CircRNA_0092516 regulates chondrocyte proliferation and apoptosis in osteoarthritis through the miR-337-3p/PTEN axis

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Research

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Abstract

Background

The dysregulation of circular RNAs (circRNAs) has been identified in various human diseases. Here, we mainly investigated the potential mechanism of circRNA_0092516 in osteoarthritis (OA).

Methods

The expression of circRNA_0092516 was detected by quantitative real-time PCR. MTT, flow cytometry, and Western blot were used to confirm the functions of circRNA_0092516 *in vitro*. Besides, RNA pull-down and dual-luciferase reporter gene experiments were used to study the potential mechanism.

Results

circRNA_0092516 was up-regulated in the tissues of OA patients and chondrocytes stimulated by IL-1β. The potential mechanism analysis revealed that circRNA_0092516 bound to miR-337-3p, and the interference with circRNA_0092516 promoted chondrocyte proliferation and repressed cell apoptosis through the miR-337-3p/PTEN axis, thereby improving OA.

Conclusions

Overall, our data showed that the interference with circRNA_0092516 promoted chondrocyte proliferation and repressed cell apoptosis through the miR-337-3p/PTEN axis, eventually slowed down the progress of OA.

1. Introduction

Osteoarthritis (OA) is a type of age-related joint disease that has been widely regarded as an irreversible disease and one of the main causes of disability in the elderly[1]. Although the clinical treatment of OA has made good progress, there are still deficiencies such as poor prognosis and expensive treatment costs[2, 3]. During the occurrence of OA, various pathological factors directly or indirectly act on chondrocytes, and emerging evidence has confirmed that the inhibition of chondrocyte proliferation, differentiation, and inflammatory response are conducive to hindering the occurrence and development of OA[4, 5]. Therefore, we urgently need to explore the underlying mechanism of chondrocyte proliferation and apoptosis in OA, which is critical for the relief of OA.

Recently, circular RNAs (circRNAs) have gradually become a popular molecule in non-coding RNA research and is characterized by a stable structure and high tissue-specific expression[6, 7]. Increasing evidence indicates that the dysregulation of circRNAs is closely related to the progress of various human

diseases. For instance, Zhao *et al*. found that circ_0136474 is significantly up-regulated in OA cartilage tissues, and this highly expressed circ_0136474 inhibits chondrocyte proliferation and promotes cell apoptosis[8]. Shen *et al*. indicated that the overexpression of circSERPINE2 impedes the progression of OA by inhibiting human chondrocyte apoptosis[9]. Here, what attracts our attention is that circRNA_0092516, also known as circRNA-NT5C2, has been reported to be up-regulated in OA[10], indicating that circRNA_0092516 might be related to the occurrence and development of OA. Besides, increasing evidence shows that circRNAs are rich in the binding sites of miRNAs, and affect the expression of downstream target mRNA by competing for endogenous RNA (ceRNA) to interact with miRNA[11, 12]. However, it is currently unclear whether circRNA_0092516 can act as a ceRNA in the progress of OA.

MicroRNAs (miRNAs) are small non-coding RNAs that cannot encode proteins, and negatively regulate target mRNAs expression by interacting with the 3 'untranslated region (UTR) of the target mRNAs[13]. Various previous studies have shown that the abnormal expressions of miRNAs are closely related to the development of OA[14, 15]. As reported, after the occurrence of OA, miR-181a-5p is abnormally downregulated and the in-depth mechanism research finds that miR-181a-5p plays an important role in the process of OA by negatively regulating SBP2[16]. Besides, Chen *et al*. found that miR-29b-3p promotes chondrocyte apoptosis and promotes the occurrence and development of OA through targeting PGRN[17]. Importantly, our previous study shows that miR-337-3p is significantly down-regulated in OA cartilage tissues[18]. However, the mechanism by which miR-337-3p plays roles in OA has not been fully elucidated.

In the current study, we mainly explored the role and potential mechanism of circRNA_0092516 in OA.

2. Materials And Methods

2.1 Patients and tissue samples

10 cases of osteoarthritis cartilage tissue were collected from patients with knee OA of total knee replacement surgery, and 10 cases of normal articular cartilage tissues were obtained from non-osteoarthritis patients who had undergone surgical repair of knee fractures and had no history of arthritis. All cartilage donors included in this study provided informed consent. This study was approved by the Human Ethics Committee of the Third Affiliated Hospital of Suchow University.

2.2 RNA extraction and quantitative real-time PCR (qRT-PCR)

qRT-PCR experiments were performed according to the previously described method with minor changes[19]. TRIzol reagent was used to isolate the total RNA from cartilage tissues and primary chondrocytes, and then RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) was used to reverse transcribe RNA into cDNA. Next, Forget-Me-Not™ EvaGreen® qPCR Master Mix (Biotium) and CFX96™ Real-Time PCR Detection Systems (BIO-RAD) were used to perform the real-time PCR. GAPDH

was used as an internal control for circRNA_0092516, and U6 was used as an internal control for miR-337-3p. Finally, the relative expressions of circRNA_0092516 and miR-337-3p were quantified using the $2^{-\Delta\Delta Ct}$ method.

