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# Role of the ciRS-7/miR-7 axis in the regulation of proliferation, apoptosis and inflammation of chondrocytes induced by IL-1β

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ABSTRACT

Osteoarthritis (OA) is a degenerative joint disease caused by articular cartilage degradation and joint inflammation, with considerable involvement of microRNAs and circular RNAs. However, the precise role of the ciRS-7/miR-7 axis within OA still requires further elucidation. In this study, quantitative reverse-transcription PCR (qRT-PCR) was utilized to determine the relative expression of ciRS-7 and miR-7 in blood samples from OA patients compared with those from healthy individuals. Human OA chondrocytes (C28/12 cell line) were transfected with ciRS-7-siRNA, ciRS-7-cDNA, inhibitor or miR-7 mimic to investigate the influence of ciRS-7/miR-7 expression on chondrocyte apoptosis, inflammation and related signaling pathways. Decreased ciRS-7 expression and increased miR-7 expression were observed in OA blood samples. IL-1β exposure of chondrocytes significantly inhibited proliferation and promoted inflammatory cytokine release. ciRS-7 was down-regulated but miR-7 was up-regulated in IL-1β-induced chondrocytes. Transfection of ciRS-7 siRNA and miR-7 mimic enhanced the impact of IL-1β on inflammatory cytokine release and cell apoptosis as quantified using ELISA and flow cytometry. Conversely, ciRS-7 cDNA and miR-7 inhibitor induced the reverse effect. These findings demonstrate that the ciRS-7/miR-7 axis can possibly serve as a regulator in mediating proliferation, apoptosis and inflammation in chondrocytes in the process of OA development.

## 1. Introduction

Osteoarthritis (OA) is a disabling degenerative joint disease that is the principal cause of incapacity in the elderly [1,2]. A number of pathological variations have been demonstrated in OA joints, including damage to articular cartilage and progressive devastation, thickening of the subchondral bone, extracellular matrix degradation, osteophyte formation, change in synovial inflammation, ligament deterioration, and hypertrophy of the knee menisci and joint capsule [3–5]. The etiology of OA is multifactorial, including mechanical, inflammatory, metabolic and genetic factors [6,7]. However, the molecular mechanisms regulating OA pathogenesis remain unclear and there are currently no effective interventions or therapies that slow or reverse OA progression [3]. As a result, an understanding of the molecular mechanisms in articular chondrocytes during OA progression is key to progressing the development of OA therapies.

Non-coding regions of DNA segments (e.g. lncRNA, miRNA and circRNA) that account for approximately 98% of the human genome, have recently been shown to participate in a diverse range of diseases [8–10]. As techniques such as RNA-Seq and bioinformatics analysis have progressed, an increasing number of circRNAs have been discovered, and the principle behind their formation and biological functions progressively revealed [11]. CircRNA is a novel, non-translated RNA comprising a covalently-closed ring structure, that is found in a variety of organisms [12]. The circRNA denoted ciRS-7, found in humans and also known as Cdr1as (cerebellar degeneration-related protein 1 antisense transcript), has been shown to be an endogenous competitive RNA inhibitor of miR-7 [13]. Furthermore, ciRS-7 has been found to have > 70 conserved binding sites that allows it to act as a "super sponge" of miR-7. It is abnormally expressed in many cancers and related to numerous "cancer-driven" pathways [14–16]. ciRS-7 acts as a controller of miR-7 via regulation of its activity in human diseases. In addition, miR-7 is involved in the pathogenesis of a number of disorders via regulation of downstream pathways. Studies have

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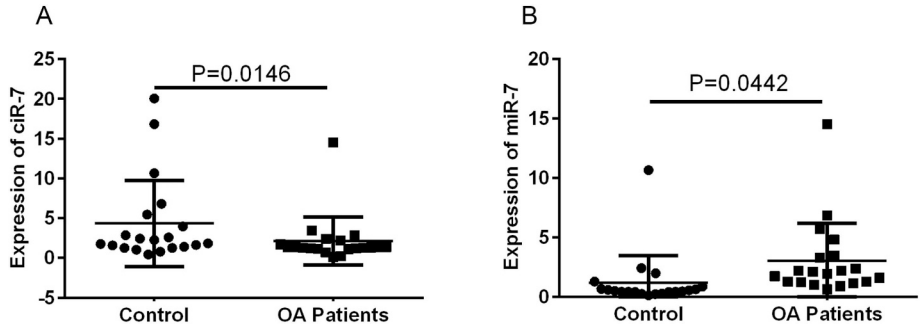


**Fig. 1.** Down-regulation of ciRS-7 and up-regulation of miR-7 in clinical blood samples. (A) ciRS-7 mRNA expression and (B) miR-7 mRNA expression in 20 OA and 20 healthy samples, as analyzed by qRT-PCR.

