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Icariin Promotes the Chondrocyte Proliferation and Stabilizes Extracellular Matrix by Regulating NEAT1/MiR-27a-3p/MAPK Signaling Pathway

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Background: Osteoarthritis (OA) is a frequently occurring degenerative joint disease. As the primary bioactive ingredient extracted from *Epimedium*, icariin (ICA) is reported to promote osteogenic differentiation and bone formation. Nonetheless, the specific mechanisms of ICA in treating OA warrant further elucidation. The current study is targeted at ascertaining the mechanism of ICA in modulating the phenotypes of chondrocytes with an *in-vitro* model.

Methods: To construct an *in-vitro* model of OA, we used interleukin (IL)-1β to induce human chondrocytes (C-28/I2). Nuclear paraspeckle assembly transcript 1 (NEAT1) and miR-27a-3p expressions were examined through qRT-PCR. Cell viability was determined through Cell Counting Kit-8 (CCK-8) assay; flow cytometry was employed to analyze cell cycle progression; cell migration was examined by Transwell assay; enzyme-linked immunosorbent assay (ELISA) was conducted to measure the levels of inflammation-related factors (IL-8 and IL-6). The binding relationship of miR-27a-3p with NEAT1 was verified through dual-luciferase reporter gene assay; Western blot assay was conducted to measure the expressions of extracellular matrix-related proteins (ADAM metallopeptidase with thrombospondin type 1 motif 5 (ADAMTS-5), collagen II, matrix metalloproteinase 13 (MMP-13), and aggrecan) and mitogen-activated protein kinase (MAPK) signaling pathway-associated proteins (p-p38, p-Jun N-terminal kinase (JNK), and phosphorylated MAPK1 and MAPK2 (p-ERK1/2)). MiR-27a-3p's downstream target genes were predicted using the miRwalk, StarBase, miR-Database (DB), TargetScan databases, and the DAVID database was adopted to perform a Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis of the target genes.

Results: ICA significantly boosted chondrocyte viability and migration, as well as suppressed the apoptosis, inflammatory response and extracellular matrix degradation of C-28/I2 cells. ICA down-regulated NEAT1 expression in chondrocytes. MiR-27a-3p was recognized as NEAT1's downstream target, and bioinformatics analysis implied that miR-27a-3p's potential target genes might be related to the MAPK signaling pathway activation. ICA could inhibit p-ERK1/2, p-p38, and p-JNK expressions through promoting miR-27a-3p expression.

Conclusions: ICA protects chondrocyte via modulating the NEAT1/miR-27a-3p/MAPK axis, and our findings partly explain the mechanism of ICA in ameliorating OA.

Keywords: osteoarthritis; icariin; NEAT1; miR-27a-3p; MAPK signaling pathway

Background

Known as a common disease, osteoarthritis (OA) is an important cause of disability among elderly people, characterized by articular cartilage damage and an increased rate of chondrocyte apoptosis [1]. Remodeling or loss of the extracellular matrix (ECM) of articular cartilage is the key pathological change in OA, and type II collagen (collagen II) and proteoglycan (aggrecan) synthesized by chondrocytes are gradually reduced during the development of OA, while some enzymes involved in ECM degradation (matrix metalloproteinase 13 (MMP-13), ADAM metallopeptidase with thrombospondin type 1 motif 5 (ADAMTS-5)) are highly activated during cartilage injury [2].

Icariin (ICA), as a flavonoid, is extracted from the traditional Chinese herb *Epimedium*, which has anti-inflammatory, tumor-suppressive and antioxidative pharmacological effects [3]. ICA can ameliorate chondrocyte injury by promoting chondrocyte viability, inhibiting apoptosis [4–7]. Specifically, ICA can enhance chondrocyte viability through boosting the expression of hypoxia-inducible factor 1α and facilitating anaerobic glycolysis [6]. Another study reports that ICA activates the NFE2 like bZIP transcription factor 2/anti-oxidant response ele-

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ment (Nrf2/ARE) pathway to attenuate interleukin (IL)-1β-induced ECM degradation [7]. Nonetheless, the underlying mechanisms of ICA in ameliorating OA have not been completely elucidated.

There are many studies suggesting that long non-coding RNAs (lncRNAs) partake in regulating biological processes [8]. Aberrant lncRNA expression is found to be linked to chondrocyte injury and OA development [9]. For example, it is revealed that lncRNA MALAT1 expression is upregulated in OA chondrocytes and facilitates OA progression by targeting the miR-150-3p/AKT3 axis [10]. LncRNA PVT1 facilitates chondrocyte apoptosis by repressing miR-488-3p [11]. LncRNA FOXD2 adjacent opposite strand RNA 1 (FOXD2-AS1) modulates cyclin D1 (CCND1) expression through absorbing miR-206, thereby regulating chondrocyte proliferation [12]. Nuclear paraspeckle assembly transcript 1 (NEAT1) is a lncRNA transcribed from chromosome 11 as a carcinogenic factor in various human cancers [13]. In OA, NEAT1 has been found to inhibit chondrocyte viability and promote apoptosis [14].

Recognized as a kind of non-coding RNA which is 22 nucleotides long, microRNAs (miRNAs) suppress mRNA expression through binding to the target mRNA 3′-UTR [15]. It has been found that miRNAs are dysregulated in OA and implicated in the development of OA [16–19]. MiR-27a-3p participates in OA pathogenesis, and it modulates chondrocyte proliferation, inflammation and ECM degradation [20]. Nevertheless, whether ICA plays a role in protecting chondrocytes by regulating miR-27a-3p is unclear.

