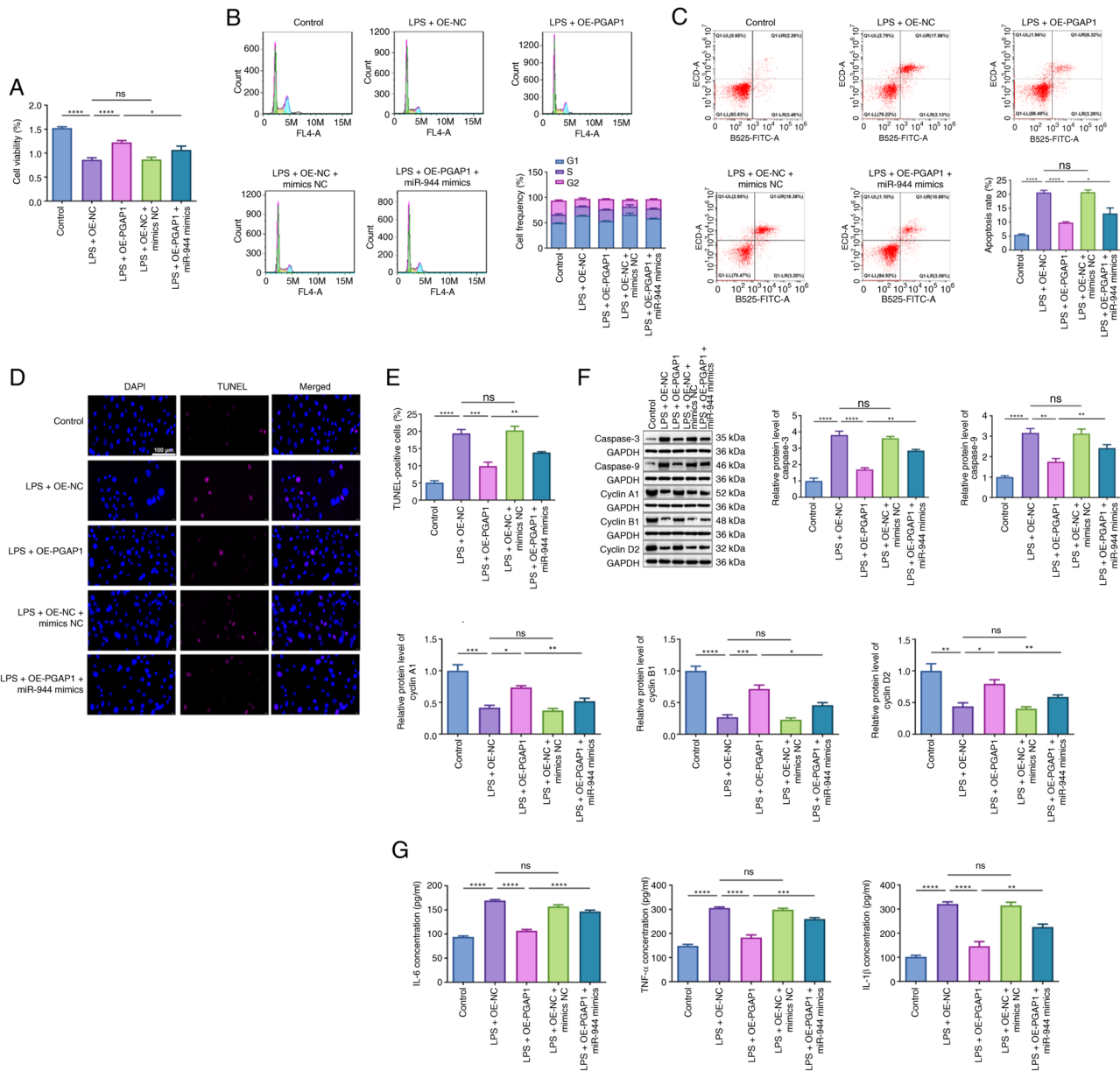


Figure 5. miR-944 interacts with PGAP1 to modulate proliferation, apoptosis and inflammation in LPS-stimulated chondrocytes. Following transfection of OE-PGAP1 or miR-944 mimics, (A) the viability of LPS-induced chondrocytes was measured by Cell Counting Kit-8 assay. (B) Cell frequency of LPS-induced chondrocytes was calculated via flow cytometric analysis. (C) The apoptotic rate of LPS-induced chondrocytes was calculated via flow cytometric analysis. (D and E) The percentages of TUNEL-positive cells were assessed by TUNEL assay. Scale bar, 100 μ m. (F) The protein levels of Caspase-3, Caspase-9, Cyclin A1, Cyclin B1 and Cyclin D2 in LPS-induced chondrocytes were determined via western blotting. (G) The levels of IL-6, TNF- α and IL-1 β in LPS-induced chondrocytes were determined via ELISA. * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001. miR, microRNA; PGAP1, post-GPI attachment to protein 1; LPS, lipopolysaccharide; OE-, overexpression; NC, negative control; ns, no significance.

this was partially restored upon the transfection of miR-944 mimics (P <0.01).