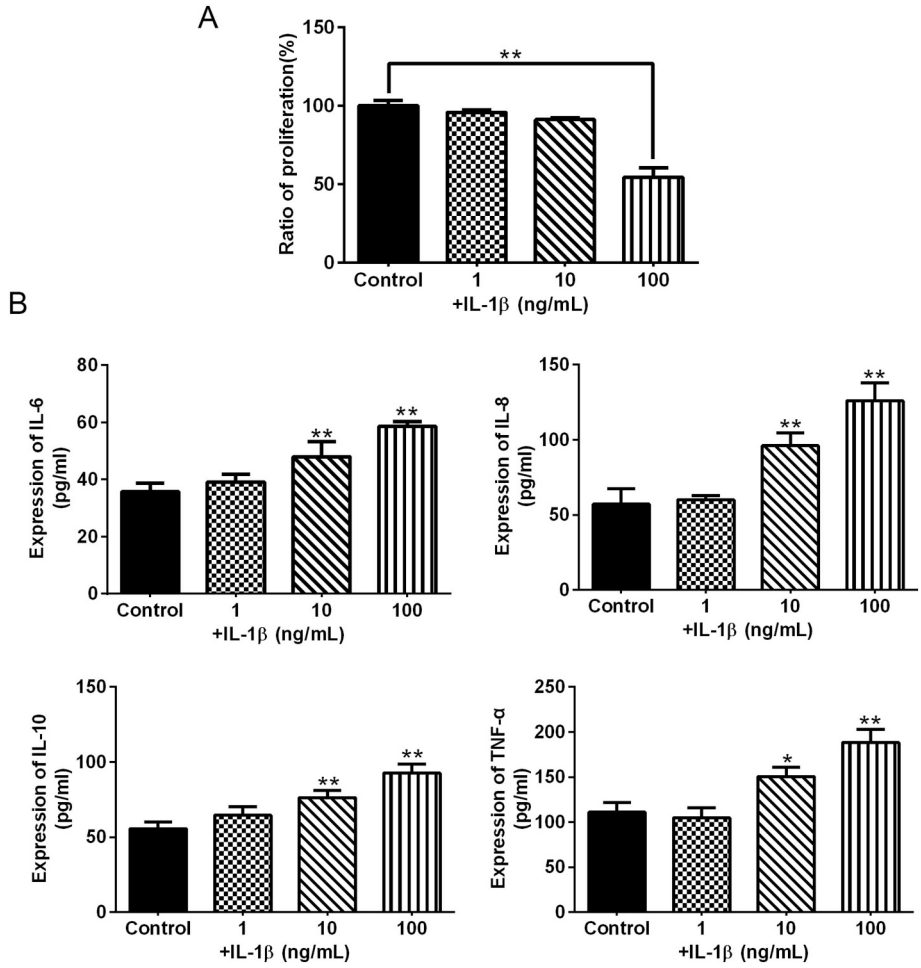


**Fig. 2.** IL-1β induced apoptosis and inflammation in chondrocytes. C28/I2 cells were cultured with various concentrations of IL-1β. (A) Influence of IL-1β on cell viability; (B) Influence of IL-1β on levels of inflammatory cytokines, including IL-6, IL-8, IL-10 and TNF-α. *P < 0.05; **P < 0.01 vs. control.

demonstrated that the ciRS-7/miR-7 axis could affect the expansion of the mesencephalon, down-regulate the expression of Alzheimer's disease-related target proteins such as ubiquitin-linked protein ligase A, and influence evolution, metastasis and the colony formation of malignant tumors [17–19]. Nevertheless, whether a correlation exists between ciRS-7 and miR-7 in osteoarthritis remains unclear.

To date, no studies that have investigated the specific characteristics of the ciRS-7/miR-7 axis in OA have been performed. This study aimed to reveal whether the ciRS-7/miR-7 axis might affect chondrocyte proliferation or apoptosis and thus contribute to the development of OA.

## 2. Material and methods

### 2.1. Collection of OA blood samples

20 OA blood samples and 20 healthy blood samples (n = 20) were obtained from Changzhou Second People's Hospital following approval by the institutional Research Ethics Committee. Patients were notified of the research objectives and consent was signed and obtained on each occasion. Blood samples were centrifuged through Ficoll at 1500 rpm for 20 min. The fraction containing monocytes was removed to a separate tube to which Trizol was added to isolate and extract RNA for