2.3 Isolation of primary chondrocytes

Cartilage slices were obtained under sterile conditions and cut into thin slices, then placed them in phosphate-buffered saline (PBS) supplemented with 1% trypsin at 37 °C for 15 min, and then centrifuged to discard the supernatant. We washed the collected cartilage 3 times to remove trypsin and continued to incubate for 12–16 h at 37 °C in PBS supplemented with 2 mg/L collagenase IV. Next, the above chondrocytes were washed with Dulbecco's Modified Eagle's medium (DMEM) supplemented with penicillin (100 U/mL)-streptomycin (100 μg/mL) and centrifuged at 200 g for 10 min to harvest the final primary chondrocytes.

2.4 Culture and different treatment of primary chondrocytes

The primary chondrocytes harvested above were placed in DMEM medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 1% glutamine, and 10% fetal bovine serum (FBS), and cultured at 37 °C, 5% CO_2. Besides, we added 10 ng/mL IL-1β to the DMEM medium to stimulate primary chondrocytes for 24 h.

2.5 Western blot analysis

RIPA lysis buffer was used to extract total protein from cartilage tissues and primary chondrocytes, and the BCA Protein Assay Kit (Sigma) was used to quantify the concentration of the above-extracted protein. Then, the same amount of protein was separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a PVDF membrane (Millipore). After blocking with 5% skim milk, the membrane was incubated with the primary antibody overnight at 4 °C. The next day, the membrane and the secondary antibody were incubated at room temperature for 1 h. Finally, according to the standard procedures of the manufacturer, the ECL chemiluminescence kit (Santa Cruz Biotechnology) was used to detect the protein, and the image was processed using ImageJ software.

2.6 Cell transfection

Briefly, primary chondrocytes were cultured in 96-well plates supplemented with DMEM. According to the reagent manufacturer's instructions, the synthetic si-circRNA_0092516, si-circRNA_0092516 + miR-337-3p inhibitor were transfected into primary chondrocytes using Lipofectamine 2000 (Invitrogen). 48 h after transfection, the cells were harvested for subsequent research.

2.7 Detection of cell proliferation

According to the manufacturer's standard protocol, the proliferation of primary chondrocytes was analyzed using the MTT Assay kit (Abcam). Briefly, primary chondrocytes with different treatments were seeded in 96-well plates and cultured for 48 h. Next, 20 μL of MTT solution was added to each well and

incubated at 37 °C for another 2 h. Then, the absorbance was measured at a wavelength of 570 nm to determine the proliferation of primary chondrocytes.

2.8 Flow cytometry assay

Based on the previously described methods[20], we performed the flow cytometry assay to detect the apoptosis of chondrocytes. After washing with cold phosphate buffer, the primary chondrocytes after different treatments were collected. Then, the cells were resuspended in the membrane conjugate buffer and incubated with 5 μL LITC Annexin V and 5 μL PI working solution for 15 min at room temperature. Finally, the flow cytometry FACSCalibur instrument (BD Biosciences) was used to analyze the apoptosis ratio of primary chondrocytes.

2.9 Dual-luciferase reporter gene assay

293T cells (1×10^4) were seeded in 24-well plates for cultivation. WT-circRNA_0092516 or MUT-circRNA_0092516 was constructed, and pre-NC or miR-337-3p mimic was co-transfected into 293T cells using Lipofectamine 2000 (Invitrogen). After 48 h, the cells were harvested, and then the luciferase activity was detected using the Dual-Luciferase Reporter Assay kit (Promega) according to the reagent manufacturer's instructions.

2.10 RNA pull-down

The primary chondrocyte lysate was incubated with biotinylated miR-337-3p at 30 °C for 30 min. Next, the biotin-coupled RNA complex was incubated with streptavidin magnetic beads for 4 h, and separated from the supernatant on a magnetic stand. Finally, the expression of circRNA_0092516 in the miR-337-3p pull-down complex was analyzed by qRT-PCR.

2.11 Statistical Analysis

SPSS 19.0 was used to analyze the statistical analysis and all data in this study were expressed as mean ± standard deviation (SD). Differences between two groups were analyzed by Student's *t*-test, and differences among more than two groups were analyzed by one-way ANOVA. Pearson correlation coefficient analysis was used to analyze the correlation between circRNA_0092516 and miR-337-3p, circRNA_0092516, and PTEN, and the r-value was used to evaluate the correlation of two variables. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 CircRNA_0092516 is up-regulated in tissues of osteoarthritis patients and chondrocytes stimulated by IL-1β