The mitogen-activated protein kinase (MAPK) signal pathway is a classical intracellular transduction pathway that transmits various extracellular stimulus signals from the cell membrane into the nucleus and plays a critical part in basic cellular processes, e.g., cell differentiation, migration, and apoptosis [21]. Growing research has demonstrated that the MAPK signal pathway partakes in the pathogeneses of varied human diseases including OA [22–24]. This study surveyed the role of ICA in IL-1β-induced chondrocyte injury. We report that, ICA can protect chondrocytes via modulating the NEAT1/miR-27a-3p axis and the MAPK signal pathway.

Materials and Methods

Cell Culture and Processing

Human embryonic kidney cells (HEK293T) and human chondrocytes (C-28/I2) were obtained from Procell (Wuhan, China) and cultured in DMEM/Nutrient Mixture (F-12) (ThermoFisher Scientific, Waltham, MA, USA) containing 10% FBS (16000-044, Gibco, Grand Island, NY, USA), 10 mg/mL streptomycin and 100 U/mL penicillin, which was incubated at 37 °C in 5% CO_2. C-28/I2 cells were induced with 10 ng/mL IL-1β (Sigma, Saint Louis, MO, USA) for 24 h to construct an *in-vitro* model of OA, while the control cells were treated with an equivalent amount of phosphate buffer saline (PBS). Mycoplasma test was used for validate that the cells were not contaminated, and STR test was used for validate the authenticity of the cell lines. The results showed that the cells were not contaminated and the cell line was authentic.

ICA Preparation and Processing

From Sigma Aldrich (Cat. I1286, Saint Louis, MO, USA), we bought ICA ($C_{33}H_{40}O_{15}$, molecular weight: 676.66, purity $\geq$94%) (Fig. 1A). We dissolved ICA in DMSO (Sigma, MO, USA) to make solutions of 2 μM, 5 μM, and 10 μM, which were stored at –20 °C. When C-28/I2 chondrocytes reached 70–80% confluency, the cells were stimulated for 2 h with ICA at different concentrations, and the control cells were subjected to treatment with 0.1% DMSO.

Cell Transfection

C-28/I2 chondrocytes in good growth condition were inoculated in 6-well plates (2×10^5 cells/mL) and cultured at 37 °C. From Genomeditech (Shanghai, China), we purchased NEAT1 overexpression plasmids and blank plasmids, miR-27a-3p mimic and inhibitor, and the negative control miRNA. When cell confluency reached 60%, we used Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) to transfect the above vectors into the C-28/I2 cells.

qRT-PCR

By the manufacturer's instructions, total RNA was extracted from C-28/I2 chondrocytes utilizing TRIzol reagent (ThermoFisher Scientific, Waltham, MA, USA) and then reverse-transcribed to cDNA employing the ABI Reverse Transcription Kit (4368814, Applied Biosystems, New York City, NY, USA). On a Biosystems 7300 Real-Time PCR system (7300, ABI, Foster City, CA, USA), a SYBR GreenMix kit was adopted to perform qRT-PCR. The $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative expressions of the target genes, with U6 and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) normalizing gene expression. The primer sequences are the following (F, Forward; R, Reverse):

NEAT1:
F, 5′-GGGTACCCGCTTGTTTCAAAGGGAGCAG-3′,
R, 5′-CCTCGAGGCTGGCTAGTCCCAGTTCAGC-3′;

miR-27a-3p:
F, 5′-CGCGTTCACAGTGGCTAAGT-3′,
R, 5′-GTGCAGGGTCCGAGGTATTC-3′;

U6:
F, 5′-CTCGCTTCGGCAGCACA-3′,
R, 5′-AACGCTTCACGAATTTGCGT-3′;

glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*):

F, 5′-TCGACAGTCAGCCGCATCTTCTTT-3′,

R, 5′-ACCAAATCCGTTGACTCCGACCTT-3′.

CCK-8 Assay

A Cell Counting Kit-8 (CCK-8) kit (Med Chem Express, Monmouth Junction, NJ, USA) was employed to detect C-28/I2 chondrocyte viability. The cells were inoculated in 96-well plates (5000 cells/well) and, after 24 h of cell culture, 10 μL of CCK-8 reagent was supplemented, and subsequently the plates were incubated at 37 °C in 5% CO_2 for 2 h. The optical density (OD) value of each well at 450 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell Cycle Assay

The chondrocytes were fixed overnight at 4 °C in pre-chilled 85% ethanol and stained at 4 °C with PI staining solution for 30 min. Flow cytometry was conducted to determine the cell cycle distribution, and the percentage of cells in each phase was analyzed by Modfit software to compare the differences among the G0/G1, S, and G2/M phases.

TUNEL Assay

Chondrocyte apoptosis was detected using Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick-End Labeling (TUNEL) assay. C-28/I2 cells were inoculated in 6-well plates (3.5×10^5 cells/well), fixed at room temperature for 15 min with 4% paraformaldehyde, and subsequently permeabilized for 20 min in 0.25% Triton-X 100. According to the instructions, the cells were cultured away from light with 50 μL of TUNEL reaction mix (45 μL of FITC-labeled dUTP & 5 μL of TdT enzyme) for 60 min, followed by incubation with DAPI for 5 min for nuclear staining. After washing the cells by PBS, the positive cells were observed and counted under the fluorescence microscope, and the apoptosis rate was calculated.

Transwell Assay

We used the Transwell chamber (pore size: 8 μM; 3422, Corning Costar, Cambridge, MA, USA) to detect C-28/I2 chondrocyte migration. We added 200 μL of cell suspension (with serum-free medium) to the upper compartment and 600 μL of complete medium to the lower compartment. The cells on the filter's lower surface were fixed 24 h later and stained with crystal violet solution, and they were counted under the inverted microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)

C-28/I2 cells were transferred to 6-well plates and cultured for 48 h. Then, the chondrocytes were lysed and the supernatant was harvested after centrifugation. Following the manufacturer's instructions, the IL-8 and IL-6 levels of the supernatant were determined utilizing the corresponding enzyme-linked immunosorbent assay (ELISA) kit (HM10222 (for IL-8) and HM10205 (for IL-6), Bio-Swamp Life Science, Shanghai, China).