Discussion

With the progressive intensification of global population aging, the number of individuals affected by OA continues to rise. Epidemiological data have confirmed that primary OA affects ~46.3% of middle-aged and elderly individuals in China (20). Globally, roughly 14% of people aged 60 years and older are diagnosed with this condition, with its incidence increasing alongside age (21). The economic impact of OA is significant, resulting in annual losses exceeding \$300 billion in terms

of income and healthcare expenses (21). In view of its high epidemiological universality, OA has become a major factor contributing to reduced quality of life and an increased strain on healthcare systems (22). Although the exact etiological origin and comprehensive pathogenic regulatory network of OA remains unclear, recent studies increasingly highlight inflammatory processes and chondrocyte apoptosis as critical elements driving disease progression (23,24). In this context, in-depth excavation and systematic clarification of the key molecular signaling mechanisms driving the pathological progression of OA are conducive to identifying reliable therapeutic targets and further facilitating the targeted research and development of novel efficient drugs for early intervention,

clinical remission and etiological blockade of OA. In the present study, a novel molecular mechanism involved in OA progression was identified, demonstrating that the lncRNA SNHG6/miR-944/PGAP1 axis is linked to chondrocyte apoptosis and inflammation, thereby mediating OA development.

Accumulating clinical and fundamental studies have consistently documented aberrant epigenetic regulation of multiple lncRNAs throughout the pathological progression of OA. Among these dysregulated transcripts, the functional implications of canonical lncRNA candidates, including HOTAIR, H19 and PVT1, in OA onset and deteriorative progression have been systematically validated and mechanistically elaborated in successive independent investigations (10,25,26). In addition to the aforementioned lncRNAs, the role of the SNHG family of lncRNAs in the pathogenesis of OA has emerged as a prominent topic in numerous studies. Multiple SNHG members have been functionally annotated as critical upstream regulatory molecules in the development of OA. Their mechanisms involve various processes, including chondrocyte apoptosis (for example, SNHG5) (27), inflammation reactions (for example, SNHG1) (28), autophagy (for example, SNHG7) (29), and the mediation of extracellular matrix (ECM) degeneration (for example, SNHG12) (30). These dysregulated SNHG family members exhibit aberrant endogenous expression levels in the synovial fluid of patients with OA, enabling differentiation between OA patients and healthy individuals. However, as an important member of SNHG family, the specific biological function and potential molecular mechanism of SNHG6 in the continuous progression of OA remain poorly elucidated and lack systematic experimental validation. In the present study, it was observed that compared with the normal controls, SNHG6 was significantly downregulated in synovial fluid of patients with OA. This finding suggested that the abnormal expression of SNHG6 may be linked to the pathogenesis of OA. Accumulating evidence has demonstrated that multiple members of the SNHG family of lncRNAs exert pivotal regulatory effects on inflammatory responses and chondrocyte apoptosis, two core pathological events that dominate the entire progression process of OA (27,28). On this basis, it was reasonably hypothesized that SNHG6 may also be associated with inflammation and chondrocyte apoptosis. Subsequently, a series of gain-of-function and loss-of-function functional validation experiments were conducted *in vitro* to systematically corroborate the aforementioned research hypothesis. It was found that silencing of SNHG6 markedly promoted apoptosis and inflammation. The overexpression of SNHG6 suppressed chondrocyte apoptosis and mitigated inflammatory responses. In addition to regulating chondrocyte apoptosis and inflammation, the present study further revealed that SNHG6 modulated chondrocyte proliferation via precise control of cell cycle progression, with G1 phase arrest and dynamic changes in core cyclins (Cyclins A1, B1 and D2) showing a strong positive correlation with flow cytometry-based cell cycle distribution data. G1 phase arrest is a classic cytological hallmark of impaired chondrocyte proliferation in OA, which impairs chondrocyte renewal and repair, and ultimately accelerates articular cartilage matrix degradation (31). Flow cytometric analysis demonstrated that LPS stimulation induced marked G1 phase accumulation of chondrocytes and a concomitant reduction in S and G2/M phase populations, indicative of