Recent studies have shown that some circRNAs are found to be dysregulated in OA and are closely related to the occurrence and development of OA[10, 21]. Here, we detected the expression of circRNA_0092516 in OA cartilage tissues by qRT-PCR and found that circRNA_0092516 was significantly up-regulated in the OA group (Fig. 1A). Pearson correlation analysis showed that the expressions of circRNA_0092516 and miR-337-3p were negatively correlated, and the expressions of circRNA_0092516 and PTEN were positively correlated (Fig. 1B). Next, chondrocytes were isolated from normal articular cartilage tissues and then stimulated the cells with 10 ng/mL IL-1β for 24 h to establish an *in vitro* OA model. As shown in Fig. 1C, circRNA_0092516 was significantly up-regulated in the IL-1β group. Besides, various previous studies have confirmed that matrix metallopeptidase 1 (MMP1) and matrix metallopeptidase 13 (MMP13), inflammatory factors IL-6 and TNF-α, and chondrocyte secreted Aggrecan (Aggrecan) and type II collagen (COL II) are all closely related to the occurrence and development of OA[22–24]. Western blot analysis indicated that the protein levels of OA marker molecules MMP1, MMP13, and inflammatory factors TNF-α, IL-6 were markedly up-regulated in the IL-1β group, while the extracellular matrix metabolism-related molecules COL II and Aggrecan were significantly down-regulated (Fig. 1D). Collectively, these data suggested that the abnormal expression of circRNA_0092516 might be related to the progress of OA.

3.2 Interference with circRNA_0092516 promotes chondrocyte proliferation and inflammatory factors release and inhibits cell apoptosis

Next, we isolated chondrocytes from normal articular cartilage tissues and transfected si-circRNA_0092516 into chondrocytes and then stimulated the cells with 10 ng/mL IL-1β for 24 h to further explore the role of circRNA_0092516 in chondrocyte proliferation, apoptosis, and inflammatory factor release. From the results of qRT-PCR, we found that we had successfully interfered with circRNA_0092516 in chondrocytes, and IL-1β stimulation down-regulated the expression of miR-337-3p, while this down-regulation was reversed after the interference with circRNA_0092516 (Fig. 2A). Western blot analysis indicated that the interference with circRNA_0092516 down-regulated the protein levels of MMP1, MMP13, and inflammatory factors IL-6 and TNF-α, and up-regulated extracellular matrix metabolism-related molecules COL II and Aggrecan (Fig. 2B). As reported, PI3K/AKT and NF-κB signaling pathways are related to OA, and PTEN can regulate PI3K/AKT signaling pathways[25, 26], so we next examined the protein levels of PTEN and NF-κB p65. Also, as shown in Fig. 2C, IL-1β stimulation up-regulated the protein levels of PTEN and p-p65, while this up-regulation was reversed after the interference with circRNA_0092516. MTT and flow cytometry results showed that IL-1β stimulation inhibited the proliferation of chondrocytes (Fig. 2D) and promoted cell apoptosis (Fig. 2E), while these effects were reversed after the interference with circRNA_0092516. Besides, the analysis of Western blot revealed that IL-1β stimulation up-regulated the protein levels of cleaved-caspase-3 and Bax, and down-regulated Bcl-2, while these effects were reversed after the interference with circRNA_0092516 (Fig. 2F). Overall, the above experimental results indicated that the interference with circRNA_0092516 promoted chondrocyte proliferation and inflammatory factors release and inhibited cell apoptosis.

3.3 circRNA_0092516 is a bait for miR-337-3p

Next, we further explored the potential mechanism of circRNA_0092516 in OA. Bioinformatics online prediction software found that there were binding sites between circRNA_0092516 and miR-337-3p (Fig. 3A), and dual-luciferase reporter gene results indicated that miR-337-3p negatively regulated the luciferase activity of circRNA_0092516 (Fig. 3B). Besides, the results of RNA pull-down experiments revealed that circRNA_0092516 was enriched in the cell lysates pulled by miR-337-3p-Biotin (Fig. 3C), suggesting that there was an interaction between circRNA_0092516 and miR-337-3p. Next, we transfected si-circRNA_0092516 into chondrocytes and then stimulated the cells with 10 ng/mL IL-1β for 24 h. As shown in Fig. 3D, the interference with circRNA_0092516 significantly up-regulated the expression of miR-337-3p. These data indicated that circRNA_0092516 could be used as a bait for miR-337-3p, and circRNA_0092516 negatively regulated the expression of miR-337-3p.

3.4 Interference with circRNA_0092516 promotes chondrocyte proliferation and inflammatory factors release and inhibits cell apoptosis through miR-337-3p

Subsequently, we transfected si-circRNA_0092516, si-circRNA_0092516 + miR-337-3p inhibitor into chondrocytes and then stimulated the cells with 10 ng/mL IL-1β for 24 h. We had previously confirmed that the interference with circRNA_0092516 down-regulated the protein levels of MMP1, MMP13, IL-6 and TNF-α, and up-regulated COL II and Aggrecan (Fig. 2B), and our further study indicated that these effects were reversed after the transfection of miR-337-3p inhibitor (Fig. 4A). Also, MTT and flow cytometry results revealed that the interference with circRNA_0092516 promoted the proliferation of chondrocytes (Fig. 2D) and repressed cell apoptosis (Fig. 2E), and our further research showed that these effects were reversed after the transfection of miR-337-3p inhibitor (Fig. 4B& Fig. 4C). Moreover, Western blot analysis indicated that the interference with circRNA_0092516 down-regulated the protein levels of cleaved-caspase-3 and Bax, and up-regulated Bcl-2 (Fig. 2F), while these effects were reversed after the transfection of miR-337-3p inhibitor (Fig. 4D). Overall, miR-337-3p reversed the effect of the interference with circRNA_0092516 on chondrocyte proliferation, apoptosis, and inflammatory factors release.

