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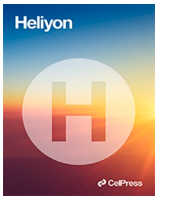

Research article

# CircRELL1 promotes osteoarthritis progression by regulating miR-200c-3p

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ABSTRACT

*Background:* There is a growing body of evidence indicating a potential association between circular RNA and the pathogenesis of human osteoarthritis (OA). Nevertheless, the precise extent of their involvement in OA remains largely unexplored. Hence, the objective of this investigation is to elucidate the function of Circular (Circ) RELL1 in the context of OA.
*Methods:* 24 OA tissue samples and 11 normal tissue samples were collected. The inflammatory OA-like conditions were established by Destabilized Medial Meniscus (DMM) operation in mice and LPS-induced C28/I2 cells. OA severity and articular cartilage degradation were assessed by Safranin-O staining, hematoxylin-eosin (H&E) staining, and International Society for Osteoarthritis Research (OARSI) criteria. CircRELL1, miR-200c-3p, and TCF4 were measured by RT-qPCR and Immunoblot. The cell viability and apoptosis rate were measured by MTT and flow cytometry, respectively. The levels of cytokines interleukin (IL)-1β, IL-6, and TNF-α were determined by ELISA. Apoptosis-associated proteins (cleaved caspase-3, Bax, and Bcl-2) and extracellular matrix (ECM) degradation-associated proteins (MMP13, collagen II, and Aggrecan) were detected by Immunoblot. The interaction between miR-200c-3p and circRELL1 or TCF4 was verified by dual luciferase reporter assay and RIP assay.
*Results:* CircRELL1 expression was upregulated in OA patients, and the results were consistent in DMM mice and LPS-treated C28/I2 cells. Silencing circRELL1 improved cartilage injury caused by DMM and contributed to a lower OARSI score. Silencing CircRELL1 increased the activity of OA chondrocytes *in vivo* and *in vitro* and inhibited cellular inflammatory responses and ECM degradation. In terms of mechanism, circRELL1 functioned by targeting miR-200c-3p, leading to the suppression of inflammatory factor production, cell apoptosis, and ECM degradation, thus inhibiting the progression of OA.
*Conclusion:* CircRELL1 may promote the progression of OA by regulating the miR-200c-3p.

## 1. Introduction

Osteoarthritis (OA) is a chronic degenerative disease of articular cartilage characterized by chronic inflammation, progressive destruction of articular cartilage, and subchondral osteosclerosis [1–3]. Chondrocytes are the only resident cells in the joint system and

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undergo a variety of changes during OA. Abnormal apoptosis and inflammatory response of chondrocytes are closely related to extracellular matrix (ECM) and OA progression [4]. At present, the exact pathogenesis of OA is still uncertain, and its therapeutic effect is limited [5,6]. Therefore, elucidating the potential molecular mechanism of OA is of great significance to search for effective targets and therapeutic strategies.

Circular RNA (circRNAs) are a class of non-coding RNAs that possess a closed covalent ring structure, resulting from the reverse splicing of precursor messenger RNA. These molecules are extensively expressed in mammals and demonstrate remarkable conservation, stability, and tissue-specific properties [7]. An increasing body of evidence indicates that circRNAs have a significant impact on cellular physiology, serving as miRNA sponges, transcription regulators, RNA-binding protein binding molecules, protein translation templates, and immune regulatory factors [8]. Previous studies have demonstrated that circRNAs function as miRNA decoys, thereby modulating the expression of target genes and impacting OA through their involvement in ECM degradation, inflammatory response, cellular apoptosis, and intracellular signaling pathways [9]. For example, circRNA_0092516 regulates chondrocyte differentiation and apoptosis by mediating miR-337-3p [10]. circSERPINE2 reduces IL-1β-induced chondrocyte apoptosis and ECM degradation by decoying miR-495 [11]. There is a limited amount of literature available regarding circRELL1, with hsa_Cir_0002194 being identified as a derivative of the RELL1 gene. In an investigation concerning the regulation of endothelial cells by circRELL1, hsa_Cir_0002194 functions as a miR-687-3p-κ B-axis sponge, thereby exerting control over MyD88/NF and subsequently influencing the progression of inflammation [12]. The study speculates that circRELL1 may also be involved in OA inflammation. Therefore, this study explored the biological function of circRELL1 in OA.

Recent studies have confirmed that miRNA is also involved in the progression of OA [13,14]. Among them, miR-200c-3p can induce chondrocyte hypertrophy, transform chondrocytes from a static state to a proliferative state, and affect cell differentiation [15]. In addition, plasma miR-200c-3p can be used as a potential diagnostic biomarker for knee OA [16]. Transcription factor 4 (TCF4) is a key risk gene on human chromosome 18 [17] and it can promote the progression of OA [18,19].

This study explored the biological function of circRELL1 in OA and its potential molecular mechanism related to miR-200c-3p. Here, inflammatory OA-like conditions *in vitro* and *in vivo* were established by using LPS-induced C28/I2 cells and mouse Destabilized Medial Meniscus (DMM) surgery, providing new targets and strategies for the clinical treatment of OA.

## 2. Materials and methods

### 2.1. Patient sample

Tissue samples from 24 patients diagnosed with OA who underwent total knee replacement and 11 healthy patients after trauma were collected and stored at −80 °C immediately. Informed consent was obtained from all patients for the study. This study was approved by the Ethics Committee of Shanghai Songjiang District Central Hospital (NO. 2016SJ3016).

### 2.2. Destabilized Medial Meniscus (DMM) model and treatment

As previously mentioned [20], the OA model was established by DMM surgery on 22–25 g male C57BL/6 mice aged 8 weeks. In brief, 20 mice were randomly divided into four groups: Sham, DMM, DMM + LV-sh-NC, DMM + LV-sh-circRELL1. After anesthesia by intraperitoneal injection of 40 mg/kg pentobarbital, mice underwent transection of the medial meniscotibial ligament of the right posterior limb, resulting in medial meniscus displacement.