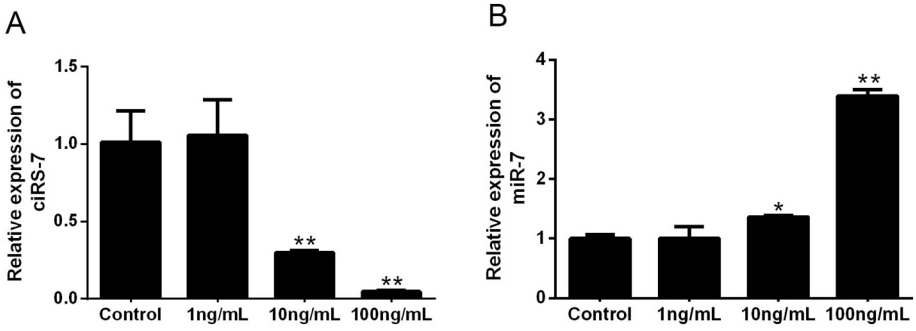


**Fig. 3.** Expression of (A) ciRS-7 and (B) miR-7 in chondrocytes in different experimental groups. *P < 0.05, and **P < 0.01 vs. control.

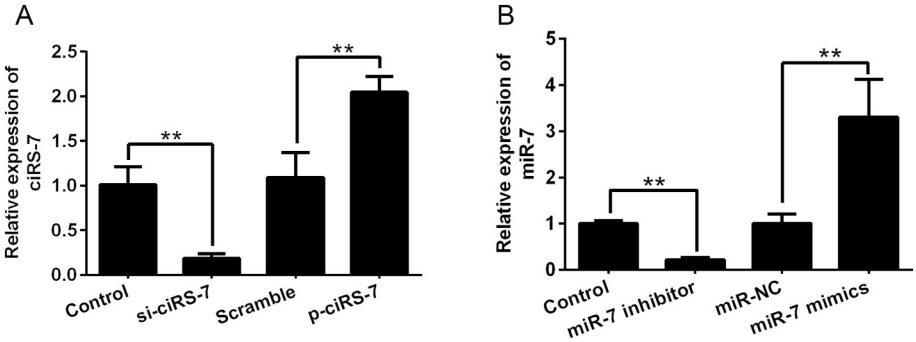


**Fig. 4.** Expression of (A) ciRS-7 and (B) miR-7 in chondrocytes with differential transfection. **P < 0.01 vs. control or scramble.

reverse transcription. Monocytes were isolated immediately following blood sample collection.

### 2.2. Extraction of RNA and quantitative reverse-transcription PCR (qRT-PCR)

Trizol reagent (Invitrogen) was added to blood or cells to extract all molecules of RNA, in accordance with the manufacturer's instructions. RNA was quantified using a NanoDrop ND-2000 microvolume spectrophotometer and gene and ciRS-7 expression analysis performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) and Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA). MicroRNA was reverse transcribed to obtain miRNA first strand cDNA synthesis (tailing reaction, Sangon Biotech, cat. No. B532451) in accordance with the manufacturer's guidelines. Quantitative RT-PCR was conducted using SYBR Green Master Mix (Applied Biosystems, A25780) to measure the expression of ciRS-7 and miR-7, with sncU6 used as an endogenous control. The primer sequences were: sncU6, forward: 5′-CTCGCTTCGGCAGCACA-3′, reverse: 5′-AACGCTTCACGAATTTGCGT-3′; miR-7, forward: 5′-GCAGTGAGGTAGTAGGTTG-3′, reverse: 5′-GGTCCAGTTTTTTTTTTTTTTTAACTATAC-3′; ciRS-7, forward: 5′-ACGTCTCCAGTGTGCTGA-3′, reverse: 5′-CTTGACACAGGTGCCATC-3′; Bcl-2, forward: 5′-CAGGAAACGGCCCGGAT-3′, reverse: 5′-CTGGGGCCTTTCATCCTCC-3′; Bax, forward: 5′-GGGTTGTCGCCCTTTTCTAC-3′, reverse: 5′-CTGGAGACAGGGACATCAGT-3′; Caspase-3, forward: 5′-TGCTATTGTGAGGCGGTTGTAG-3′, reverse: 5′-GGCACACCCACCGAAAAC-3′.

### 2.3. Establishing circRNA and miRNA plasmids and transfection

Human ciRS-7 cDNA was prepared and replicated into a pcDNA3.1 expression vector, based on the description of Hansen et al. [20]. Only a 562-bp DNA fragment was inserted, with subsequent insertion of 1 kb upstream and 200 bp downstream of the nonlinear splice sites. Similarly, an 800-bp DNA extension was placed upstream of the splice acceptor site and inserted downstream in the opposite orientation. Inhibitor and negative control (NC) miRNAs, miR-7 mimics and ciRS-7 siRNAs were purchased from Drom GenePharma (China). Cells were transfected with plasmids (2 ng/mL), siRNAs (50 nM) or miRNAs (50 nM) using Lipofectamine 2000 (Thermo Fisher Scientific) in accordance with the manufacturer's instructions.