Dual-Luciferase Reporter Gene Assay

The NEAT1-wide type (WT) and NEAT1-mutant (MUT) luciferase reporter plasmids were constructed by amplifying WT NEAT1 sequence and MUT NEAT1 sequence containing the potentially complementary binding site to miR-27a-3p, and then they were integrated into the luciferase reporter plasmid pmirGLO. HEK293T cells were co-transfected with the above luciferase reporter plasmid and miR-27a-3p mimic or the negative control miR-NC. We collected the cells 48 h later, and the luciferase activity was determined on a dual-luciferase reporter detection system (e1960, Promega Corporation, Madison, WI, USA).

Western Blot Assay

Protein was extracted from C-28/I2 cells employing RIPA lysis buffer (AR0105, Boster, Beijing, China) and quantified utilizing the BCA kit (23250, Pierce, Rockford, IL, USA). SDS-PAGE (Sigma-Aldrich, Saint Louis, MO, USA) was performed, and the protein samples were transferred onto PVDF membranes (IPVH00010, Millipore, Billerica, MA, USA). The membranes, after being blocked at room temperature with 5% skim milk for 2 h, were incubated at 4 °C overnight with primary antibodies. The primary antibodies were as follows: anti-MMP-13 (1:1000; ab315267, Abcam, Cambridge, UK), anti-ADAMTS-5 (1:1000; ab41037, Abcam, Cambridge, UK), anti-collagen II (1:1000; ab307674, Abcam, Cambridge, UK), anti-phosphorylated MAPK1 and MAPK2 (p-ERK1/2) (1:1000; ab201015, Abcam, Cambridge, UK), anti-aggrecan (1:1000, ab313636, Abcam, Cambridge, UK), anti-p-p38 (1:1000; ab178867, Abcam, Cambridge, UK), anti-p-JNK (Jun N-terminal kinase) (1:1000, ab307802, Abcam, Cambridge, UK) and anti-GAPDH (1:1000, ab8245, Abcam, Cambridge, UK). Then the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:2000; ab6940, Abcam, Cambridge, UK) at room temperature for 2 h. Ultimately, the ECL luminescence reagent (P0018S, Beyotime, Shanghai, China) was adopted for developing the protein bands, with GAPDH as the internal reference.

Bioinformatics Analysis

Prediction of miR-27a-3p's downstream target genes was conducted using the miRWalk, starBase, miR-Database (DB), TargetScan databases, and an analysis of intersecting target genes was conducted employing the online website (http://bioinformatics.psb.ugent.be/webtools/Venn/). The DAVID (https://david.ncifcrf.gov/home.jsp) database is an online site for gene function annotation and analysis, with

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which we performed a Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis of the intersecting target genes.

Statistical Aanalysis

The tool for statistical analysis and graphing was GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Each experiment was carried out in triplicate and repeated 3 times, and mean ± standard deviation was the expression form of the results. The comparisons between/among groups were conducted via Student's *t*-test or one-way ANOVA with post-hoc test, with $p < 0.05$ indicating a difference of statistical significance.

Results

ICA's Impacts on Chondrocyte Viability, Cell Cycle Progression and Apoptosis

To investigate ICA's (Fig. 1A) effects on chondrocytes in the OA condition, human C-28/I2 chondrocytes were stimulated with IL-1β for 24 h to construct an *in-vitro* cell model of OA, and then the chondrocytes were treated with 2 μM, 5 μM, and 10 μM ICA for 2 h. The CCK-8 assay manifested that, as against the control group, the cell viability of the IL-1β treatment group was markedly lowered, whereas both 5 μM ICA and 10 μM ICA remarkably elevated the chondrocyte viability, and 5 μM ICA was chosen for follow-up experiments (Fig. 1B). The TUNEL assay suggested that in the IL-1β treatment group C-28/I2 chondrocyte apoptosis rate was notably higher, while as against the IL-1β-treated group, the apoptosis rate was noticeably lowered in the IL-1β/ICA group (Fig. 1C). The flow cytometry analysis of ICA's effect on C-28/I2 chondrocyte cycle progression showed a significant rise in the proportion of G0/G1 phase in the IL-1β-treated group in contrast with the control group, while the proportion of G0/G1 phase in the IL-1β/ICA group was observably diminished in contrast with the IL-1β-treated group (Fig. 1D). The above findings show that IL-1β successfully induces chondrocyte injury and ICA significantly promotes chondrocyte viability and inhibits apoptosis.

Effects of ICA on Chondrocyte Migration, Inflammatory Response and ECM Degradation

To delve deeper into ICA's effect on IL-1β-induced chondrocyte injury, chondrocytes were treated with ICA and the migration of chondrocytes was examined using Transwell assay, which indicated that, as against the IL-1β-treated group, the cell migration in the IL-1β/ICA group was dramatically higher (Fig. 2A). The ELISA revealed that, as against the IL-1β-treated group, IL-8 and IL-6 levels were dramatically reduced in the cells of the IL-1β/ICA group (Fig. 2B,C). Besides, the expressions of MMP-13, ADAMTS-5, and extracellular matrix (ECM)-related proteins (aggrecan, collagen II) were determined via Western blotting, and it was unveiled that MMP-13 and ADAMTS-5 were noticeably downregulated in the IL-1β/ICA group as opposed to the IL-1β treatment group; relative to the IL-1β treatment group, aggrecan and collagen II expressions were significantly elevated in the IL-1β/ICA group (Fig. 2D,E), indicating that ICA remarkably facilitates chondrocyte migration and suppresses IL-1β-induced inflammatory response and ECM degradation.