Short hairpin RNA (shRNA) specifically targeting circular RELL1, as well as interfering shRNA (Sh NC) was obtained from Shanghai Genechem (Genechem Incorporation, Shanghai, China). Subsequently, these shRNAs were incorporated into lentiviral particles. The Sham-operated mice underwent excision of the skin of the right knee joint without damaging the ligaments. One week after DMM operation, lentivirus-packaged sh-circRELL1 and sh-NC (5 μL, $1 \times 10^9$ PFU) were injected into the knee joint in the DMM + LV-sh-circRELL1 and DMM + LV-sh-NC groups. Mice in the Sham group and DMM group were injected with 0.9 % normal saline in the same amount [21]. The injection was given once a week for three weeks. The mice were euthanized after the third injection, and the knee tissues were collected for histological analysis. All animal experiments were approved by Shanghai Songjiang District Central Hospital Animal Ethics Committee (NO.2016SJ3022).

### 2.3. Cell culture and treatment

Human normal chondrocytes C28/I2 (Cell Bank of Chinese Academy of Sciences) were cultured with PMI-1640 (Gibco-BRL, USA) containing 10 % FBS (Gibco-BRL, USA) at 37 °C and 5 % $CO_2$. The inflammatory OA-like conditions was established by stimulating C28/I2 cells with 5 μg/mL LPS (Sigma, USA) for 12 h [22]. Following this, the aforementioned cells were utilized for the purpose of conducting cell function assessments, encompassing the evaluation of cell proliferation activity through MTT detection, the identification of cell apoptosis via flow cytometry detection, and the detection of inflammatory factor release by cells through enzyme-linked immunosorbent assay (ELISA).

### 2.4. Cell transfection

si-circRELL1, oe-TCF4, and miR-200c-3p mimic/inhibitor, and their negative controls (si-NC, vector, mimic NC, and inhibitor NC) were synthesized in GenePharma (Shanghai, China). Corresponding oligonucleotides and plasmids were transfected using

Lipofectamine 2000 (Invitrogen, USA). A new medium was replaced 8 h after transfection and cells were obtained after 48 h and assessed by RT-qPCR and Immunoblot to verify transfection efficiency.

### 2.5. Real time quantitative PCR (RT-qPCR)

Total RNA was extracted from cartilage tissues and chondrocytes using TRIzol® reagent (Invitrogen). Total RNA concentration and quality were measured using NanoDrop 2000. Reverse transcription was conducted using Superscript II (Invitrogen), followed by PCR assay using SYBR Green Universal Master Mix reagent (Roche, USA). U6 and GAPDH were considered internal references, and the relative expression was calculated by $2^{-\Delta\Delta Ct}$. Primers are shown in Table 1.

### 2.6. Dual luciferase reporter assay

According to starbase3.0 (https://starbase.sysu.edu.cn/), miR-200c-3p binding sites with circRELL1 or TCF4 were predicted. The 3′untranslated region (UTR) sequences of circRELL1 and TCF4 wild and mutant types were inserted into the pmirGLO vector (Promega, USA) to construct WT-circRELL1, MUT-circRELL1, WT-TCF4 and MUT-TCF4. The above plasmids and miR-200c-3p mimic or mimic NC were transfected into C28/I2 cells using Lipofectamine 2000 (Invitrogen). Luciferase activity was assessed after 48 h using a dual luciferase reporter assay kit (Promega) and detected with Synergy 2 Multidetector Microplate Reader (BioTek Instruments Inc. USA).

### 2.7. RNAse R

After extracting total RNA from C28/I2 cells, 10 μg sample was co-incubated with RNAse R (3 U/g, Epicentre Technologies, USA) at 37 °C for 30 min and assayed for circRELL1 and circRELL1 mRNA stability using RT-qPCR.

### 2.8. Actinomycin D experiment

C28/I2 cells ($4 \times 10^5$) were plated into a 6-well plate, treated with 2 μg/mL actinomycin D (MCE, HY-17559), and harvested at 0, 4, 8, 12, and 24 h, respectively to measure circRELL1 and RELL1 mRNA by RT-qPCR.

### 2.9. Subcellular separation experiment

Subcellular separation experiment was performed using PARIS™ Kit (Life Technologies, USA). C28/I2 cells ($1 \times 10^6$) were suspended in the separation buffer and centrifuged to separate RNA from the nucleus and cytoplasm. Gene expression was detected by RT-qPCR, with U6 as the internal reference of the nucleus and GAPDH as that of the cytoplasm.

### 2.10. MTT experiment

The transfected C28/I2 cells were inoculated in 96-well culture dishes ($5 \times 10^3$ cells/well) and added with 10 % MTT solution (Guduo Biotechnology, Shanghai, China) for 4 h. The supernatant was collected and incubated with 100 μL dimethyl sulfoxide (Sigma) for 10 min and absorbance at 490 nm was read on the microplate reader (Thermo Fisher Scientific, USA).

### 2.11. Flow cytometry

Flow cytometry analysis was performed using the membrane associated protein V-FITC/PI cell apoptosis detection kit (Solarbio, CA1020). C28/I2 cells were washed twice with pre-cooled PBS and resuspended with 1 mL of 1 × binding buffer to achieve $1 \times 10^6$

**Table 1**
PCR primer sequences.

| Genes | Sequences (5′– 3′) |
|---|---|
| circRELL1 | F: GGCACAGAGTAGCAGCCC |
| | R: GTGACAAAGGCCCAGGACTC |
| miR-200c-3p | F: GCCTAATACTGCCGGGTAAT |
| | R: GCAGGGTCCGAGGTATTC |
| TCF4 | F: CGACTTCCCCTGACCTGAAC |
| | R: TTCTCACGCTCTGCCTTCTG |
| U6 | F: CTCGCTTCGGCAGCACA |
| | R: AACGCTTCACGAATTTGCGT |
| GAPDH | F: CACCCACTCCTCCACCTTTG |
| | R: CCACCACCCTGTTGCTGTAG |

Note: F, forward; R, reverse; circRELL1, circular RNA RELL1; miR-200c-3p, microRNA-200c-3p; TCF4, transcription factor 4; GAPDH, glyceraldehyde-3-phosphatedehydrogenase.

