MiR-140-3p Ameliorates the Progression of Osteoarthritis via Targeting CXCR4

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Abstract

Osteoarthritis is a common disease character with progressive destruction of cartilage. MiR-140-3p was validated as a biomarker for osteoarthritis. However, the mechanism by which miRNA-140-3p regulates osteoarthritis remains unclear. Thus, this study aims to evaluate the potential function of miRNA-140-3p during the pathogenesis of osteoarthritis. MiRNA-140-3p expression in tissue and CHON-001 chondrocyte cells was determined with qRT-PCR. *In vitro* osteoarthritis model was established by treatment of the chondrocyte cells CHON-001 with IL-1β. Cell proliferation and apoptosis were measured with CCK8 and Annexin V/PI apoptosis assay, respectively. Protein expressions were evaluated using western blot. The target gene of miR-140-3p was predicted using Targetscan and miRDB. MiR-140-3p was downregulated in knee tissue from patients with osteoarthritis. IL-1β inhibited the proliferation of CHON-001 cells via inducing apoptosis. In addition, IL-1β significantly inhibited the expressions of collagen II and aggrecan and increased the level of MMP13. However, the effects of IL-1β could be ameliorated by the addition of miR-140-3p mimics. Moreover, luciferase reporter assay demonstrated CXCR4 as a target gene of miR-140-3p. IL-1β-induced upregulation of CXCR4 could be blocked by miR-140-3p mimics. Our study indicated that miR-140-3p could suppress the progression of osteoarthritis by directly targeting CXCR4. Therefore, miR-140-3p might serve as a potential therapeutic target for the treatment of osteoarthritis.

**Keywords:** MicroRNA-140-3p, Interleukin-1β, chemokine (C-X-C motif) receptor 4, proliferation, apoptosis, osteoarthritis
Introduction

Osteoarthritis (OA) is the most common type of disease in joint, which could lead to joint pain and disability.\textsuperscript{1} Osteoarthritis is characterized by continuous articular cartilage destruction, which leading to a loss of balance between the synthesis and degradation of extracellular matrix of articular cartilage cells. Factors associated with occurrence of osteoarthritis include aging, genetic, mechanical and environmental components.\textsuperscript{2,3} IL-1\textsubscript{β} plays a central role in the pathophysiology of cartilage injury and degradation in arthritis.\textsuperscript{4} In addition, IL-1\textsubscript{β} can participate in the persistence of arthritis by directly stimulating synoviocytes and enhancing the matrix degradation.\textsuperscript{4} Despite the high prevalence of osteoarthritis, studies focusing on its pathobiology and associated genetic factors in the pathogenesis of osteoarthritis remains very limited.

MicroRNAs (miRNAs) are a type of small (about 22 nucleotides) non-coding RNAs. It could regulate gene expression via targeting mRNAs complementary and blocking mRNA cleavage or protein translation.\textsuperscript{5} Emerging evidences have suggested that miRNAs are associated with several key cellular processes including apoptosis, differentiation, development and lipid metabolism. In addition, miRNAs have also been validated to be correlated with pathogenesis of OA. Song and his colleagues reported that miR-222 regulated MMP-13 through targeting HDAC-4 during the progression of osteoarthritis.\textsuperscript{6} Meanwhile, Wang et al. reported miR-142-3p inhibited apoptosis of chondrocyte and inflammation in osteoarthritis by blocking NF-kB signaling pathway mediated by HMGB1.\textsuperscript{7} A recent study using analysis of serum microRNA array identified that miR-140-3p was a potential biomarker of osteoarthritis.\textsuperscript{8} In non-small cell lung cancer (NSCLC), miR-140-3p functioned as a tumor suppressor. It inhibited cell growth and migration via induced apoptosis in NSCLC.\textsuperscript{9} It was reported that dysregulation of miR-140-3p in synovial fluid was correlated with severity of osteoarthritis; meanwhile, miR-140-3p inhibited chondrocyte apoptosis and inflammation in osteoarthritis.\textsuperscript{7,10} However, the mechanism by which miRNA-140-3p regulates osteoarthritis remains unclear. Thus,
this study aims to evaluate the potential function of miRNA-140-3p during the pathogenesis of osteoarthritis.