### 2.4. Cell lines and cell culture

C28/I2 chondrocytes were cultured and stimulated with a range of concentrations of IL-1β to simulate the OA environment. C28/I2 cells were cultivated in DMEM (HyClone) supplemented with 1% penicillin/streptomycin (HyClone) and 10% fetal bovine serum (FBS, Gibco) in an atmosphere containing 5% $CO_2$ at a temperature of 37 °C. The C28/I2 cells were stimulated by the addition of 1, 10 and 100 ng/mL of IL-1β in the culture medium for 12 h. Chondrocytes without stimulation acted as controls.

### 2.5. MTT assay

Into each well of a 96-well plate, 100 μL of a suspension of 5000 C28/I2 cells were added, then cultured in 5% $CO_2$ at 37 °C. MTT dye (10 μL of 2.5 mg/mL in PBS) was added to the wells then incubated for 4 h. The supernatants were then removed and 150 μL DMSO added. The optical density (OD) of each well was measured using a microplate reader (Thermo, USA) at a wavelength of 570 nm. The inhibition rate (IR) of cell proliferation was calculated according to the following formula: $IR = (OD\ value_{control\ well} - OD\ value_{testing\ well}) / OD\ value_{control\ well} * 100\%$.

### 2.6. ELISA assay

The levels of TNF-α, IL-6, IL-8 and IL-10 in the culture medium of the C28/I2 cells induced by IL-1β were quantified using ELISA kits (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's instructions.