3.5 Interference with circRNA_0092516 affects PTEN expression and NF-κB signaling pathway through miR-337-3p

Furthermore, si-circRNA_0092516, si-circRNA_0092516 + miR-337-3p inhibitor was transfected into chondrocytes and then stimulated the cells with 10 ng/mL IL-1β for 24 h. The results of qRT-PCR had indicated that the interference with circRNA_0092516 down-regulated the expression of circRNA_0092516 and up-regulated miR-337-3p (Fig. 2A), and our further research showed that these effects were reversed after the transfection of miR-337-3p inhibitor (Fig. 5A). Besides, Western blot analysis revealed that the interference with circRNA_0092516 significantly down-regulated the protein levels of PTEN and p-p65, and our further research showed that this down-regulation was reversed after the transfection of miR-337-

3p inhibitor (Fig. 5B). The above data indicated that the interference with circRNA_0092516 regulated PTEN expression and NF-κB signaling pathway through miR-337-3p.

4. Discussion

At present, there is still a lack of effective strategies for the treatment of OA[27]. To explore effective treatment options, we urgently need to explore the underlying molecular mechanisms that promote the progress of OA and identify new biological targets. Here, we determined that circRNA_0092516 was a key up-regulator of circRNAs related to the progression of OA, and our in-depth studies found that the interference with circRNA_0092516 promoted chondrocyte proliferation and repressed cell apoptosis through miR-337-3p/PTEN axis, thereby alleviating OA.

CircRNAs are a class of endogenous non-coding RNAs that are widely distributed and characterized by tissue specificity and conservation[28]. More and more research shows that circRNAs can act as a ceRNA under different physiological and pathological conditions, and then play regulatory roles in various human diseases including OA[29, 30]. Zhang *et al*. found that circRNA-CDR1as acts as a sponge of microRNA-641 to regulate extracellular matrix metabolism and inflammation to promote the development of OA[31]. Also, Liu *et al*. found that circRNA-CER regulates MMP13 expression by competitively binding to miR-136 with MMP13, and participates in the degradation process of chondrocyte ECM in OA[32]. In this study, we found that circRNA_0092516 was markedly up-regulated in the tissues of osteoarthritis patients and chondrocytes stimulated by IL-1β, and then we verified the interaction between circRNA_0092516 and miR-337-3p through the dual-luciferase reporter gene assay. Besides, we also found that circRNA_0092516 negatively regulated the expression of miR-337-3p. Therefore, the above data of our experiments indicated that circRNA_0092516 targeted miR-337-3p by acting as a sponge.

So far, increasing miRNAs have been found to play core functions in the pathogenesis and process of OA by interacting with the 3 'UTR of their target mRNAs [33, 34]. As reported, miRNA-15a-5p regulates the development of OA by targeting PTHrP in chondrocytes[35]. Another study points out that miRNA-93 alleviates OA by targeting TLR4 to inhibit chondrocyte apoptosis and inflammation[36]. Moreover, our previous study has been found that miR-337-3p is significantly down-regulated in OA cartilage tissues[18], suggesting that miR-337-3p might be related to the progress of OA. Phosphatase and tensin homolog (PTEN) is widely regarded as a tumor suppressor and has been reported to have significantly low expression in OA[37, 38]. Importantly, our previous study also indicated that miR-337-3p is involved in the occurrence and development of OA by regulating PTEN to promote chondrocyte proliferation and inhibit apoptosis[18]. Here, our results further confirmed that the expressions of circRNA_0092516 and miR-337-3p were negatively correlated, and the expressions of circRNA_0092516 and PTEN were positively correlated, and miR-337-3p reversed the effect of the interference with circRNA_0092516 on the down-regulation of PTEN and the proliferation and apoptosis of chondrocytes and the NF-κB signaling pathway.

In summary, our research showed that circRNA_0092516 was markedly up-regulated in the tissues of osteoarthritis patients and chondrocytes stimulated by IL-1β, and the interference with circRNA_0092516

promoted chondrocyte proliferation and inhibited cell apoptosis through miR-337-3p/PTEN axis, thereby improving OA. This might provide new strategies and directions for the treatment of OA, which was of great significance.

5.Declarations

Ethics approval and consent to participate

This study was approved by the Human Ethics Committee of the Third Affiliated Hospital of Suchow University.

Consent for publication

The study was undertaken with the patient's consent.

Availability of data and materials

Not applicable.

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Availability of data and materials

Not applicable.

Conflict of Interest

The authors declare that they have no conflict of interest.

Competing Interests

The authors declare that they have no competing interests.