Methods

**Patient samples**

Osteoarthritis samples were collected from 30 patients experienced total knee replacement surgery at The First Affiliated Hospital of Soochow University. Cartilage samples from 30 traumatic amputees without known history of joint disease were used as control. Osteoarthritis was diagnosed according to the American College of Rheumatology criteria. Informed consent was obtained from all patients. The study was conducted following the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of The First Affiliated Hospital of Soochow University.

**Cell culture**

CHON-001, a chondrocyte cell line of human, was purchased from ATCC (ATCC, Rockville, MD, USA). CHON-001 was culture in Dulbecco's Modified Eagle's Medium supplemented with 0.1 mg/mL G-418 (Gibco, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). Cells were incubated in humidified atmosphere with 5% CO₂ at 37°C. CHON-001 cells were passaged at a ratio of 1:5. For OA model establishment, CHON-001 were treated with IL-1β (10 ng/ml) for 24 h before experiments.

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

Total RNA was obtained using Trizol reagent (Invitrogen, Carlsbad, CA, USA). SuperScript II RNase H Reverse Transcriptase Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to synthesize cDNA according to the manufacturer’s instructions. cDNA expression was quantified by real-time PCR using 7900HT Fast
Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The procedure of qPCR: initial denaturation (95°C, 10 min), denaturation (95°C, 15 s, 40 cycles), and annealing extension (60°C, 30 s). The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ (Ct means cycle threshold) method. U6 was used as control. All experiments were carried out at least in triplicate. Primers of miR-140-3p and U6 were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

**Western blot assay**

CHON-001 cells were lysed with SDS buffer (50 mM Tris-HCl pH 6.8, 5 mM EDTA, 2% SDS and 5% glycine). Protein concentration was measured by BCA Protein Assay Kit (Thermo Fisher, Waltham, MA, USA). Equal amounts (30 μg) of protein from each sample were separated in a 10% SDS-PAGE at 80V for 2 h. The separated proteins were transferred onto PVDF membranes (Millipore, Billerica, MA, USA) at 200 mA for 3 h. Next, membranes were blocked with 5% non-fat milk in TBST (20 mM Tris pH 7.6, 150 mM NaCl and 0.05% Tween-20) for 1 h, and followed by incubation with primary antibodies at 4°C for overnight. Then, membrane was washed thrice with TBST and incubated with HRP (horseradish peroxidase)-conjugated goat anti-rabbit secondary antibody (Santa Cruz, dilution 1:5000) for 2 h at room temperature. For tissue samples, dissect the tissue with clean scissors on ice. 300 μL of ice cold NP40 lysis buffer (150 mM sodium chloride, 1.0% NP-40, and 50 mM Tris pH 8.0) was added rapidly to the tube, and homogenized with an electric homogenizer (PowerLyzer 24 Homogenizer, Qiagen, Chadstone, VIC, Australia). Each sample was added with more lysis buffer (400 μL) and maintained constant agitation for 2 h at 4°C on an orbital shaker in the fridge. Then, tubes were centrifuge for 20 min at 12,000 rpm at 4°C. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice. Then the tissue lysate was used for western blot following above procedures. The primary antibodies were: MMP-13 (E4W3T) Rabbit mAb (1:1000, cat. #69926, Cell Signaling Technology, Danvers, MA, US), MMP-3 (D7F5B) Rabbit mAb (1:1000, cat. #14351, Cell Signaling...
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Technology, Danvers, MA, US), MMP-9 (D6O3H) XP® Rabbit mAb (1:1000, cat. #13667, Cell Signaling Technology, Danvers, MA, US), β-Actin (13E5) Rabbit mAb (1:1000, cat. #4970, Cell Signaling Technology, Danvers, MA, US), Anti-Collagen II antibody (1:1000, cat. #ab34712, Abcam, Cambridge, MA, US), Rabbit Anti-Aggre can antibody (1:1000, cat. #ab36861, Abcam, Cambridge, MA, US), and CXCR4 antibody (12G5) (1:1000, cat. #35-8800, Invitrogen, Waltham, MA, US), Anti-p-STAT3 antibody (1:1000, cat. #ab76315, Abcam), Anti-STAT3 antibody (1:1000, cat. #ab119352, Abcam), Anti-p-JAK2 antibody (1:1000, cat. #ab195055, Abcam), Anti-JAK2 antibody (1:1000, cat. #ab108596, Abcam).