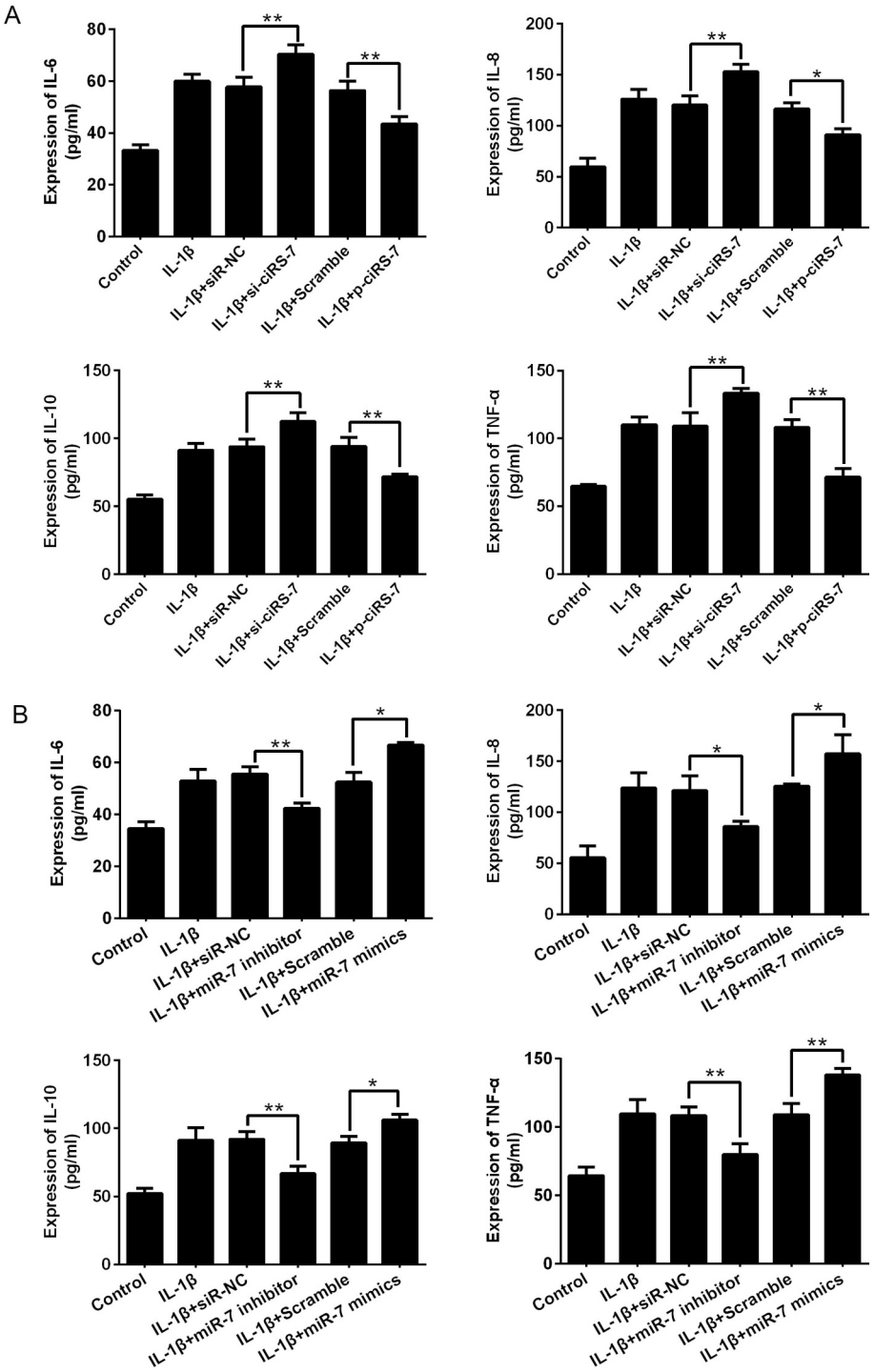


**Fig. 5.** Effects of (A) ciRS-7 and (B) miR-7 axis on inflammatory cytokines in different experimental groups. *P < 0.05, **P < 0.01 vs. control or scramble.

### 2.7. Annexin V/propidium iodide (PI) staining

Cell apoptosis was examined using a FITC-labeled Annexin V (Annexin V-FITC) apoptosis measurement kit. After 48 h of transfection, cells were harvested and washed twice with ice-cold PBS, then re-suspended in 1× binding buffer and stained in the dark with Annexin V-FITC and PI. The proportion of cells that were apoptotic was calculated using flow cytometry in accordance with the manufacturer's instructions.

### 2.8. Western blot analysis

Western blot analysis was conducted following standard techniques in the literature. Total protein was extracted from cell lysates and the quantity in each specimen tested using a BCA Kit (Beyotime Biotechnology, Nanjing, China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate the proteins, which were then transferred to PVDF membranes. The PVDF membranes were blocked with 5% skim milk for 2 h then incubated with the following