ICA Regulates MiR-27a-3p Expression through NEAT1

To analyze the mechanism of action of ICA, the expression of NEAT1 in chondrocytes was assessed through qRT-PCR, and we found that NEAT1 expression was noticeably up-regulated in the C-28/I2 cells of the IL-1β treatment group as opposed to the control group, while NEAT1 was notably downregulated in cells of the IL-1β/ICA group as opposed to the IL-1β treatment group (Fig. 3A). Moreover, we used the starBase database to predict NEAT1's downstream targets, and miR-27a-3p was discovered to have the binding site complementary to NEAT1. MiR-27a-3p mimics were confirmed by dual-luciferase assay to significantly inhibit NEAT1-WT reporter's luciferase activity, whereas having no influence on that of NEAT1-MUT reporter (Fig. 3B). qRT-PCR indicated that miR-27a-3p was markedly downregulated in C-28/I2 cells of the IL-1β treatment group in comparison to the control group, yet miR-27a-3p expression was noticeably higher in C-28/I2 cells of the IL-1β/ICA group as opposed to the IL-1β treatment group (Fig. 3C). To confirm whether ICA regulates miR-27a-3p expression through NEAT1, NEAT1 overexpression plasmid was transfected into chondrocytes. qRT-PCR showed that overexpression of NEAT1 partially weakened the facilitating effect of miR-27a-3p expression induced by ICA (Fig. 3D). The aforementioned results validate that miR-27a-3p is NEAT1's downstream target and ICA can promote miR-27a-3p expression through NEAT1.

ICA Protects Chondrocytes by Regulating MiR-27a-3p

Next, miR-27a-3p inhibitors were transfected into C-28/I2 chondrocytes. CCK-8 experiment manifested that, in contrast to that of the ICA treatment group, the cell viability of the miR-27a-3p inhibitor group was observably inhibited (Fig. 4A); flow cytometry manifested that, as opposed to the ICA treatment group, the proportion of cells in the G0/G1 phase was observably elevated in the miR-27a-3p inhibitor group (Fig. 4B); TUNEL assay suggested that the cell apoptosis rate of the miR-27a-3p inhibitor group was markedly higher in comparison to the ICA treatment group (Fig. 4C). Transwell assay unveiled that miR-27a-3p inhibitor partially rescued the promoting effect of ICA on chondrocyte migration (Fig. 4D); ELISA showed that miR-27a-3p inhibitors counteracted in part the suppressive effect of ICA on the inflammatory response of chondro-

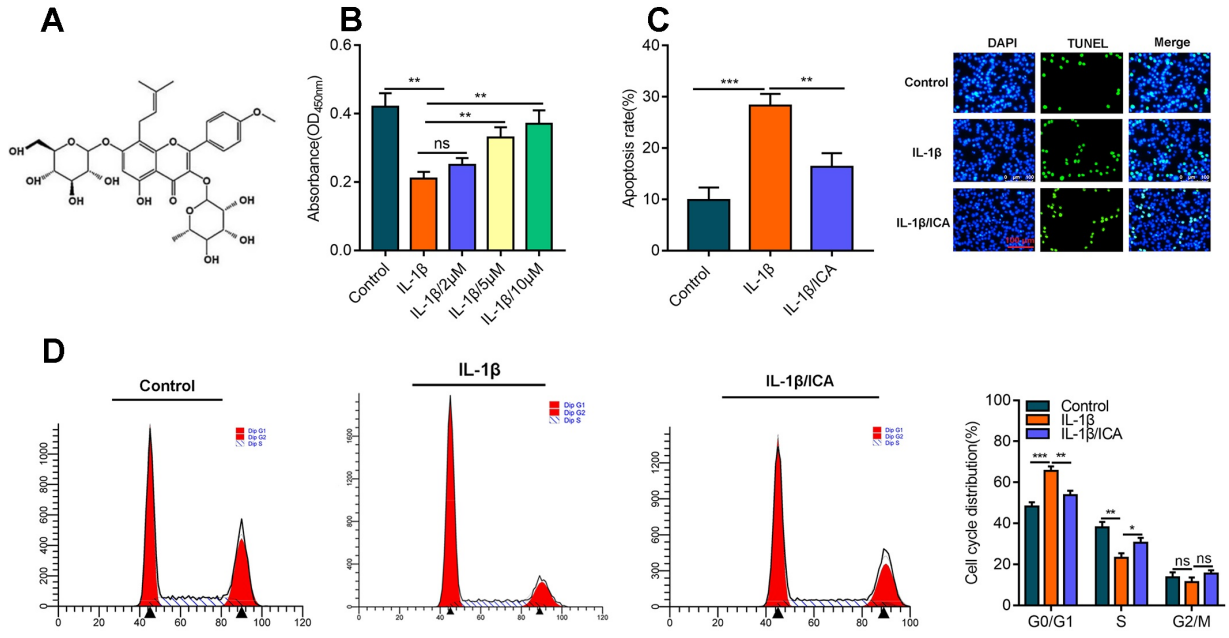

Fig. 1. ICA's effects on chondrocyte viability, cell cycle and apoptosis. (A) The molecular structure of ICA. (B) Detection via CCK-8 of C-28/I2 cell viability after IL-1β and ICA treatment. (C) Detection through TUNEL assay of C-28/I2 cell apoptosis after ICA and IL-1β treatment. (D) Analysis by flow cytometry of C-28/I2 cell cycle distribution after ICA and IL-1β treatment. The black triangle indicates the cell peak of G1 stage and G2 stage. ns. $p > 0.05$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. OD, optical density; IL, interleukin; ICA, icariin; CCK-8, Cell Counting Kit-8; TUNEL, Terminal Deoxynucleotidyl Transferase mediated dUTP Nick-End Labeling.