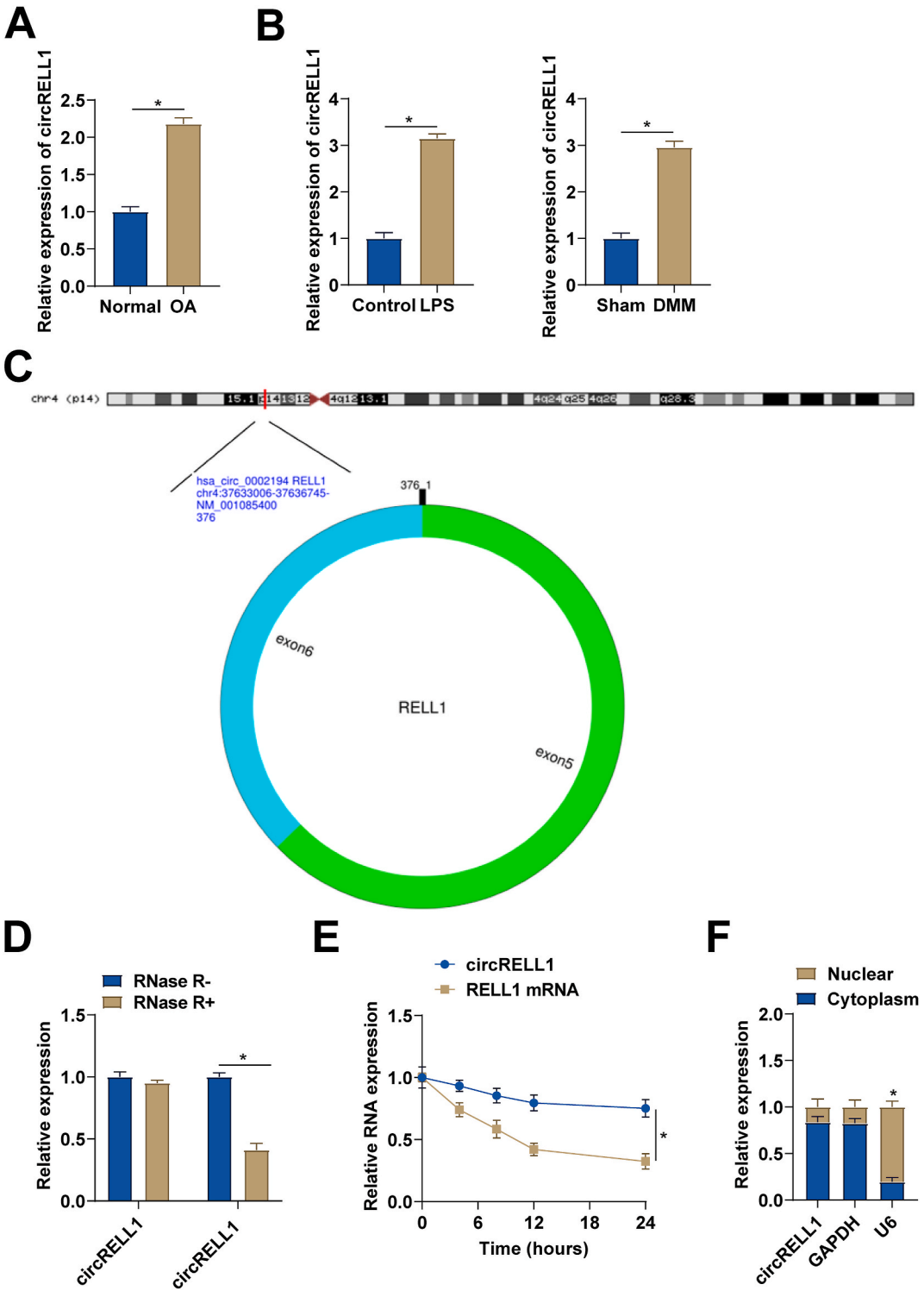


**Fig. 1.** circRELL1 is a circRNA with up-regulated expression in OA.
A: RT-qPCR measured circRELL1 in cartilage tissues of OA patients and healthy controls. B: RT-qPCR measured circRELL1 in LPS-induced C28/I2 cells and DMM mice. B: Basic information of circRELL1. D: RNAse R verifies the ring structure of circRELL1. E: Actinomycin D analyzes the stability of circRELL1. F: Subcellular isolation assayed expression position of circRELL1 in C28/I2 cells. Data were expressed as mean ± SD (N = 3), $P < 0.05$.

cells/ml. Then, 100 μl suspension was mixed with 5 μl Annexin V-FITC in the dark for 10 min and then with 5 μl PI solution for 5 min. The proportion of apoptotic cells was determined by FACScan flow cytometry (BD Biosciences).

### 2.12. ELISA

After a 48-h period of transfection, the cell supernatant were be collected. Subsequently, the corresponding ELISA kit (Beyotime, China) should be employed to detect the presence of IL-1β, IL-6, and TNF-α within the supernatant. The optical density at 450 nm (OD value = 450 nm) should be measured using ELISA reader (BioTek ， USA). To determine cell viability, the following formula should be utilized: % viability = (treatment group OD 450nm/negative control OD 450 nm) × 100 %.

### 2.13. Immunoblot

Total protein was extracted from chondrocytes or cartilage tissues using a pre-cooled RIPA cell lysis solution (Beyotime, China) and protein concentrations were determined using a BCA protein assay kit (Pierce, USA). The protein was isolated on 12 % SDS-PAGE and subsequently transferred to a PVDF membrane (Thermo Fisher Scientific) by electrophoresis. Blocked with 5 % BSA, the membrane was added with cleaved caspase-3 (1:1000, CST, USA), Bax (1:1000, CST), Bcl-2 (ab32124, Abcam, USA), MMP13 (1:2000, Abcam), collagen II (1:3000, Abcam), Aggrecan (1:1000, Abcam) and GAPDH (1:1000, Abcam) overnight at 4 °C and then with the corresponding secondary antibody for 1 h. The images were developed using the enhanced chemiluminescence kit (ultrassignal, China).

### 2.14. RNA immunoprecipitation (RIP)

RIP experiments were conducted using the Magna RIP Kit(Millipore). The cells were lysed with RIP lysis buffer, and the supernatant was mixed with an appropriate amount of Ago2 or IgG antibody bound to magnetic beads and co-incubated at 4 °C for 6 h. The bound immunoprecipitates were eluted with elution buffer and analyzed by RT-qPCR.