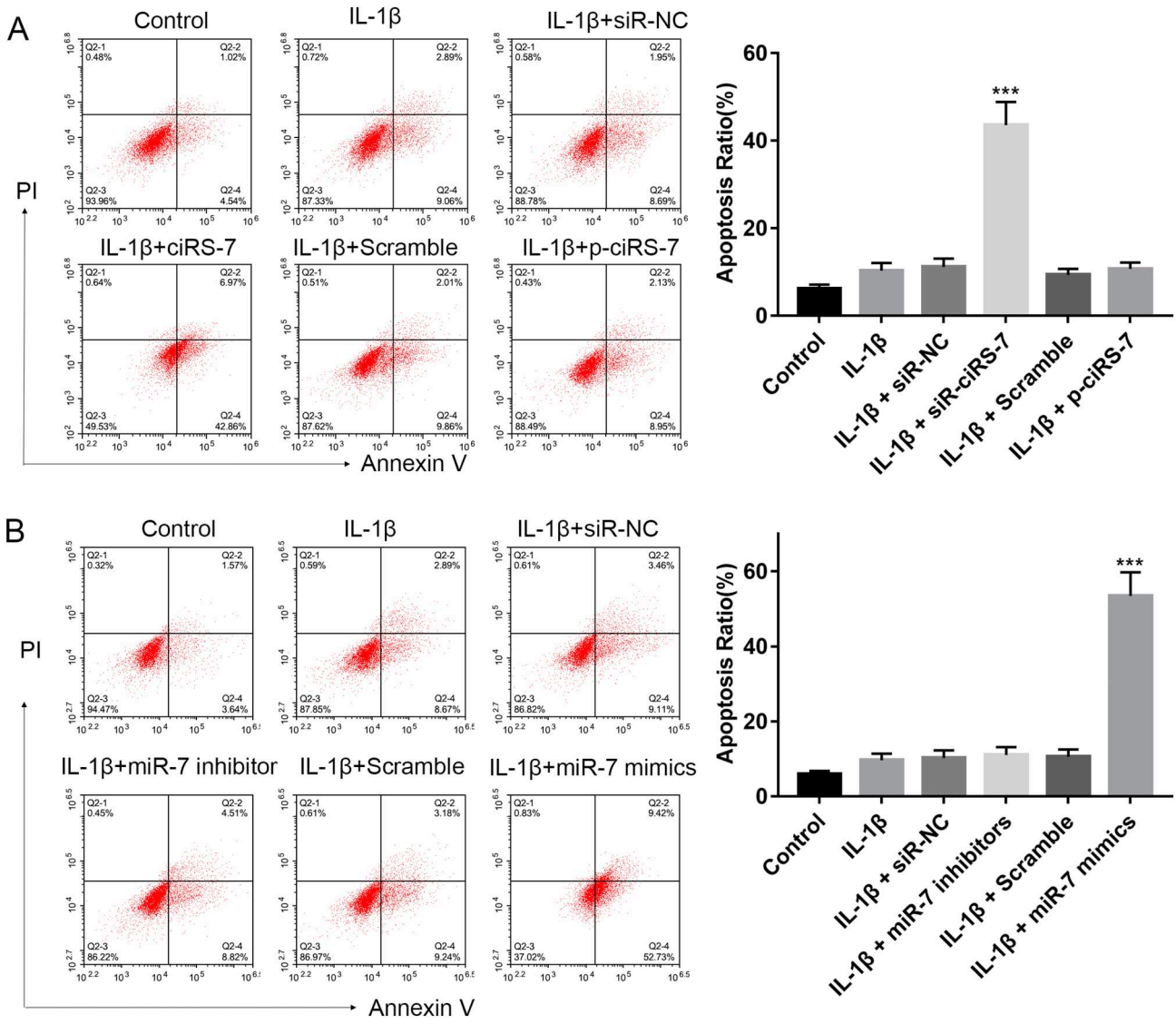


**Fig. 6.** Effects of (A) ciRS-7 and (B) miR-7 expression on apoptosis in different experimental groups as measured by flow cytometry. ***P < 0.001 vs. control for three experiments.

primary antibodies, all sourced from Abcam: anti-Bcl-2 (cat. no. ab182858, dilution 1:2000), anti-Caspase-3 (cat. no. ab4051, dilution 1:500), anti-active caspase-3 antibody (cat. no. ab32042, dilution 1:1000), anti-caspase-9 (cat. no. ab2013, dilution 1:1500), anti-active caspase-9 antibody (cat. no. ab2324, dilution 1:2000) overnight. The PVDF membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. ab205718, dilution 1:2000) and the protein bands visualized using ECL. All results were conducted in triplicate.

### 2.9. Statistical analysis

The assays were conducted and studied in triplicate. Data were analyzed using SPSS17.0 statistical software. The results are represented as means ± SD. Analysis of two groups was achieved using a *t*-test. The statistical significance of variances from multiple groups was calculated using analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Decreased expression of ciRS-7 and increased expression of miR-7 was detected in OA patients

To evaluate the difference in ciRS-7 and miR-7 expression in healthy subjects and OA patients, quantitative reverse-transcription PCR analysis of blood samples was conducted, 20 in each group. The results in Fig. 1 reveal that the expression of ciRS-7 was significantly down-regulated in OA patients compared to healthy subjects, while miR-7 was up-regulated ($P = 0.0146$ and 0.0442, respectively).

### 3.2. IL-1β exposure inhibited proliferation and promoted inflammation in chondrocytes

C28/I2 chondrocytes were stimulated using different concentrations of IL-1β to simulate the OA environment. From the MTT results in Fig. 2A, we found that cell proliferation decreased after treatment with IL-1β, inhibition occurring in a concentration-dependent manner,