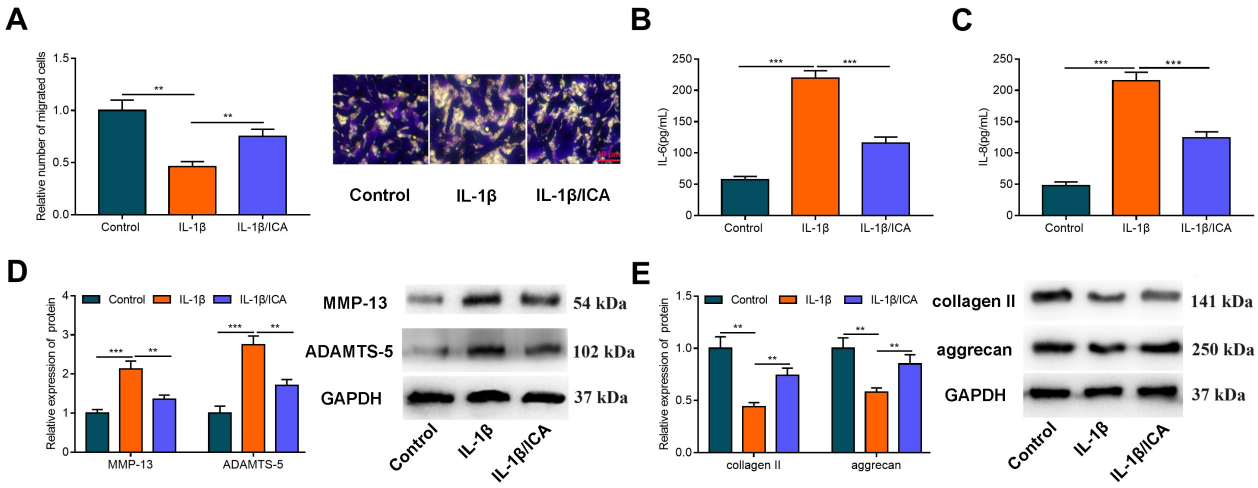

Fig. 2. Effects of ICA on chondrocyte migration, inflammatory response, and ECM degradation. (A) Detection of C-28/I2 cell migration after IL-1β and ICA treatment by Transwell assay. (B,C) Detection via ELISA of IL-8 and IL-6 levels in C-28/I2 cells after IL-1β and ICA treatment. (D,E) Western blot was conducted to examine the expressions of ECM-associated proteins (ADAMTS-5, aggrecan, MMP-13, and collagen II) in C-28/I2 cells after ICA and IL-1β treatment. $^{**}p < 0.01$, $^{***}p < 0.001$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP-13, matrix metalloproteinase 13; ADAMTS-5, ADAM metallopep-tidase with thrombospondin type 1 motif 5; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay.

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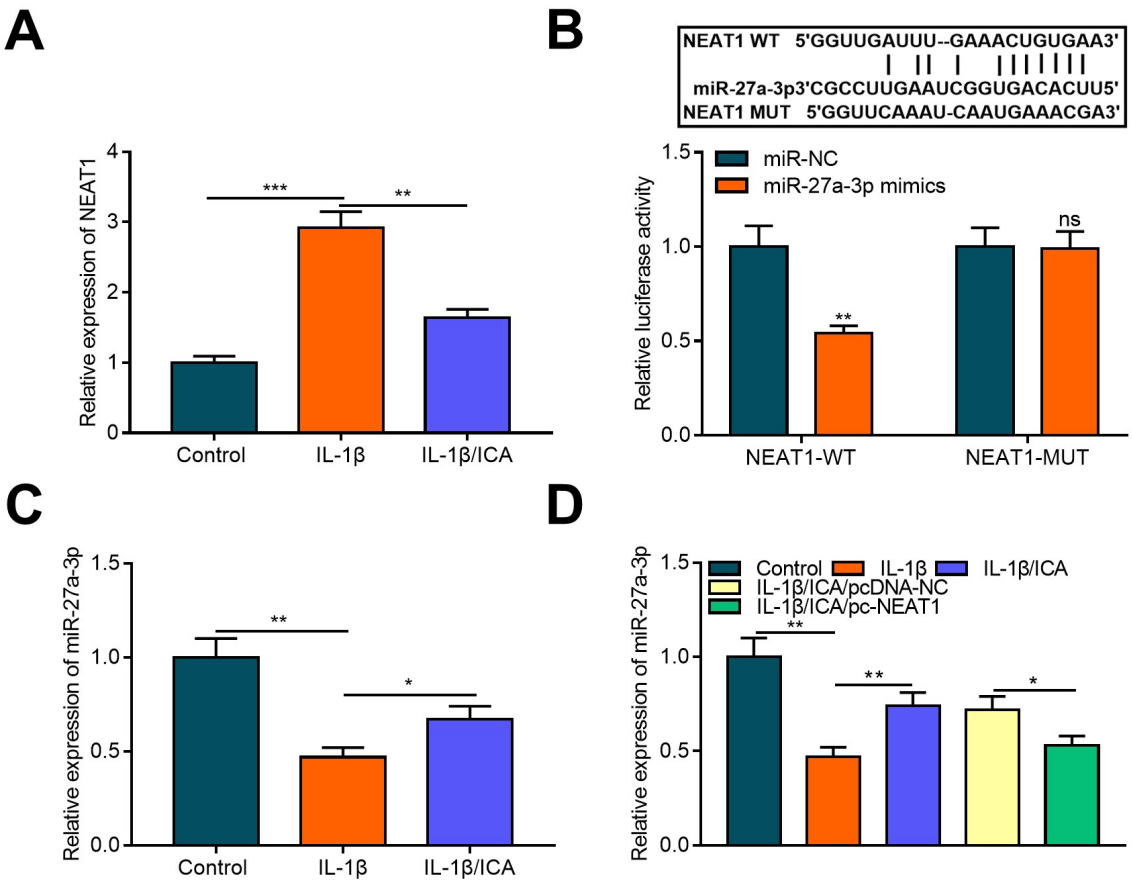