### 2.15. Histological examination

Cartilage tissues were fixed at 4 °C in 4 % paraformaldehyde for 2 days and placed in 15 % ethylenediaminetetraacetic acid for 2 weeks. After dehydration with ethanol and xylene, the cartilage tissue was embedded in paraffin and sliced by a microtome with a thickness of 5 μm. The slices were stained with HE and Modified Safranine O-Fast Green FCF Cartilage Stain Kit (bioss, China). In short, HE staining was to dye the slices with hematoxylin for 3 min, then dye with eosin for 30 s, and then seal the slices after dehydration. Safranine O Stain, on the other hand, stains slices with a rapid green stain solution for 5 min, followed by Safranin O stain for 2 min. Cartilage can be dyed red and osteoblasts green. Damage to cartilage tissue can result in light or no staining of saffranine O. OARSI score was performed on cartilage tissue injury [23].

### 2.16. Data analysis

Data analysis was evaluated using SPSS software (SPSS 22.0 for Windows; SPSS, Inc, Chicago). GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA) was used for graphing. All data were presented as mean ± standard deviation (SD). Student's t-test analyzes differences between the two groups, and one-way analysis of variance (ANOVA) detects multiple comparisons. All experiments were repeated at least 3 times independently with 3 repetitions within each experiment. Each assay was conducted in triplicate. $p < 0.05$ was statistically significant.

## 3. Results

### 3.1. circRELL1 is a circRNA with upregulated expression in OA

First, RT-qPCR measured circRELL1 expression in 24 OA tissue samples and 11 healthy control samples collected. Results showed that circRELL1 expression was elevated in cartilage tissues of OA patients compared with healthy controls (Fig. 1A). In addition, cell and mouse models of OA were established using LPS-induced C28/I2 cells and DMM surgery. RT-qPCR results detected the increased trend of circRELL1 expression in LPs-induced C28/I2 cells and DMM mice (Fig. 1B). From the online bioinformatics website circBank (http://www.circbank.cn/), circRELL1 is in the exon 5 and 6 derived from the RELL1 gene, with a length of about 376 nt (Fig. 1C). The stability of circRELL1 tested by RNAse R and actinectin D experiments showed that circRELL1 was resistant to the digestion of RNAse R and had a longer half-life than linear RNA (Fig. 1D and E). Subcellular isolation experiments showed that circRELL1 was mainly present in the cytoplasm of C28/I2 cells (Fig. 1F).

### 3.2. circRELL1 depletion inhibits LPS-induced chondrocyte injury

circRELL1 expression was designed to be knocked down in chondrocytes. circRELL1 expression was effectively down-regulated after transfection of si-circRELL1 into C28/I2 cells (Fig. 2A). MTT assay manifested that LPs-induced cell activity was reduced in C28/I2 cells, while circRELL1 knockdown increased cell viability (Fig. 2B). Flow cytometry determined that the apoptosis rate of C28/

I2 cells was induced by LPS, while circRELL1 silencing decreased the apoptosis rate (Fig. 2C). Subsequently, the expression levels of inflammatory cytokines IL-1β, IL-6 and TNF-α in the supernatant of cell culture were detected by ELISA assay. Results found that LPS stimulated C28/I2 cells to secrete more inflammatory cytokines, but circRELL1 knockdown effectively inhibited this phenomenon (Fig. 2D). Meanwhile, Immunoblot analysis showed that LPS promoted cleaved caspase-3, Bax, and MMP13, and inhibited Bcl-2, collagen II, and Aggrecan protein expressions. However, when circRELL1 was down-regulated in C28/I2 cells, the protein expression of these factors was reversed (Fig. 2E).

### 3.3. *In vivo silencing circRELL1 improves OA damage*

Lentivirus-packaged sh-circRELL1 was injected into the joints of DMM mice. RT-qPCR results confirmed that circRELL1 expression was effectively down-regulated (Fig. 3A). Safranine O-Fast Green staining observed that the chondrocytes of DMM mice were reduced and the cartilage tissue damage was serious, while circRELL1 knocked down could significantly improve the cartilage tissue damage (Fig. 3B). At the same time, H&E staining demonstrated decreased thickness of hyaline cartilage (HC) and disordered chondrocyte arrangement of articular cartilage in DMM mice, while circRELL1 downregulation could increase HC thickness and alleviate chondrocyte disorder to a certain extent (Fig. C). Low circRELL1 decreased OARSI score in DMM mice (Fig. 3D). Apoptosis- and ECM-related proteins in the knee cartilage of mice were analyzed by Immunoblot. The findings suggested that circRELL1 knockdown could effectively block the promoting effect of DMM surgery on apoptosis and ECM degradation of mouse knee chondrocytes (Fig. 3E).

### 3.4. *circRELL1 targets miR-200c-3p*

Based on the subcellular localization of circRELL1 and the mechanism of circRNA as ceRNA, the study used starBase3.0 database to predict and analyze the potential downstream targets of circRELL1. circRELL1 and miR-200c-3p shared potential binding sites (Fig. 4A). RIP experiments confirmed that circRELL1 and miR-200c-3p were enriched in Ago2 (Fig. 4B). After transfecting miR-200c-3p mimic into C28/I2 cells, RT-qPCR detection results showed that miR-200c-3p levels were enhanced (Fig. 4C). Dual luciferase assay results confirmed that the activity of luciferase was blocked after co-transfection of miR-200c-3p mimic and WT-circRELL1 (Fig. 4D). In addition, RT-qPCR detection of miR-200c-3p expression in DMM mice and LPS-induced C28/I2 cells showed that miR-200c-3p expression was reduced (Fig. 4E), and depleting circRELL1 effectively restored miR-200c-3p expression in C28/I2 cells (Fig. 4F).

### 3.5. *circRELL1/miR-200c-3p synergy aggravates inflammatory like-OA progression*

The study design co-transfected si-circRELL1 and miR-200c-3p inhibitor into C28/I2 cells for study. The results of RT-qPCR showed that si-circRELL1 could promote miR-200c-3p levels, while miR-200c-3p inhibitor could reverse this promotion effect (Fig. 5A). MMT and flow cytometry results evaluated that miR-200c-3p inhibitor could reduce the proliferation promotion and apoptosis inhibition of si-circRELL1 on LPS-treated C28/I2 cells (Fig. 5B and C). ELISA results further detected that si-circRELL1 inhibited the expression of LPS-induced inflammatory cytokines in C28/I2 cells, and this inhibition was suppressed by miR-200c-3p inhibitor (Fig. 5D). Immunoblot results also confirmed that miR-200c-3p inhibitor obstructed si-circRELL1-mediated inhibition of LPS-induced apoptosis of C28/I2 cells and levels of ECM degradation-related proteins (Fig. 5E).