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Authors' contributions

ZH conceived and designed the study and drafted the manuscript. WM collected the data and JX contributed to the statistical analysis. XD interpreted the data. WL put forward the concept of the study and reviewed the manuscript. All authors read and approved the final manuscript.**Acknowledgements**

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Figures

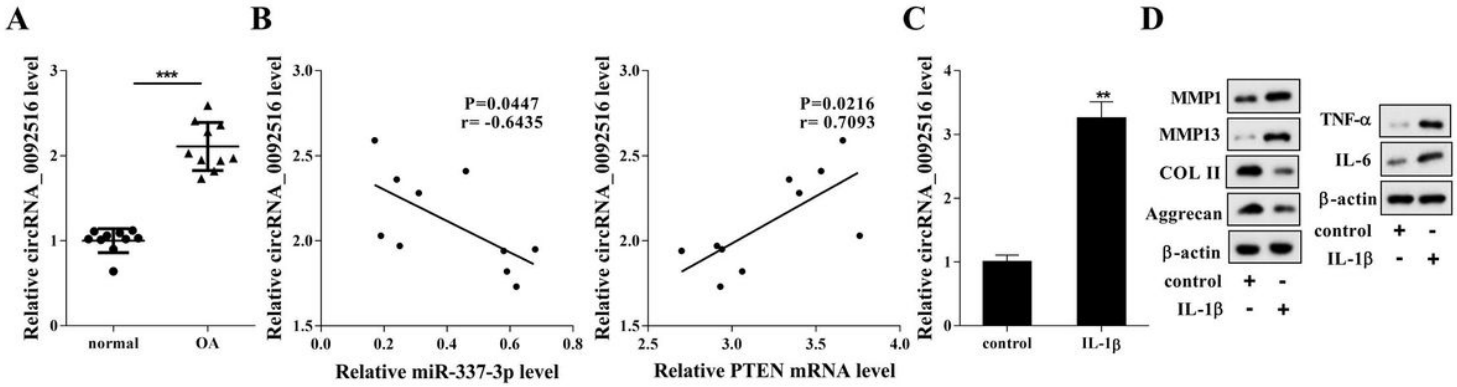

Figure 1

Expression of circRNA_0092516 in the tissues of osteoarthritis patients and chondrocytes stimulated by IL-1β. (A) qRT-PCR was used to detect the expression of circRNA_0092516 in OA cartilage tissues. (B) Pearson correlation analysis was used to analyze the correlation between the expressions of circRNA_0092516 and miR-337-3p, and the expressions of circRNA_0092516 and PTEN. Next, chondrocytes were isolated from normal articular cartilage tissues and then stimulated the cells with 10 ng/mL IL-1β for 24 h to establish an in vitro OA model. (C) Detection of circRNA_0092516 expression. (D) Western blot was used to detect the protein levels of OA marker molecules matrix metallopeptidase 1 (MMP1) and matrix metallopeptidase 13 (MMP13), extracellular matrix metabolism-related molecules COL II and Aggrecan, and inflammatory factors TNF-α, IL-6. ***P<0.001 vs. normal group. **P<0.01 vs. control group.