Fig. 3. ICA modulates miR-27a-3p expression through NEAT1. (A) Detection by qRT-PCR of NEAT1 expression in C-28/I2 cells after ICA and IL-1β treatment. (B) The starBase database and dual-luciferase assay confirmed that NEAT1 bound directly to miR-27a-3p. (C) Detection of miR-27a-3p expression in C-28/I2 cells after ICA and IL-1β treatment through qRT-PCR assay. (D) Detection through qRT-PCR of miR-27a-3p expression after C-28/I2 cells were transfected with NEAT1 overexpression plasmids. ns. $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. NEAT1, nuclear paraspeckle assembly transcript 1; WT, wide type; MUT, mutant.

cytes (Fig. 4E,F). Besides, the Western blot results showed that ADAMTS-5 and MMP-13 were dramatically upregulated, and the collagen II and aggrecan levels were notably decreased in C-28/I2 cells transfected with miR-27a-3p inhibitors, compared to the ICA treatment group (Fig. 4G,H), indicating that miR-27a-3p inhibition counteracts in part the protective effect of ICA on chondrocytes.

ICA Regulates the Expressions of MAPK Signaling Pathway-Related Proteins through MiR-27a-3p

To further explore miR-27a-3p's downstream regulatory mechanism, miR-27a-3p's downstream target genes were predicted using the miRWalk, starBase, miR-DB, TargetScan databases, and a Venn diagram was plotted (Fig. 5A). Furthermore, a KEGG enrichment analysis was conducted on the target genes within the intersection using the DAVID database, and it was discovered that these target genes were probably associated with the MAPK signaling pathway (Fig. 5B). To confirm whether ICA plays a role in protecting chondrocytes via modulating the MAPK signaling pathway, the expressions of MAPK pathway-associated proteins (p-JNK, p-ERK1/2, and p-p38) were examined by Western blot after treating C-28/I2 cells with IL-1β/ICA, and it was shown that, in contrast with the control group, p-ERK1/2, p-p38 and p-JNK expressions were markedly enhanced in the IL-1β treatment group, whereas they were remarkably decreased in the ICA treatment group as against those in the IL-1β treatment group (Fig. 5C). We subsequently transfected miR-27a-3p inhibitor into C-28/I2 chondrocytes, and the results showed that, in comparison to the ICA treatment group, p-p38, p-ERK1/2 and p-JNK levels in C-28/I2 chondrocytes were significantly increased after miR-27a-3p was inhibited (Fig. 5C). These data suggest that ICA can repress the activation of MAPK signaling through miR-27a-3p.