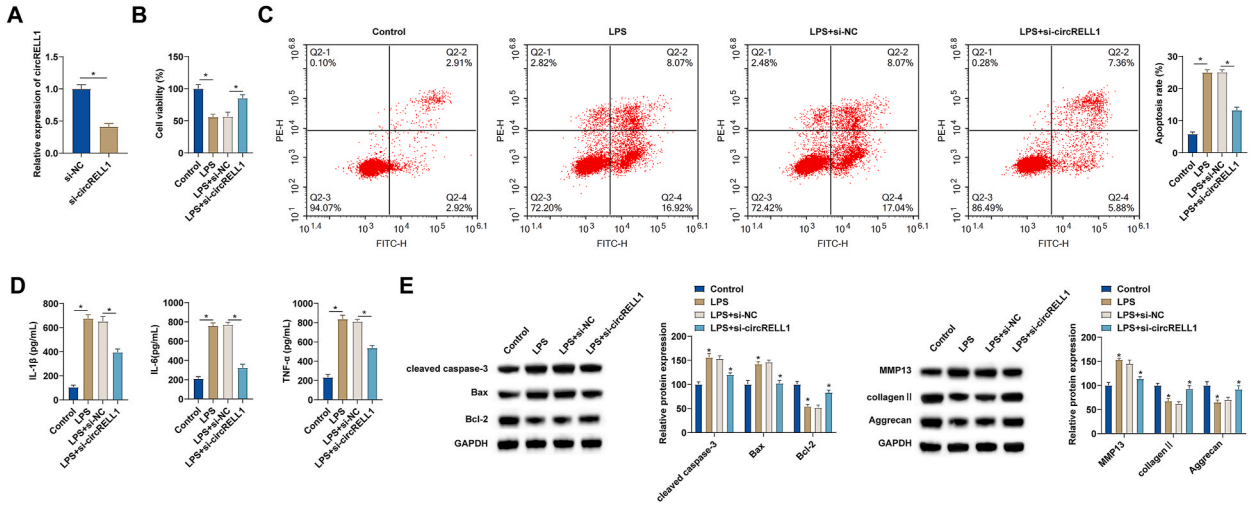


**Fig. 2.** Reducing circRELL1 inhibits LPS-induced chondrocyte injury
si-circRELL1 or si-NC was introduced into C28/I2 cells. A: RT-qPCR measured circRELL1 in C28/I2 cells. B: MTT assay detected cell viability. C: Flow cytometry measured cell apoptosis rate. D: ELISA assayed inflammatory cytokines. E: Immunoblot detected related proteins in apoptosis and ECM. Data were expressed as mean ± SD (N = 3), $P < 0.05$.

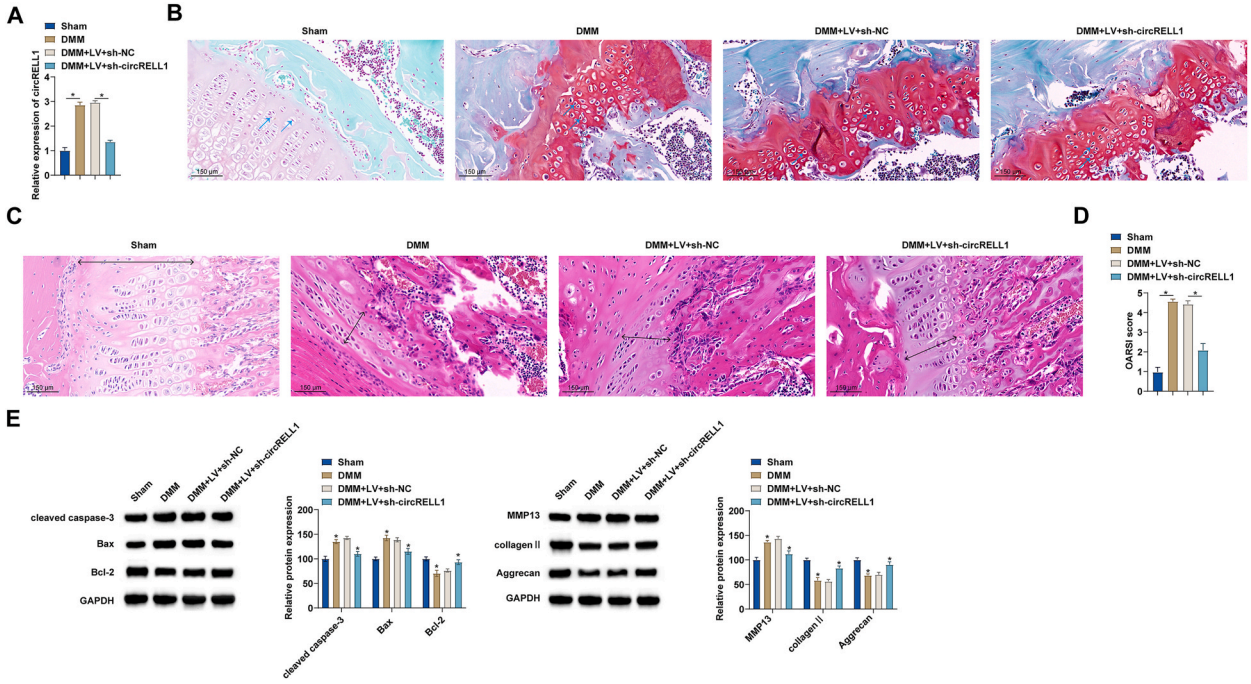


**Fig. 3.** *In vivo* silencing circRELL1 improves OA injury
Lentivirus-packaged sh-circRELL1 was injected into the knee joint of DMM mice. A: RT-qPCR measured circRELL1. B: Safranine O-Fast Green staining evaluated the number of chondrocytes and the degree of tissue injury (the blue arrows serve to denote the presence of chondrocytes). C: HE staining evaluated the morphological changes of cartilage tissue (black double arrows are utilized to indicate the thickness of the transparent cartilage). D: OARSI score evaluated the degree of cartilage injury. E: Immunoblot analyzed related proteins in apoptosis and ECM in the knee joint of mice. Data were expressed as mean ± SD (N = 3), $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

### 3.6. *TCF4 is regulated by miR-200c-3p*

starbase 3.0 database predicted the target genes of miR-200c-3p and screened out TCF4, whose potential binding sites are shown in Fig. 6A. RIP experiment confirmed the enrichment of miR-200c-3p and TCF4 in Ago2 (Fig. 6B). Silencing miR-200c-3p elevated levels of TCF4, while overexpressing miR-200c-3p was contrary to the results (Fig. 6C and D). Dual luciferase assay confirmed that miR-200c-3p regulated TCF4 expression (Fig. 6E). TCF4 was abnormally elevated in patients with OA and in OA animal and models of inflammatory like-OA conditions (Fig. 6F–H).