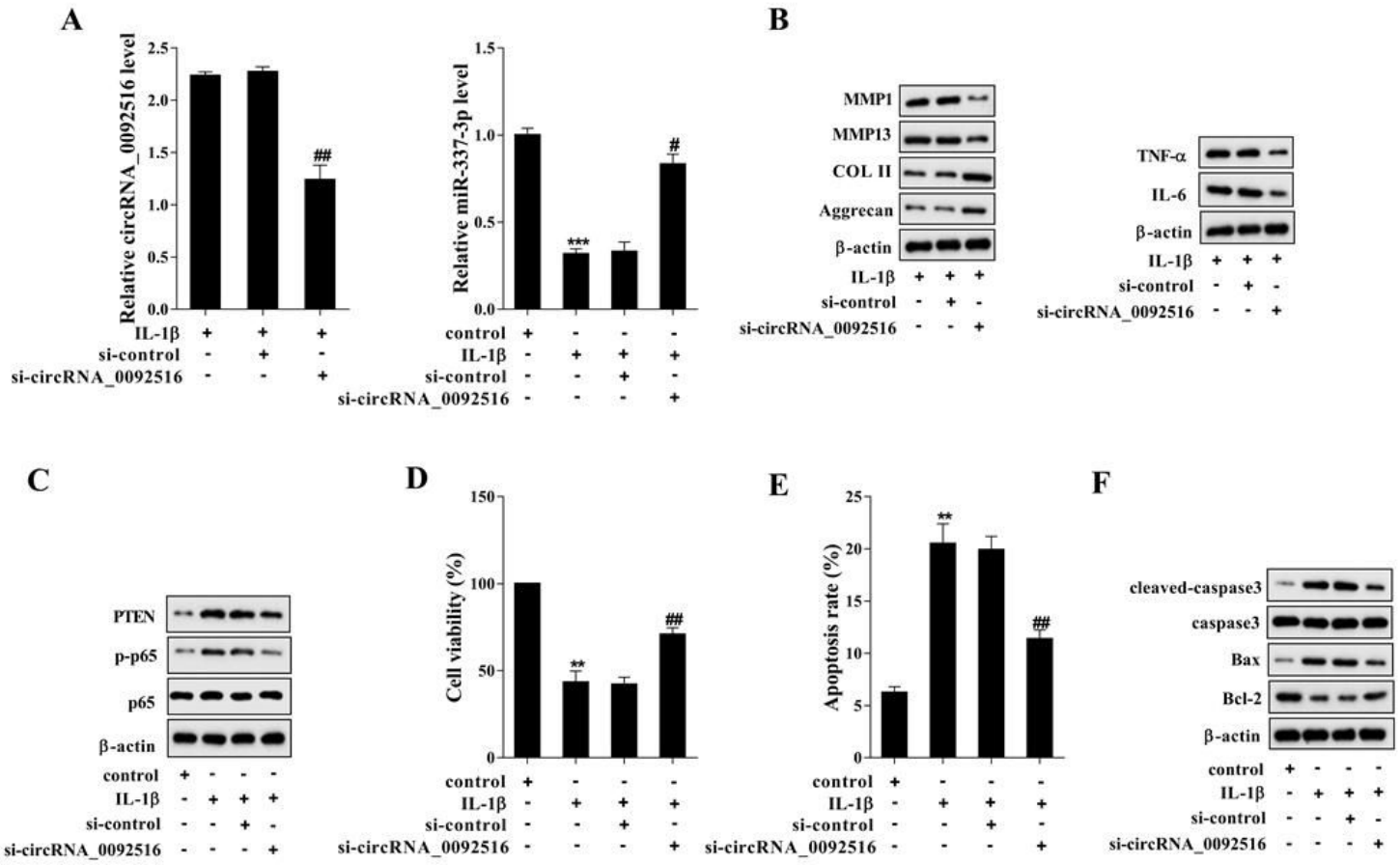

Figure 2

Effect of circRNA_0092516 on chondrocyte proliferation, apoptosis, and inflammatory factors release. Chondrocytes were isolated from normal articular cartilage tissues and transfected si-circRNA_0092516 into chondrocytes and then stimulated the cells with 10 ng/mL IL-1β for 24 h. (A) Detection of circRNA_0092516 and miR-337-3p expressions. (B) Detection of the protein levels of MMP1, MMP13, and inflammatory factors IL-6 and TNF-α, and extracellular matrix metabolism-related molecules COL II and Aggrecan. (C) Detection of the protein levels of PTEN, p-p65, and p65. (D) MTT was used to detect the proliferation of chondrocytes. (E) Flow cytometry was used to detect the apoptosis of chondrocytes. (F) Detection of the protein levels of cleaved-caspase-3, caspase3, Bax, and Bcl-2. #P<0.05, ##P<0.01 vs. IL-1β + si-control group. **P<0.01, ***P<0.001 vs. control group.