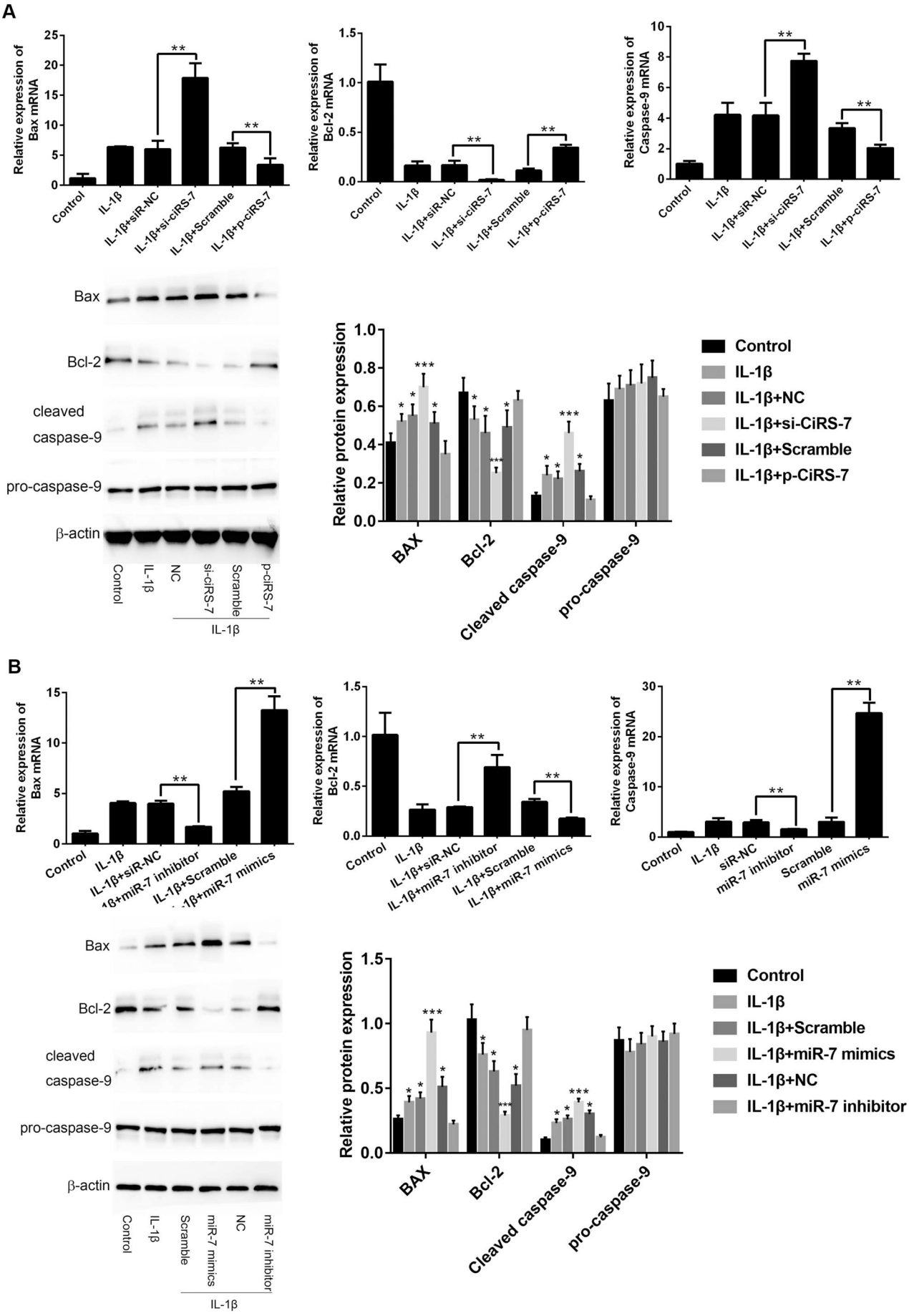


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**Fig. 7.** Effect of (A) ciRS-7 and (B) miR-7 axis on BAX, Bcl-2, cleaved caspase-9 and pro-caspase-9 expression as determined by qRT-PCR and Western blot analysis in 10 ng/mL IL-1β-induced chondrocytes. For relative protein expression, *P < 0.05, **P < 0.01, ***P < 0.001 vs. control for three experiments.

although a concentration of 10 ng/mL had no inhibitory effect on cell viability. After treatment with 10 ng/mL IL-1β, secretion of the pro-inflammatory cytokines TNF-α, IL-6, IL-8 and IL-10 increased (Fig. 2B). As a result, 10 ng/mL IL-1β was selected for subsequent studies.

It can be seen from Fig. 3 that the expression of ciRS-7 in chondrocytes induced by IL-1β (at concentrations of 10 and 100 ng/mL) declined significantly compared to that of the control ($P < 0.05$), while the opposite was true for miR-7 expression. 10 ng/mL IL-1β caused the same trend in ciRS-7 and miR-7 expression as observed in the blood of OA patients.

### *3.3. Impact of the ciRS-7/miR-7 axis on chondrocyte inflammation induced by IL-1β*

The previous experimental results suggested that 10 ng/mL IL-1β was suitable for simulating an OA environment. Hence, the influence of the ciRS-7/miR-7 axis on OA was conducted with chondrocytes induced by 10 ng/mL IL-1β. Firstly, isolated chondrocytes were transfected with ciRS-7-siRNA or p-ciRS-7 for ciRS-7, and miR-7 mimic or inhibitor for miR-7. As shown in Fig. 4, the expression of ciRS-7 and miR-7 changed significantly after transfection, demonstrating that the procedure was successful.

The results in Fig. 5A reveal that a down-regulation of ciRS-7 expression promoted the release of inflammatory cytokines (IL-6, IL-8, IL-10 and TNF-α), while miR-7 exhibited the opposite effect (Fig. 5B).

### *3.4. Impact of the ciRS-7/miR-7 axis on chondrocyte apoptosis induced by IL-1β*

Whether the ciRS-7/miR-7 axis had an effect on cell apoptosis was ascertained by flow cytometry. Reduced ciRS-7 and increased miR-7 expression had pro-apoptotic effects on chondrocytes induced by 10 ng/mL IL-1β compared with their respective controls. As evident in Fig. 6A–B, the percentage of apoptotic cells increased considerably in the IL-1β + si-ciRS-7 ($P < 0.05$) and IL-1β + miR-7 mimic ($P < 0.05$) groups compared with the corresponding scramble group treated with IL-1β, whereas apoptosis decreased significantly in chondrocytes with elevated ciRS-7 and suppressed miR-7 expression ($P < 0.05$).