**Immunofluorescence**

Cells were washed with PBS three times and fixed in pre-cold methanol for 10 min at -20°C. Next, cells were incubated with primary antibodies for anti-Ki67 (Abcam; ab15580) (1:1000), DAPI (ab104139) at 4°C overnight. Subsequently, cells were incubated with goat anti-rabbit IgG second antibody (Abcam; ab150077) (1:5000) at 37°C for 1 h. Cells were observed by fluorescence microscope (Olympus CX23 Tokyo, Japan) and cell number was counted by Image J software.

**Cell proliferation assay**

Cell proliferation assay was conducted on the basis of colorimetric assay using cell counting kit-8 (CCK8, Dojindo, Kumamoto, Japan). Cells were incubated for 24, 48 and 72 h at 37°C. After that, each well was mixed with 10 μl CCK-8 solution. Next, cells were incubated for another 1 h and cell absorbance (OD450) was detected using microplate reader.

**Cell apoptosis assay**

A total of 5×10⁵ cells were collected by centrifuging (10000 g) for 10 min at 4°C. Double staining with annexin V–fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (BioVision, St Lake Worth, FL, USA) was performed following
manufacturer’s protocol, and cell apoptosis was analyzed by flow cytometry. The percentage of apoptotic cells was measured using Cell Quest software (Becton, Franklin Lakes, NJ, USA).

**Luciferase reporter assay**

P-MIR-report plasmid (0.25 mg, Ambion, Austin, TX, USA) containing the 3’-UTR of CXCR RNA (mouse) and miR-140-3p (50 nM) were transfected into chondrocytes using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Luciferase activity was detected 48 h later using luciferase assay kit (Promega, Madison, WI, US).

**ROS analysis**

ROS generation was determined by using 29,79-dichlorodihydrofluorescein (DCFH-DA; Sigma Aldrich, St. Louis, MO, USA). CHON-001 cells (2×10^4 cells per well) were plated onto fluorescent 96-well plates overnight at 37°C. After that, 10 nM miRNA mimics were transfected into CHON-001 cells for 12 h, followed by treatment of IL-1β (10 ng/ml) for 48 h. Later on, cells were incubated with 10 mM DCFH-DA in the dark for 30 min. Finally, the fluorescent signals were measured by flow cytometry.

**Statistical analysis**

GraphPad Prism (GraphPad software, Inc., San Diego, CA, USA) was used to conduct data analysis. Comparisons for two groups were analyzed by Student’s t-test. Comparisons among multiple groups were analyzed with ANOVA followed by Tukey’s test. Each group was executed at least three independent experiments and all data was presented as the mean ± SD. P < 0.05 indicates a significant difference statistically.
Results

Differentiate expressions of miR-140-3p and inflammatory mediators in human osteoarthritic cartilage and control tissues

In order to explore the function of miR-140-3p in OA, qPCR was used to assess the expression of miR-140-3p in knee OA cartilage tissues (n=30) and normal cartilage tissues (n=30). We found that the level of miR-140-3p was significantly suppressed in OA tissue than that in normal tissue (Figure 1A, p < 0.01). Next, we examined the protein expression of cartilage extracellular matrix components (aggrecan and collagen II) and bone metabolic genes (MMP13) in three knee OA cartilage tissue samples and three normal cartilage tissue samples. As expected, protein levels of aggrecan and collagen II were markedly decreased, while protein level of MMP13 were strongly increased in OA tissues compared with control (Figure 1B). Protein expression levels of aggrecan, collagen II and MMP13 were quantified and presented in Figure 1C-E. These data suggested the level of miR-140-3p was notably downregulated in OA cartilage tissues.