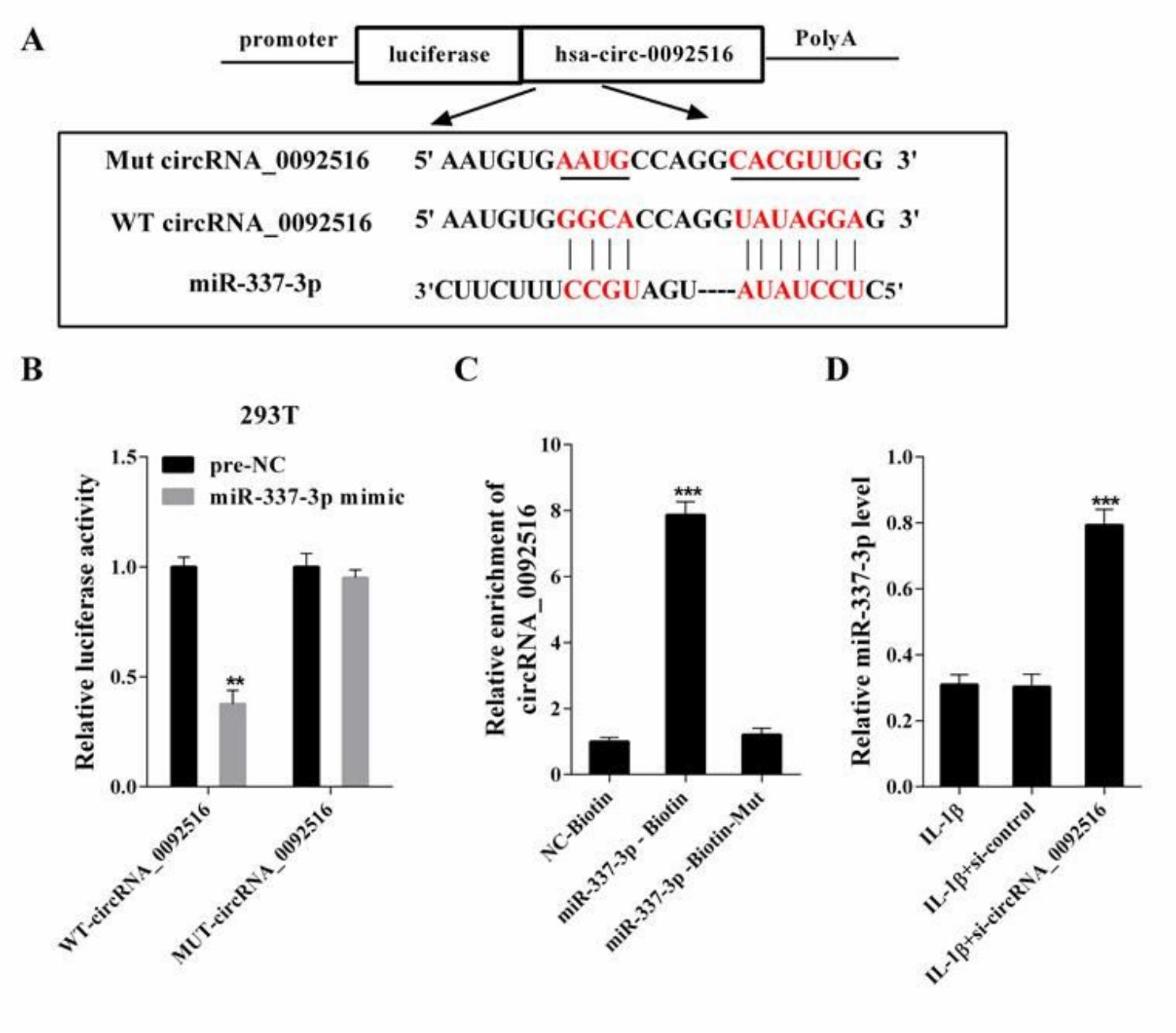

Figure 3

Interaction between circRNA_0092516 and miR-337-3p. (A) Bioinformatics online prediction software was used to analyze the binding sites between circRNA_0092516 and miR-337-3p. (B) Dual-luciferase reporter gene assay was used to detect the effect of miR-337-3p on the luciferase activity of circRNA_0092516. (C) RNA pull-down experiment was used to analyze the interaction between circRNA_0092516 and miR-337-3p. Next, si-circRNA_0092516 was transfected into chondrocytes and then stimulated the cells with 10 ng/mL IL-1β for 24 h. (D) Detection of miR-337-3p expression. **P<0.01 vs. pre-NC group. ***P<0.001 vs. NC-Biotin or IL-1β + si-control group. NC: negative control.