The concentrations of apoptosis-associated proteins including Bcl-2, Bax and Caspase-9 were measured in each group of treatments by qRT-PCR and Western blot analysis. From Fig. 7A and B, up-regulation of ciRS-7 suppressed BAX mRNA and protein expression and cleaved caspase-9 in OA model cells, while Bcl-2 expression was promoted. Down-regulation of miR-7 resulted in an influence similar to that of up-regulation of ciRS-7.

## 4. Discussion

The discovery of crucial molecules and regulatory pathways of key genes connected with the initiation and evolution of OA promises great potential for preventing joint devastation and stimulating repair in the future. These molecular mechanisms, which govern the protein levels related to OA, are potential tools for possible therapeutic intervention [21,22].

Changes in the expression of miRNAs were related to well-known clinicopathological structures and disease findings. The central function of miRNAs in biological progression has been previously recognized [23]. Thus, the various key factors in OA progression should also be explored. It has been recognized that ncRNAs (e.g. lncRNA, circRNA and miRNA) change biological processes via modification of DNA structures, RNA transcription and protein translation [24]. In fact, a number of circRNAs compete with endogenous RNA (ceRNA) for regulation of gene expression [25]. They also directly bind to target miRNAs to inhibit their expression levels. In addition, circRNA may also be an important class of posttranscriptional regulatory factors that can modify the levels of other RNAs and protein activity, acting as templates for protein synthesis, thereby reducing the ability of target genes to influence the progress of disease [26]. This study aimed to ascertain the impact of the ciRS-7/miR-7 axis on inflammation and apoptosis in OA model cells. Additionally, we analyzed apoptosis-related proteins in OA cells.

A natural antisense transcript of cerebellar degeneration-associated protein 1 (CDR1as, also known as ciRS-7), consisting of approximately 70 metal-responsive elements (MREs) is miR-7, a recently-discovered circular RNA associated with human disease [27]. Studies have found that CDR1as, together with miR-7, may play a role in the pathogenesis of cancers and Alzheimer's disease [28,29]. However, few studies have linked ciRS-7 with miR-7 in research studies associated with OA.

In this study, the expression of ciRS-7 and miR-7 was first quantified in the plasma of OA patients and healthy subjects, which indicated that the expression of ciRS-7 was significantly lower, and that of miR-7 significantly higher in OA patients than in healthy subjects. The ciRS-7/miR-7 axis possibly participates functionally in the incidence and progression of OA. It has been established that 10 ng/mL IL-1β can stimulate an environment similar to that of OA [30]. Under these conditions, exposure to IL-1β in chondrocytes was shown to inhibit proliferation and promote inflammatory cytokine release and cell apoptosis. Furthermore, experiments that investigated the function of ciRS-7 and miR-7 in OA model cells were performed. However, the response of miR-7 was contradictory to that of ciRS-7 regarding the factors studied. Inflammatory factors were significantly elevated after down-regulation of ciRS-7 and up-regulation of miR-7, while a pro-apoptotic effect was inhibited by high expression of ciRS-7 and low expression of miR-7 in chondrocytes stimulated by IL-1β. Further investigation of apoptosis-related proteins [31], the results demonstrated that Bcl-2, Bax and cleaved caspase-9 exhibited a trend in expression similar to that of the apoptosis results. Up-regulation of ciRS-7 lead to a suppression of BAX and cleaved caspase-9 mRNA and protein expression in model OA cells, with concurrent promotion of Bcl-2 expression. Down-regulation of miR-7 resulted in a similar response to up-regulation of ciRS-7.

In summary, OA patients were found to have reduced expression of ciRS-7 and increased expression of miR-7. The ciRS-7/miR-7 axis has a regulatory effect on apoptosis and inflammation in chondrocytes induced by 10 ng/mL IL-1β as OA model cells. Furthermore, knockdown of ciRS-7 and upregulation of miR-7 promoted inflammation and apoptosis in OA chondrocytes, indicating that p-ciRS-7 and si-miR-7 may act in a protective capacity in cartilaginous tissues. More importantly, this is the first time that the effect of the ciRS-7/miR-7 axis on inflammation and apoptosis in OA model cells has been revealed, which may provide novel insights into treatments for OA, the further mechanisms of which will be investigated in future studies.

## Author contributions

Xindie Zhou, Jin Li and Lifeng Jiang carried out the main experiments and statistical analysis and prepared the manuscript. Haoyu Yang, Guoming Fan and Lidong Wu designed the study and prepared the manuscript. Xindie Zhou wrote the main protocol and prepared the manuscript. Yong Huang and Nanwei Xu supervised the study and prepared the manuscript. All authors contributed to and have approved the final manuscript.

## Conflict of interest

The authors declare that they have no competing interests.

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