MiR-140-3p mimics alleviated IL-1β-induced proliferation inhibition in CHON-001 cells

To investigate whether miR-140-3p affected IL-1β-induced matrix degradation, we transfected miR-140-3p mimics into the chondrocytes. The result indicated miR-140-3p mimics increased the level of miR-140-3p by 7-8 folds, indicating the successful transfection of miR-140-3p in CHON-001 cells (Figure 2A). In addition, the CCK8 assay showed that IL-1β significantly inhibited the cell viability of CHON-001 cells compared with control group, which could be reversed by miR-140-3p mimics (Figure 2B). Meanwhile, the data of immuno-staining of Ki67 was consistent with CCK8 assay (Figure 2C, 2D). These results indicated miR-140-3p mimics could alleviate IL-1β-induced proliferation inhibition in CHON-001 cells.
MiR-140-3p mimics attenuated IL-1β-induced apoptosis of CHON-001 cells

Next, we evaluated the effect of IL-1β or/and miR-140-3p mimics on the expressions of aggrecan, collagen II and MMP13 in CHON-001 cells. Western blot showed that addition of IL-1β inhibited expressions of aggrecan and collagen II and increased the level of MMP13 compared with control group (Figure 3A). Meanwhile, addition of miR-140-3p mimics completely abrogated the effects of IL-1β on the expressions of aggrecan, collagen II and MMP13 (Figure 3A). However, miR-140-3p mimics alone treatment had very limited effects on the expressions of these proteins. Quantification of western blot data was shown in Figure 3B-D. This result indicated that miR-140-3p mimics might function in alleviation of osteoarthritis progression.

We further measured the effect of miR-140-3p mimics on cell apoptosis. Treatment of IL-1β markedly stimulated the apoptosis of CHON-001 cells and addition of miR-140-3p mimics reversed such stimulation induced by IL-1β (Figure 3E, 3F). In addition, IL-1β-induced ROS production in CHON-001 cells was significantly reversed following transfection with miR-140-3p mimics (Supplementary figure 1A and 1B). In summary, above results indicated that miR-140-3p mimics promoted the proliferation, inhibited the apoptosis and suppressed the ROS production induced by IL-1β in CHON-001 cells, indicating the potential of miR-140-3p in the pathogenesis of OA.

MiR-140-3p directly targeted CXCR4

To further investigate the mechanism by which miR-140-3p regulated the progression of OA, we predict the target genes of miR-140-3p using online tools Targetscan (http://www.targetscan.org/vert_71/) and miRDB (http://www.mirdb.org/). We found that CXCR4 3’-UTR contains putative miR-140-3p seed site, indicating that CXCR4 may be a putative target gene of miR-140-3p (Figure 4A). Next, we conducted the luciferase assay to assess the association of miR-140-3p and CXCR4. Two constructs which expressed wildtype CXCR4 (WT-CXCR4) and mutant RET
(MT-CXCR4) were established (Figure 4B). After 24 h of transfection, the promoter activities of WT-CXCR4 and MT-CXCR4 constructs were determined. Co-transfection of WT-CXCR4 with miR-140-3p mimics markedly reduced the expression of luciferase compared with control group, whereas such reduction of luciferase expression was not found in cells co-transfected with MT-CXCR4 and miR-140-3p mimics (Figure 4C). Next, we further validated the correlation of CXCR4 and miR-140-3p using qRT-PCR. The result indicated that transfection of miR-140-3p mimics could significantly decrease the mRNA level of CXCR4 with or without IL-1β (Figure 4D). These observations suggested that CXCR4 was a putative target of miR-140-3p in chondrocyte cells.

**MiR-140-3p mimics inhibited the expressions of CXCR4 in IL-1β stimulated chondrocytes**

Next, we evaluated the effect of IL-1β on protein level of CXCR4 with western blot. The result showed that an inverse correlation between miR-140-3p and CXCR4 expression was observed (Figure 5A). In addition, miR-140-3p mimics significantly suppressed protein expression p-JAK2, p-STAT3, MMP3 and MMP9 in chondrocytes with or without IL-1β (Figure 5A-5F). This result further confirmed that CXCR4 a direct target of miR-140-3p and upregulation of miR-140-3p may alleviate the pathogenesis of OA.