### 3.7. *TCF4 promotes OA progression*

si-circRELL1 and oe-TCF4 were co-transfected into C28/I2 cells for experiment. si-circRELL1 inhibited TCF4 levels, while oe-TCF4 reversed the inhibition (Fig. 7A). Moreover, oe-TCF4 mitigated the stimulative effect of si-circRELL1 on LPS-induced C28/I2 cell proliferation (Fig. 7B) and inhibition of apoptosis rate (Fig. 7C), inflammation (Fig. 7D), and protein expression of genes related to apoptosis and ECM degradation (Fig. 7E).

## 4. Discussion

OA can cause irreversible damage to articular cartilage tissue [24]. Chondrocyte dysfunction is closely related to OA progression [25]. Currently, the main treatment plan for OA is to control inflammation and relieve pain [26]. Therefore, this study explored the underlying molecular mechanism of OA progression to provide new strategies for OA treatment. Further experiments confirmed that circRELL1 was upregulated in inflammatory like-OA conditions, and could decoy miR-200c-3p to regulate TCF4, affect chondrocyte vitality, apoptosis, and inflammatory response, as well as ECM degradation process.

circRNA indicates a key role in the progression of various diseases such as OA and can be considered diagnostic biomarkers and therapeutic targets [7,27–31]. CircRELL1 can be involved in regulating inflammatory response in endothelial cells [12]. This study assessed the upregulation of circRELL1 in the tissues of patients with OA, indicating that it was associated with O development. The results were consistent with the OA mouse and inflammatory like-OA conditions we constructed *in vivo* and *in vitro*. Abnormal chondrocyte metabolism is the main factor leading to articular cartilage degeneration [32]. Studies have believed that circRNAs mediate chondrocyte ECM degradation, inflammatory response, and apoptosis by detecting the changes of pro-inflammatory cytokines

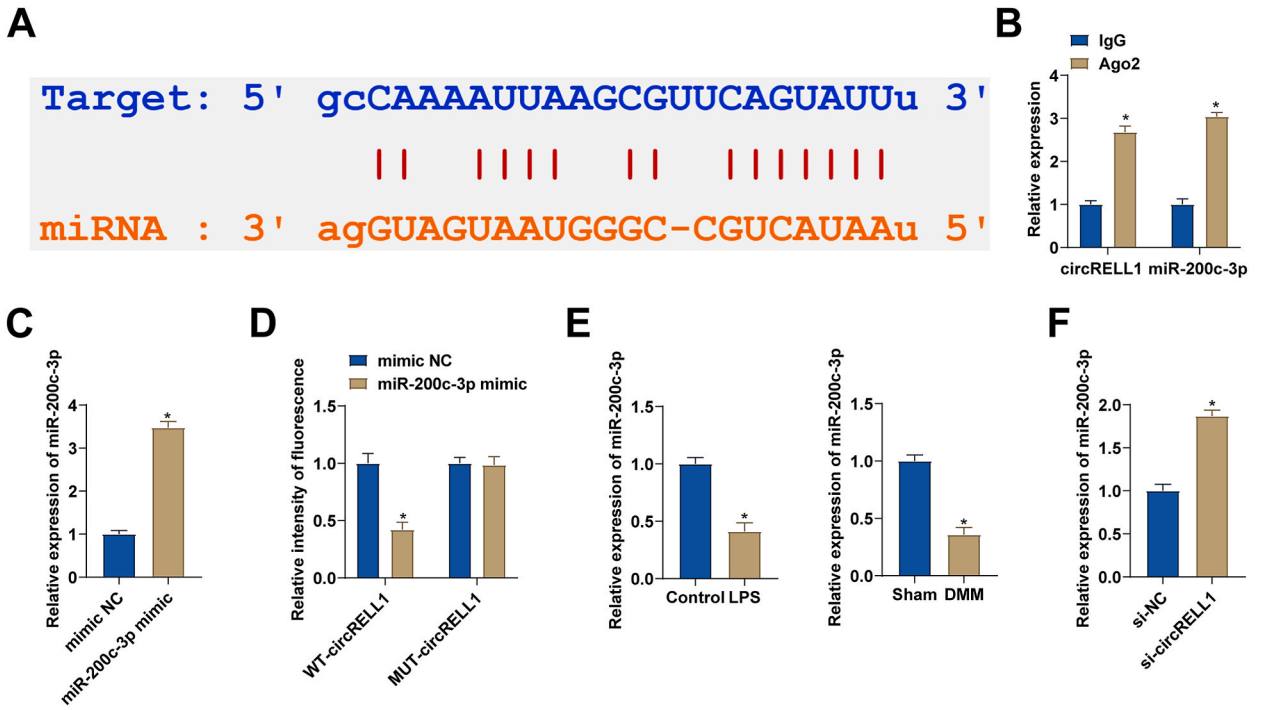


**Fig. 4.** Targeting regulation of miR-200c-3p by circRELL1.
A: Potential binding sites of circRELL1 and miR-200c-3p. B: RIP experiment analyzed the enrichment levels of circRELL1 and miR-200c-3p in Ago2. C: RT-qPCR measured transfection efficiency of miR-200c-3p mimic. D: Dual luciferase assay verified the relationship between circRELL1 and miR-200c-3p. E: RT-qPCR measured miR-200c-3p in DMM mice and LPS-induced C28/I2 cells. F: RT-qPCR measured miR-200c-3p after transfection with si-circRELL1. Data were expressed as mean ± SD (N = 3), $P < 0.05$.