typical G1 phase arrest in OA-like chondrocytes. SNHG6 silencing further exacerbated this G1 phase block, while SNHG6 overexpression effectively reversed LPS-induced G1 phase arrest and restored normal cell cycle distribution. These phenotypic changes were tightly aligned with western blot data for Cyclins A1, B1 and D2; core cell cycle regulators with non-redundant roles in driving cell cycle progression. Cyclin D2 initiates G1 phase progression and is essential for G1/S transition via activation of CDK4/6 (32); Cyclin A1 regulates both G1/S and S/G2 transitions to maintain DNA replication and S phase completion (33); Cyclin B1 is the master regulator of G2/M transition, and its upregulation is a prerequisite for mitotic entry (34). In the present study, LPS stimulation significantly downregulated Cyclin A1, B1 and D2 expression in chondrocytes, and SNHG6 silencing further suppressed their levels; conversely, SNHG6 overexpression significantly upregulated these cyclins. This inverse correlation between cyclin expression and G1 phase cell proportion is a direct reflection of molecular cell cycle regulation: Reduced cyclin expression impairs key cell cycle transitions (G1/S, S/G2 and G2/M), leading to G1 phase arrest and inhibited proliferation, while restored cyclin expression relieves these molecular blocks, reduces G1 phase accumulation, and rescues chondrocyte proliferative capacity. These results confirmed that the SNHG6 modulated chondrocyte cell cycle progression by fine-tuning expression of Cyclins A1, B1 and D2. This finding deepens our mechanistic understanding of SNHG6-mediated regulation of OA progression, suggesting that SNHG6 may act as a pathogenic factor in the progression of OA.

Notably, emerging studies have validated that SNHG family members serve as critical miRNA sponges to mediate in the pathogenesis of OA, such as SNHG1-miR-16-5p (35), SNHG9-miR-34a (36) and SNHG14-miR-214-3p (37). In line with these findings, it was further hypothesized that SNHG6 could also function as a miRNA sponge to participate in the progression of OA. Starbase online software was utilized to screen and predict the potential downstream target miRNAs of SNHG6, and the analytical results identified miR-944 as a candidate downstream binding partner of SNHG6. Meanwhile, SNHG6 was confirmed to negatively modulate the expression levels of miR-944 in LPS-induced chondrocytes. Notably, clinical and cellular phenotypic detection also revealed significantly elevated expression levels of miR-944 in synovial samples from patients with OA and LPS-induced chondrocytes. Functional rescue experiments further verified that the inhibition of miR-944 effectively attenuates chondrocyte apoptotic damage and overactivates inflammatory responses. Collectively, it was considered that SNHG6 can negatively regulate miR-944 expression to inhibit chondrocyte apoptosis and mitigate inflammatory responses, thereby ameliorating the progression of OA.

In fact, extensive crosstalk among lncRNAs, miRNAs and mRNAs has been well documented throughout the pathological progression of OA (38). Mechanistically, lncRNA can bind to miRNA, reduce the inhibitory effect of miRNA on mRNA, participate in regulating the progress of inflammation, chondrocyte apoptosis and proliferation. Of note, accumulating evidence confirms that multiple members of SNHG family also exert this regulatory effect in the development of OA, such as SNHG5-miR-26a-SOX2(39), SNHG7-miR-34a-5p-SYVN1 (29)

and SNHG15-miR-141-3p-BCL2L13 (40). In the present study, PGAP1 was identified as a potential target mRNA of miR-944. PGAP1 encodes a functional protein consisting of 922 amino acid residues, which exerts the biological enzymatic activity of glycosylphosphatidylinositol inositol-deacylase (41). It is located on the membrane of the endoplasmic reticulum (41). In previous studies, PGAP1 has been reported to be involved in NEAT1-mediated miR-374b-5p network to regulate the development of OA (42). It was therefore hypothesized that there may be some interactions between the SNHG6/miR-944 axis and PGAP1. In the present study, it was observed that overexpression of SNHG6 significantly promoted PGAP1 expression, while transfection of miR-944 mimics exhibited the opposite regulatory effect. Meanwhile, elevated miR-944 expression was capable of abrogating the inhibitory effects of PGAP1 overexpression on chondrocyte apoptosis and inflammation. Collectively, these findings confirmed the authors' hypothesis that the SNHG6-miR-944-PGAP1 axis may be a novel regulatory cascade that participates in the pathogenesis of OA.