**Discussion**

In this study, we demonstrated that expression of miR-140-3p was significantly down-regulated in knee OA cartilage. Upregulation of miR-140-3p efficiently affected on OA-associated cartilage extracellular matrix components and bone metabolic genes, implied that miR-140-3p was possibly involved in pathogenesis of OA. In addition, we found miR-140-3p mimics reversed IL-1β-induced proliferation inhibition in CHON-001 via decreasing cell apoptosis. The novelty of current study is that CXCR4 was a putative target of miR-140-3p and could be regulated in IL-1β
stimulated chondrocytes.

The functions of miR-140-3p in cell survival, proliferation and invasion have already been reported in several studies. Yang and his colleagues reported that miR-140 was stimulated by Sox9 and then targeted Sp1 to maintain the proliferation of chondrocytes.\(^{12}\) It was also reported that miR-140-3p inhibited cell proliferation, migration and invasion of lung cancer cells by targeting ATP6AP2.\(^{13}\) Based on these above evidences that miR-140-3p was closely correlated with cell survival, proliferation and invasion. We confirmed that miR-140-3p stimulated cell proliferation and decreased cell apoptosis in CHON-001 chondrocyte cells.

Several miRNAs have been confirmed to be functional in chondrocyte cells and pathogenesis of OA besides of miR-140-3p. In patients with osteoarthritis, the expression of endogenous miR-21 is up-regulated, and its over-expression could weaken cartilage formation by targeting GDF-5.\(^{14}\) MiR-27b was down-regulated by NF-kappa B and MAPK and regulated the expression of matrix metalloproteinase 13 in human osteoarthritis chondrocytes.\(^{15}\) Although modulation of miR-140-3p was reported in chondrocyte cells, there is barely report on the mechanism underlying effect of miR-140-3p on OA. In this study, upregulation of miR-140-3p markedly increased proliferation and reduced apoptosis in CHON-001 chondrocyte cells, suggesting that miR-140-3p may be a potential target for the treatment of OA.

CXCR4 is a G-protein-coupled receptor which expressed by articular chondrocyte.\(^{16}\) It was reported that CXCL12/CXCR4 axis regulated activation of aggrecanase and degradation of cartilage in a rat model with post-traumatic osteoarthritis.\(^{17}\) Osteoarthritis could be attenuated via blocking of the SDF-1/CXCR4 signaling pathway.\(^{18}\) CXCR4 was confirmed to be regulated by microRNAs. MiR-494-3p could target CXCR4 to suppress invasion, migration and proliferation of prostate cancer cell.\(^{19}\) MiR-150 regulated migration and mobilization of monocytes derived from bone marrow through miR-150/CXCR4 dependent mechanism.\(^{20}\) In this study, we validated that CXCR4 functioned as a putative target of miR-140-3p and
could be regulated in IL-1β-stimulated chondrocytes. In addition, we observed that miR-140-3p mimics significantly suppressed protein expression of CXCR4, as well as p-JAK2, p-STAT3, in IL-1β-stimulated chondrocytes. Moreover, overexpression of miR-140-3p markedly upregulated the expressions of extracellular matrix (ECM)-related proteins aggrecan and collagen II, and downregulated the expressions of matrix metalloproteinases MMP3, MMP9 and MMP13 in IL-1β-stimulated chondrocytes. Liu et al found that CXCR4 could activate the JAK2/STAT3 signaling in breast cancer.\textsuperscript{21} Yao et al indicated that DUSP19 could suppress IL-1β-induced expressions of MMP3 and MMP9 in chondrocytes via through inactivating JAK2/STAT3 pathway.\textsuperscript{22} In addition, leptin increased the production of MMP enzymes and induced the apoptosis of chondrocytes via activating JAK2/STAT3 pathway.\textsuperscript{23} Furthermore, Li et al indicated that omentin-1 upregulated the expressions of aggrecan and collagen II in IL-1β-stimulated CHON-001 cells via blocking the JAK-2/STAT3 pathway.\textsuperscript{24} Therefore, our results indicated that overexpression of miR-140-3p ameliorated the progression of osteoarthritis via suppressing CXCR4, thereby leading to inactivating JAK2/STAT3 signaling, and then inhibiting the expressions of matrix metalloproteinases, as well as increasing the expressions of ECM-related proteins.