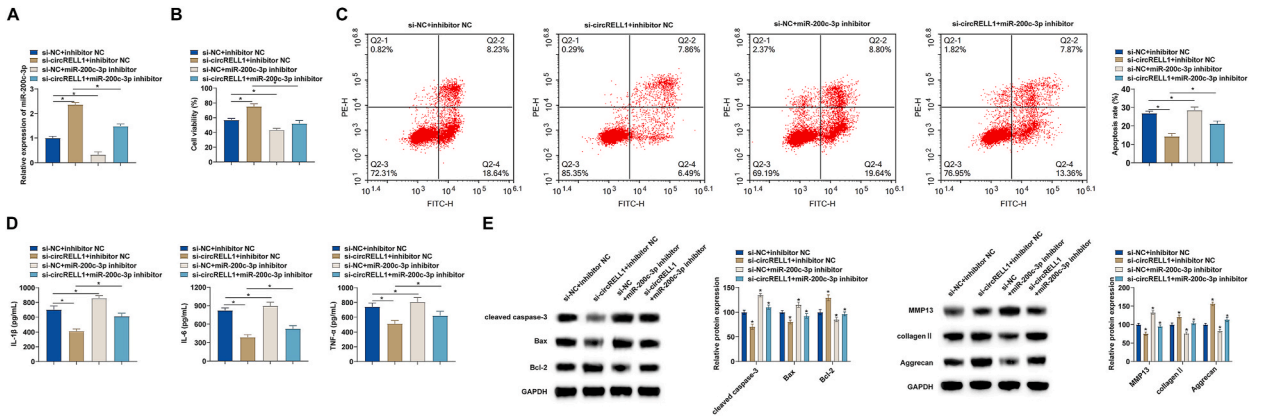


**Fig. 5.** circRELL1/miR-200c-3p axis promotes OA progression
si-circRELL1 and miR-200c-3p inhibitor were co-treated in C28/I2 cells. A: RT-qPCR measured miR-200c-3p. B: MTT assay detected cell viability. C: Flow cytometry measured cell apoptosis rate. D: ELISA assayed inflammatory cytokines. E: Immunoblot detected related proteins in apoptosis and ECM. Data were expressed as mean ± SD (N = 3), $P < 0.05$.

and the markers of metabolic decomposition and synthesis-related factors [33,34]. The findings of Jia et al. demonstrate that CircRNA-MSR exacerbates LPS-induced C28/I2 chondrocyte injury by promoting inflammatory factors IL-1β, IL-6, and TNF-α, as well as disrupting the balance of ECM synthesis and degradation [35]. Furthermore, the outcomes of our research have also demonstrated similar findings. Significantly, the present study successfully established an OA mouse model using DMN technique. The findings of this study demonstrated that the knockout of circRELL1 notably ameliorated cartilage damage in OA mice. Specifically, it resulted in an augmentation of the transparent cartilage layer thickness and a mitigation of the disruption in chondrocyte arrangement. These findings suggest that circRELL1 is a potential therapeutic target for OA.

circRNAs have been identified to act as miRNA sponges to regulate gene expression [36–38]. This study emphasized that circRELL1