Beyond PGAP1, miR-944 is predicted to target a repertoire of additional functional genes and may potentially synergize with SNHG1/5/7-mediated regulatory networks to orchestrate chondrocyte homeostasis in physiological and pathological conditions of OA. As a typical miRNA with pleiotropic regulatory functions, miR-944 is not restricted to a single target gene in its biological regulatory network; bioinformatic analysis based on databases including Starbase, miRDB and TargetScan has identified multiple putative target genes of miR-944 that are implicated in chondrocyte ECM metabolism, proliferation and inflammatory response, such as genes encoding key regulators of the NF- κ B and MAPK signaling pathways that are central to OA progression. This multi-target regulatory characteristic is consistent with the universal functional features of miRNAs, which enables a single miRNA to fine-tune multiple biological processes simultaneously. Notably, apart from SNHG6, SNHG1, SNHG5 and SNHG7, as key members of the SNHG lncRNA family, have been well-documented to mediate chondrocyte homeostasis in OA through distinct ceRNA regulatory networks: SNHG1 modulates chondrocyte apoptosis and inflammation via the miR-195/IKK- α axis (28), SNHG5 regulates chondrocyte proliferation and apoptosis through the miR-10a-5p/H3F3B axis (27), and SNHG7 orchestrates chondrocyte proliferation, apoptosis and autophagy via the miR-34a-5p/SYVN1 axis (29). These SNHG-mediated networks independently regulate chondrocyte biological behaviors, and miR-944 may serve as a novel molecular node that intersects with these networks, forming synergistic regulatory crosstalk to collectively maintain the balance of chondrocyte survival, proliferation and inflammatory response under physiological conditions. This synergistic effect also highlights the complexity of the molecular regulatory network in OA. Therefore, it is considered that individual lncRNA/miRNA/mRNA axis does not act in isolation but are intertwined to form a multi-layered regulatory network governing chondrocyte homeostasis. Given the novelty of the SNHG6/miR-944/PAP1 axis in OA reported in the present study, the current research focuses on the regulatory relationship between miR-944 and its validated target PGAP1. The identification of additional target genes of miR-944 as well as the in-depth exploration of the synergistic regulatory

mechanisms between miR-944 and SNHG1/5/7-mediated networks remain to be elucidated in future studies. Such investigations will help to further unravel the intricate molecular regulatory network underlying chondrocyte dysfunction in OA and provide more comprehensive molecular targets for the development of disease-modifying therapies for OA.

Additionally, it is imperative to acknowledge that the present study is subject to several other limitations. First, the small sample size may limit the generalizability of the present clinical findings, and larger cohorts are needed in future studies to validate these results. Second, a single cell model cannot capture the complex pathological microenvironment associated with OA, including the interactions between the degradation of the chondrocyte ECM, synovial hyperplasia, and subchondral bone remodeling. Classic IL-1 β -induced, TNF- α -induced and IL-1 β + TNF- α co-induced chondrocyte models should be established to confirm whether SNHG6/miR-944/PAP1 axis has a universal regulatory effect on chondrocyte damage induced by endogenous pro-inflammatory cytokines. Third, *in vitro* cell line assays inherently fall short of recapitulating complex clinical scenarios. The current study was restricted to preliminary cellular-level analyses, lacking verification of biological fidelity, simulation of the *in vivo* microenvironment, and assessment of translational potential. Further exploration is also warranted to elucidate the functional roles of these targets in animal model systems.

In conclusion, the present study constitutes a novel and preliminary exploratory research focusing on OA pathological mechanisms. It systematically elucidates the biological function and intrinsic molecular regulatory mechanism of SNHG6 during OA progression. Functional experiments validated that SNHG6 could competitively bind to regulate the miR-944/PAP1 signaling cascade, thereby repressing excessive chondrocyte apoptosis and abnormal inflammatory activation in OA. Taken together, the aforementioned findings to some extent deepen the mechanistic understanding of the functional role of SNHG6 in OA pathogenesis. Meanwhile, the present study provides reliable experimental basis and novel targeted theoretical references for the subsequent development of molecular targeted intervention strategies and precise clinical adjuvant treatment of OA.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

FH made substantial contributions to the conception and design of the study. FH, ZS, JY and XZ made substantial

contributions to the acquisition, analysis and interpretation of the data. FH drafted the manuscript. All authors critically revised the manuscript for intellectual content and confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All the individuals had been informed the study details and provided their written consent in advance. Ethical approval was obtained from the Ethics Committee of Wenzhou People's Hospital (approval No. KY-2023-251; Wenzhou, China) and this research adheres to the principles outlined in the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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