In sum, we suggested that miR-140-3p was down-regulated in OA cartilage tissues. Manipulation of miR-140-3p was closely correlated with proliferation and apoptosis of IL-1β stimulated chondrocytes via targeting CXCR4. This study indicated miR-140-3p might serve as a potential therapeutic target for the treatment of osteoarthritis.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials.
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Figure 1. MiR-140-3p was down-regulated in cartilage with OA. (A) The levels of miR-140-3p in cartilage tissues from 30 patients with osteoarthritis and 30 traumatic amputees were measured by real-time PCR. (B) Comparisons of protein expressions of Aggrecan, Collagen II and MMP13 in cartilage tissues from one patient with osteoarthritis and one non-OA donor. (C-E) Quantification of aggrecan, collagen II and MMP13 in three knee OA cartilage tissue and three normal cartilage tissue samples by normalizing to β-actin. ** indicates P<0.01, compared with control group. All experiments were conducted in triplicate.
Figure 2. MiR-140-3p mimics alleviated IL-1β-induced proliferation inhibition in CHON-001 cells. (A) CHON-001 cells were transfected with miR-140-3p mimics for 12 h. QRT-PCR was applied to detect the level of miR-140-3p in CHON-001 cells. (B) 10 nM miRNA mimics or 10 nM scrambled nucleotides were transfected into CHON-001 cells for 12 h, followed by treatment of IL-1β (10 ng/ml) for 24 h, 48 h and 72 h. Cell viability was detected with CCK8 assay. (C) CHON-001 cells were immunostained with Ki-67 and DAPI for cell proliferation evaluation. Scale bar = 100 μm. (D) Ki-67 positive cells were quantified. ** indicates P<0.01, compared to control group; ## indicates P<0.01, compared to IL-1β group. All experiments were conducted in triplicate.
Figure 3. MiR-140-3p mimics attenuated IL-1β-induced apoptosis of CHON-001 cells. 10 nM miRNA mimics were transfected into CHON-001 cells for 12 h, followed by treatment of IL-1β (10 ng/ml) for 48 h. (A) Western blot were conducted to measure the protein levels of aggrecan, collagen II and MMP13 in IL-1β-treated CHON-001 cells. (B-D) Quantification of aggrecan, collagen II and MMP13 expressions in CHON-001 cells by normalizing to β-actin. (E-F) Apoptosis were evaluated with Annexin V/PI staining. ** indicates P<0.01, compared to control group; ### indicates P<0.01, compared to IL-1β group. All experiments were conducted in triplicate.
Figure 4. CXCR4 was a target of miR-140-3p. (A) Prediction of a putative miR-140-3p target site in the 3'-UTR of CXCR4 mRNA. (B) Establishment of WT-CXCR4 and MT-CXCR4 3'-UTR constructs for luciferase assay. (C) Luciferase activity of CHON-001 cells transfected with miR-140-3p mimics/miR-Scr and WT-CXCR4/MT-CXCR4 3'-UTR constructs. Luciferase activity was detected at 48 h. (D) 10 nM miRNA mimics were transfected into CHON-001 cells for 12 h, followed by treatment of IL-1β (10 ng/ml) for 48 h. Effect of miR-140-3p on the protein level of CXCR4 in the presence or absence of IL-1β. ** indicates P<0.01, compared to miR-Scr groups. All experiments were conducted in triplicate.
Figure 5. MiR-140-3p mimics inhibited the expressions of CXCR4, MMP3 and MMP9 in IL-1β stimulated chondrocytes. (A) Effect of miR-140-3p mimics on the protein level of CXCR4. (B) Quantification of CXCR4 expression in CHON-001 cells by normalizing to β-actin. (C, D) Quantification of p-JAK2 and p-STAT3 expressions in CHON-001 cells by normalizing to JAK2 and STAT3 respectively. (E, F) Quantification of MMP3 and MMP9 expressions in CHON-001 cells by normalizing to β-actin. ** indicates P<0.01, compared to control group; ## indicates P<0.01, compared to IL-1β group. All experiments were conducted in triplicate.