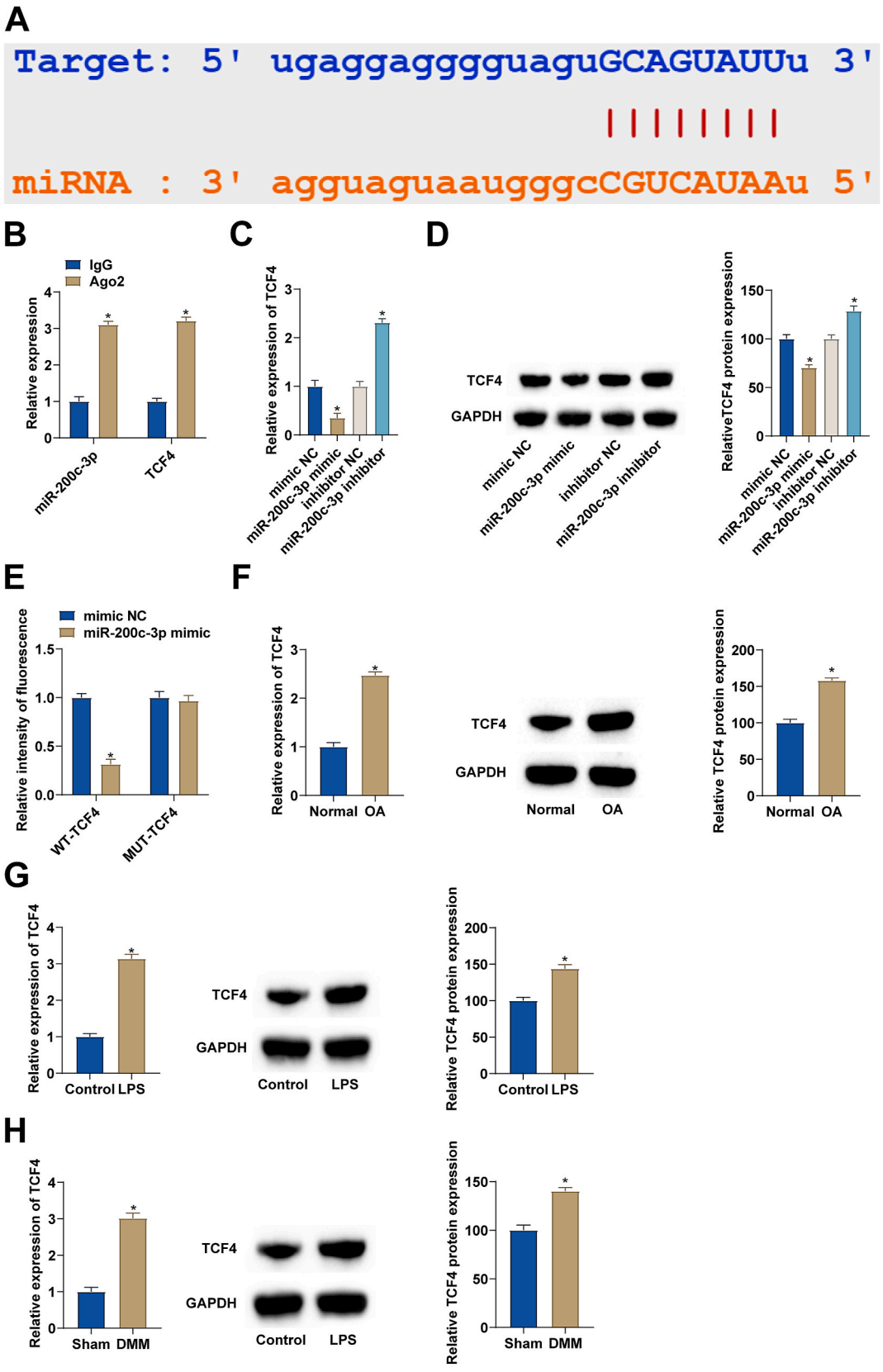


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**Fig. 6.** TCF4 is mediated by miR-200c-3p.
A: Potential binding sites of miR-200c-3p and TCF4. B: Ago2 enrichment levels of miR-200c-3p and TCF4 in C28/I2 cells in RIP assay. C–D: RT-qPCR and Immunoblot measured TCF4 in C28/I2 cells transfected with miR-200c-3p mimic and miR-200c-3p inhibitor. E: Dual luciferase assay verified the targeting relationship between miR-200c-3p and TCF4. F–H: RT-qPCR and Immunoblot measured TCF4 in OA patients and animal and cell models of OA. Data were expressed as mean ± SD (N = 3), $P < 0.05$.

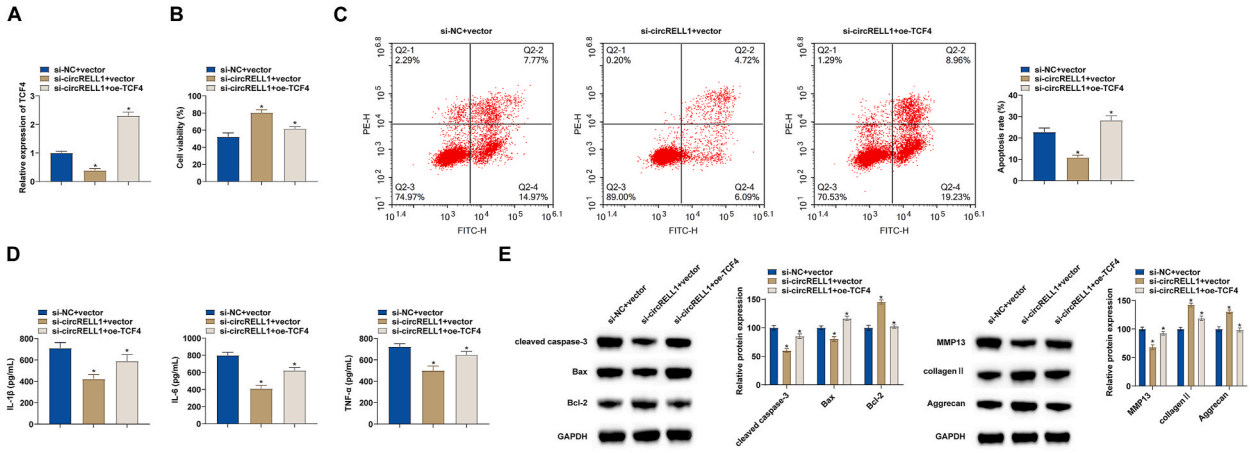


**Fig. 7.** TCF4 promotes OA progression
C28/I2 cells were co-transfected with si-circRELL1 and oe-TCF4. A: RT-qPCR measured TCF4. B: MTT assay detected cell viability. C: Flow cytometry measured cell apoptosis rate. D: ELISA assayed inflammatory cytokines. E: Immunoblot detected related proteins in apoptosis and ECM. Data were expressed as mean ± SD (N = 3), $P < 0.05$.

was mainly present in the cytoplasm and can be utilized as miRNA sponge. miR-200c-3p was analyzed as a potential downstream target of circRELL1. The study demonstrated experimentally that silencing miR-200c-3p could mitigate the silencing of circRELL1's promoting effect on the growth of inflammatory like-OA chondrocytes, as well as its inhibiting effect on apoptosis, inflammatory response, and ECM degradation. Our results confirmed that TCF4 was elevated in patients with OA, which was consistent with previous studies by Wang et al. [39]. Based on a previous study, inhibiting TCF4 could enhance cell viability of LPS-treated chondrocytes, and inhibit apoptosis, inflammatory response, and ECM degradation [22]. The current study further confirmed that overexpressing TCF4 blocked the impact of silencing circRELL1 on the progression of OA.

However, some limitations exist in the study. We have yet to explore the mechanisms of how circrell1-mediated TCF4 further promotes the inflammatory response and ECM degradation in OA. At the same time, more experiments are needed to explore whether circRELL1 affects OA by acting on other molecules and pathways. Therefore, future experiments will use bioinformatics methods and molecular experimental techniques to study the potential mechanism of circRELL1 in OA, so as to provide new targets and therapeutic strategies for the clinical treatment of OA.

## 5. Conclusion

The inhibition of CircRELL1 has been observed to impede OA progression in both *in vivo* and *in vitro* settings. Additionally, it has been found to suppress the inflammatory secretion, proliferation, and ECM disruption of chondrocytes *in vitro*, induced by LPS. This regulatory impact of circRELL1 on OA is mediated through its role as an endogenous miR-200c-3p sponge. These findings are expected to enhance the comprehension of OA formation and aid in the development of novel circRELL1-targeted therapies for OA treatment.

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Not applicable.

## Ethical statement

This study was approved by the Human Research Ethics Committee of Shanghai Songjiang District Central Hospital (NO. 2016SJ3016). Written informed consent was obtained from all the participants.

And the Animal experiments were approved by Shanghai Songjiang District Central Hospital Animal Experimental Ethics Committee (NO. 2016SJ3022), and all animal experiments were complied with the ARRIVE guidelines and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

## Data availability statement

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

## CRediT authorship contribution statement

**HongZhi Ding:** Writing – original draft, Supervision, Resources, Project administration, Formal analysis, Conceptualization. **HaiJu Chen:** Writing – original draft, Software, Methodology, Investigation, Conceptualization. **LianRong Dou:** Writing – review & editing, Resources, Investigation, Data curation. **Yang Li:** Methodology, Formal analysis, Data curation.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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