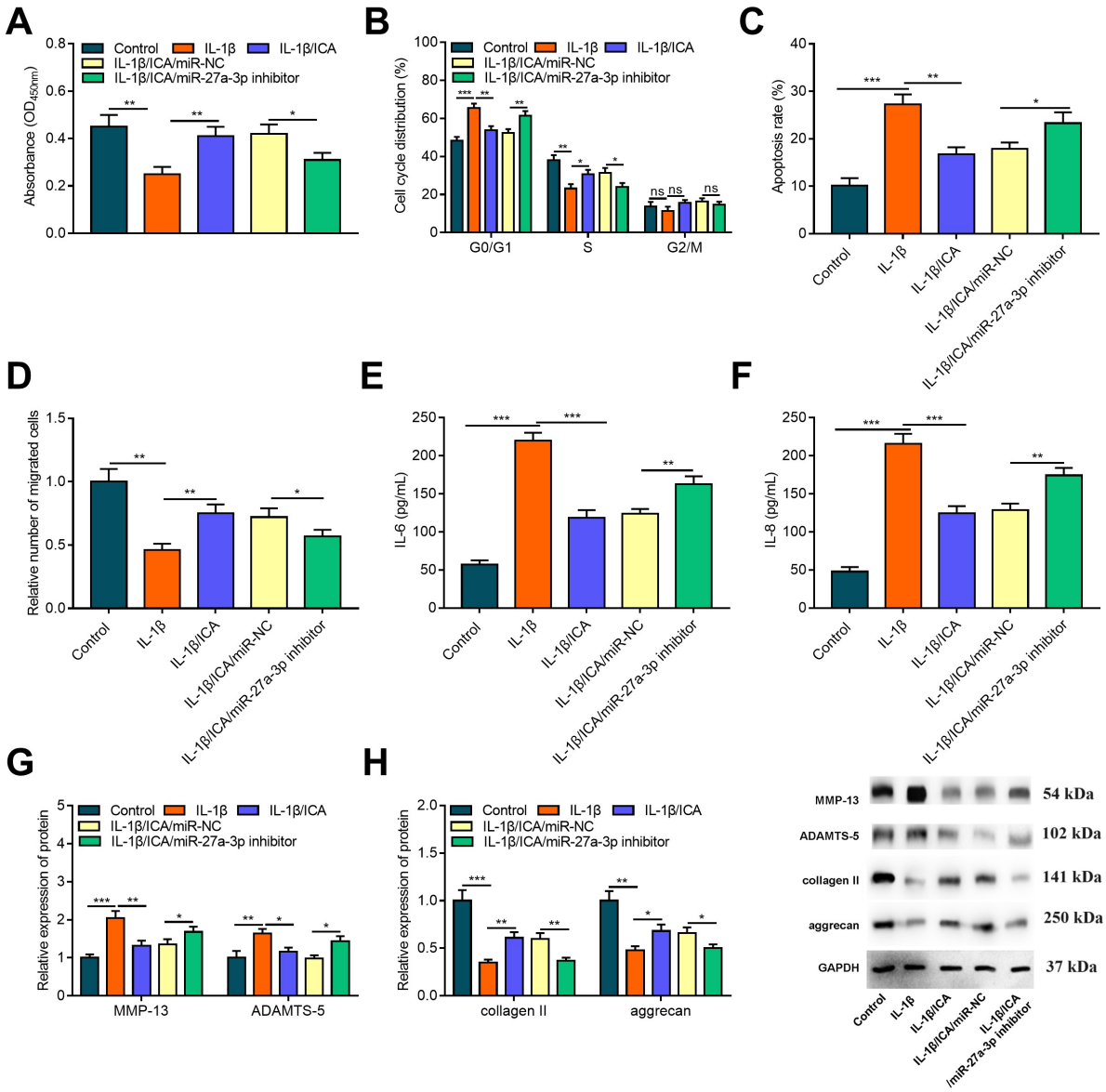

Fig. 4. MiR-27a-3p inhibitor reverses the inhibitory effect of ICA on the phenotypes of chondrocytes. (A) CCK-8 assay was conducted to evaluate C-28/I2 cell viability after transfection of miR-27a-3p inhibitors. (B) Analysis by flow cytometry of C-28/I2 cell cycle distribution after transfection with miR-27a-3p inhibitors. (C) TUNEL assay was carried out to assess C-28/I2 cell apoptosis after transfection of miR-27a-3p inhibitors. (D) Detection via Transwell assay of C-28/I2 cell migration after transfection of miR-27a-3p inhibitor. (E,F) Detection by ELISA of the levels of proinflammatory factors in C-28/I2 cells after transfection with miR-27a-3p inhibitor. (G,H) Western blotting was conducted to determine the expressions of ECM-associated proteins in C-28/I2 cells after transfection of miR-27a-3p inhibitor. ns. $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Discussion

OA is a progressive joint disease, and current drugs can only relieve pain but cannot cure OA [25]. Recent studies have revealed that OA pathogenesis is highly correlated with chondrocyte injury, inflammation, and imbalance of ECM degradation and production [26]. Therefore, there is a need to find new drugs to inhibit the progression of OA. ICA is an isoprenoid flavonol glycoside extracted from *Epimedium*, which has antioxidant, anti-neuroinflammatory and anti-apoptotic effects, and has been shown to exert anti-inflammatory and anti-osteoporotic effects on bone diseases [27]. ICA regulates the TDP-43 signaling pathway to inhibit chondrocyte apoptosis and angiogenesis [4]. For example, ICA can inhibit the inflammation of rat chondrocytes and increase collagen synthesis in cells by regulating the NLRP3/caspase-1 signaling pathway, thus reducing chondrocyte injury and ameliorating OA devel-

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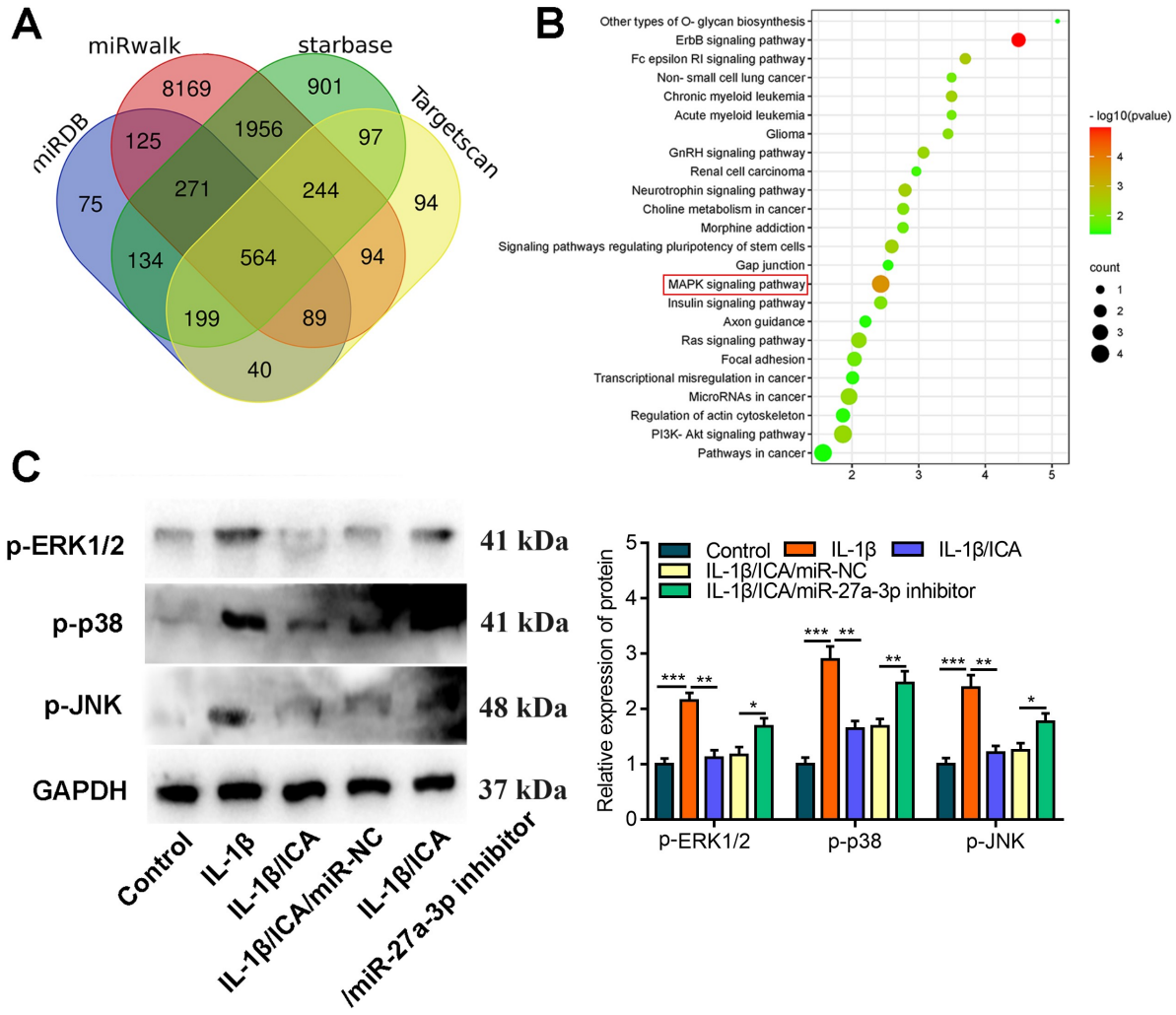