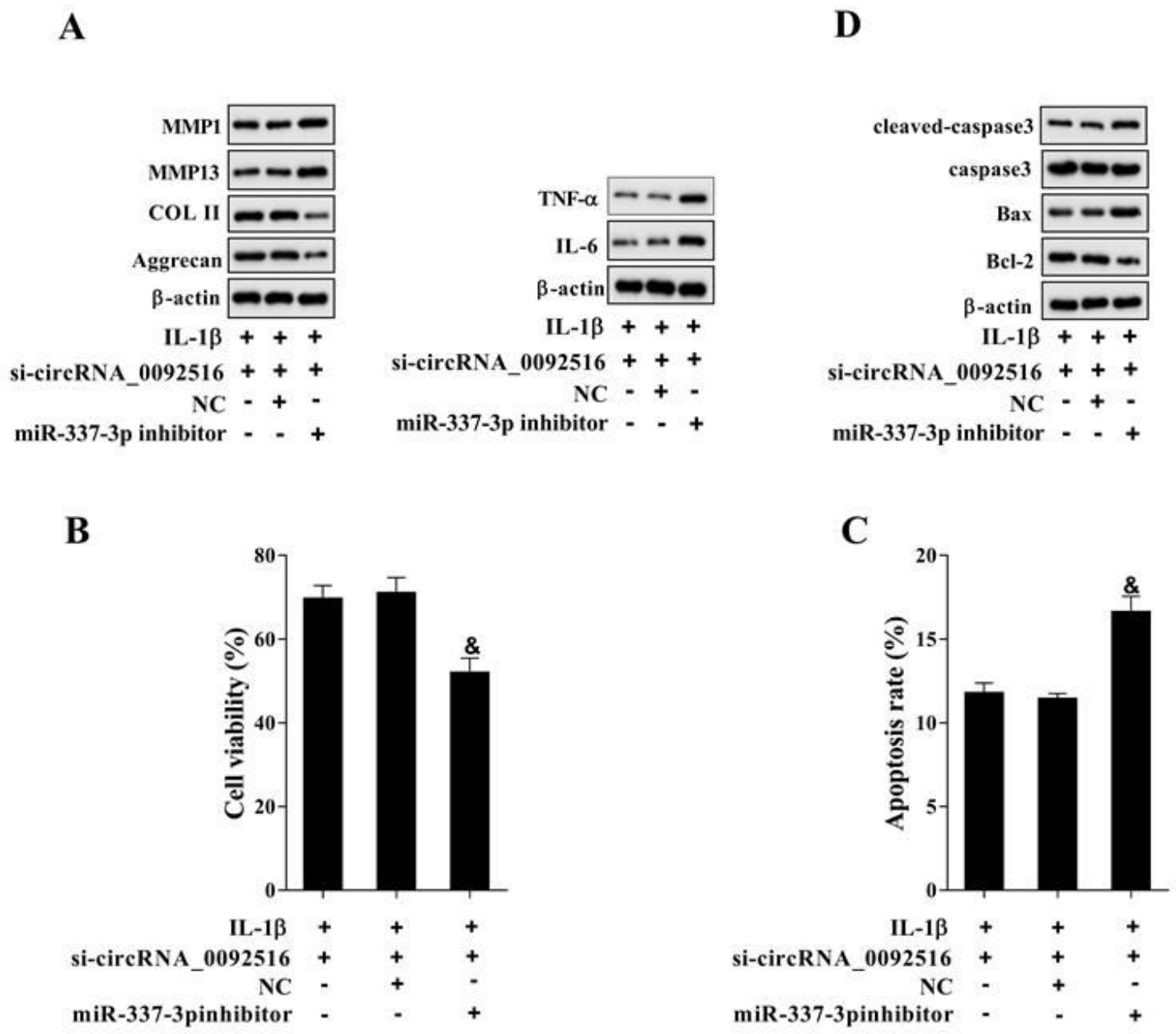

Figure 4

circRNA_0092516 affects chondrocyte proliferation, apoptosis and inflammatory factors release through miR-337-3p. si-circRNA_0092516, si-circRNA_0092516 + miR-337-3p inhibitor was transfected into chondrocytes and then stimulated the cells with 10 ng/mL IL-1β for 24 h. (A) Detection of the protein levels of MMP1, MMP13, IL-6, TNF-α, COL II, and Aggrecan. (B) Detection of the proliferation of chondrocytes. (C) Detection of the apoptosis of chondrocytes. (D) Detection of the protein levels of cleaved-caspase-3, caspase3, Bax, and Bcl-2. &P<0.05 vs. IL-1β + si-circRNA_0092516 + NC group. NC: negative control.

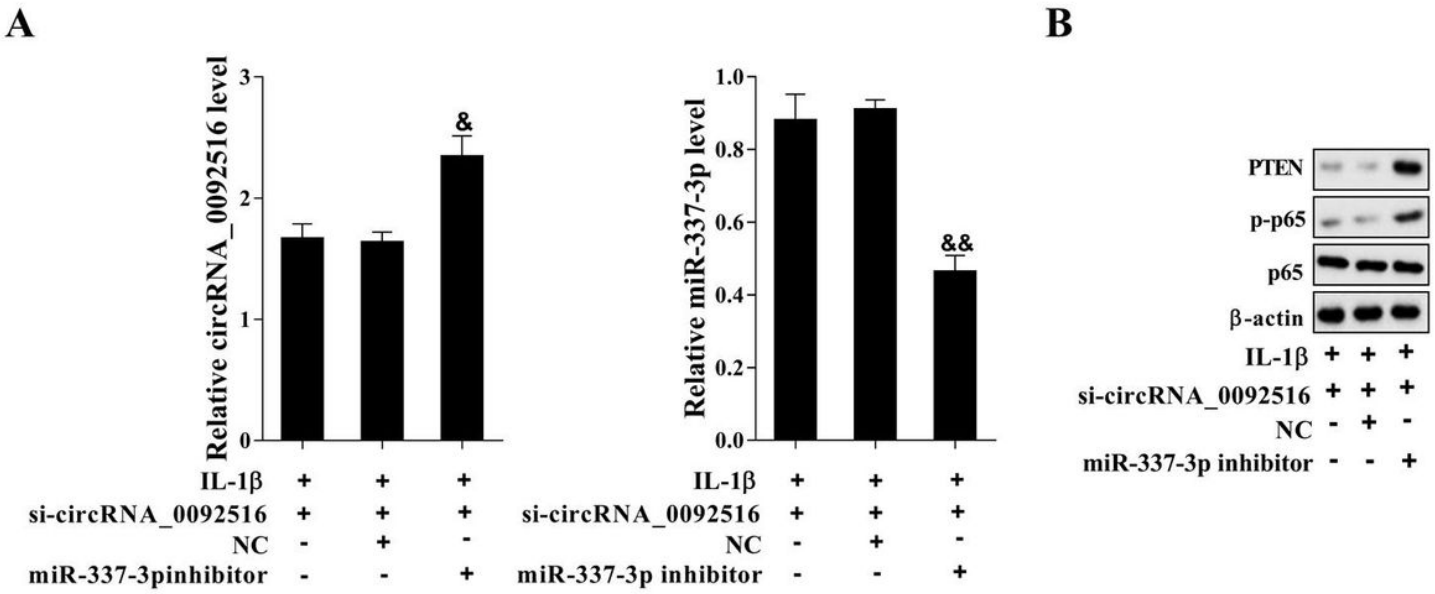

Figure 5

Effect of circRNA_0092516 on PTEN expression and NF-κB signaling pathway through miR-337-3p. si-circRNA_0092516, si-circRNA_0092516 + miR-337-3p inhibitor was transfected into chondrocytes and then stimulated the cells with 10 ng/mL IL-1β for 24 h. (A) Detection of circRNA_0092516 and miR-337-3p expressions. (B) Detection of the protein levels of PTEN, p-p65 and p65. &P<0.05, &&P<0.01 vs. IL-1β + si-circRNA_0092516 + NC group. NC: negative control.