Fig. 5. ICA regulates the expressions of MAPK signal pathway-associated proteins via miR-27a-3p. (A) The miRWalk, starBase, miR-DB, TargetScan databases were employed to predict miR-27a-3p's downstream target genes and to plot the Venn diagram. (B) KEGG enrichment analysis of intersecting target genes in the Venn diagram. Horizontal coordinates indicate gene proportions and vertical coordinates indicate the enriched pathways. (C) Western blotting was employed to examine the expressions of p-p38, p-ERK1/2, and p-JNK proteins in C-28/I2 cells after transfection with miR-27a-3p inhibitors. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. DB, Database; KEGG, Kyoto encyclopedia of genes and genomes; p-ERK1/2, phosphorylated MAPK1 and MAPK2; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

opment in rats [28]. In this work, we found that ICA significantly facilitated chondrocyte viability and migration, as well as restrained apoptosis, inflammatory response and ECM degradation, which conforms to the findings of the previous studies.

Previous studies have demonstrated that lncRNA NEAT1 is linked to inflammatory diseases. For instance, NEAT1 can promote pathological changes such as corneal neovascularization by targeting miR-1246 to promote NF-κB-mediated inflammatory responses [29]. In epilepsy, NEAT1 promotes IL-1β-induced inflammatory responses by suppressing miR-129-5p and modulating the Notch signaling pathway [30]. In sepsis, knockdown of NEAT1 can inhibit LPS-induced inflammatory response and oxidative stress through repressing the TLR2/NF-κB signaling pathway, which in turn ameliorates myocardial injury [31]. It is found that NEAT1 is strongly associated with OA progression [14,32,33]. Specifically, NEAT1 is found to be up-regulated in OA tissues and can boost chondrocyte viability by down-regulating miR-16-5p [32]. Another study reports that NEAT1 is high-expressed in OA chondrocytes and tissues, and modulates chondrocyte multiplication and apoptosis by targeting the miR-543/PLA2G4A axis and the miR-193a-3p/SOX5 axis [14,33]. This work also discov-

ered that NEAT1 was upregulated in IL-1β-induced chondrocytes, whereas ICA treatment decreased NEAT1 expression.

Additionally, miR-27a-3p reportedly plays a crucial role in various diseases. MiR-27a-3p inhibits bronchiectasis development via Smad3 [34]. MSC-derived extracellular vesicles can transfer miR-27a-3p to alveolar macrophages to relieve acute lung injury [35]. Importantly, miR-27a-3p expression is found to be down-regulated in OA cartilage tissue, and IL-1β-mediated MAPK and NF-κB pathways inhibit miR-27a-3p expression [36]. This study revealed that miR-27a-3p was NEAT1's downstream target; miR-27a-3p was downregulated in IL-1β-induced chondrocytes and miR-27a-3p inhibition reversed in part ICA's effects on chondrocytes. The MAPK signaling pathway comprises JNK, ERK, p38 and so on, which can be activated by proinflammatory factors such as IL-1β and TNF-α in response to extracellular stress, resulting in increased phosphorylation levels of ERK, JNK and p38 proteins [37]. Targeting MAPK signaling is believed to block the progression of OA. Specifically, KLF11 is found to inhibit oxidative stress, apoptosis and endoplasmic reticulum stress in chondrocytes via suppressing the MAPK pathway, thus inhibiting OA progression [38]. Also, it has been shown that gardenia glycosides exert anti-inflammatory effects on OA through inhibiting the MAPK signaling pathway activation [39]. MiR-142-5p is found to inhibit SDF-1-induced chondrocyte apoptosis and ECM degradation by targeting CXCR4 and restraining the MAPK signal pathway [40]. Our research found that ICA downregulated the expressions of MAPK pathway-associated proteins, and that miR-27a-3p inhibitor partially counteracted the inhibitory impact of ICA on the expressions of MAPK signaling pathway-related proteins. Collectively, we suppose that ICA can protect chondrocytes via regulating the MAPK signaling pathway through miR-27a-3p.

Conclusions

To sum up, ICA protects chondrocytes through regulating the NEAT1/miR-27a-3p/MAPK axis. Our study offers a theoretical foundation for the clinical application of ICA in treating OA and other bone diseases. Notably, there exist several limitations in the present work. Firstly, the regulatory effects of ICA on NEAT1/miR-27a-3p/MAPK needs to be validated with an *in-vivo* model. Secondly, other downstream miRNA targets of NEAT1 wait to be explored.

Availability of Data and Materials

The data used for supporting the findings of this study are available from the corresponding author upon request.

Author Contributions

QW, ML, CZ and LX designed the research study. QW and JD performed the research. QW and LX analyzed the data. QW, ML and CZ drafted the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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