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miR-138 suppressed the progression of osteoarthritis mainly through targeting p65

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Abstract. – OBJECTIVE: MicroRNAs are reported to play key roles in regulating the main risk factors for osteoarthritis (OA) chondrogenesis. In the current study, we focused on miR-138, which has never been explored in OA.

PATIENTS AND METHODS: The expression of miR-138 and p65 was explored in the cartilage tissues of OA patients and compared with those of normal controls. We then explored the effects of miR-138 on NF-κB signaling activation in both human OA chondrocytes and chondrogenic SW1353 cells in the presence of 10 nM TNFα. The protein levels of p65, COX-2 and IL6 were determined using Western blot analysis. To validate the target gene of miR-138, a dual luciferase reporter assay was performed.

RESULTS: The level of miR-138 was markedly reduced in the OA cartilage tissues compared with those of normal controls. Real-time PCR analysis demonstrated that the level of miR-138 decreased after TNFα treatment for 3, 6, and 12 h in the normal chondrocytes and OA chondrocytes. Furthermore, overexpression of miR-138 suppressed the protein levels of p65, COX-2 and IL6 in human OA chondrocytes and chondrogenic SW1353 cells. A dual luciferase reporter assay demonstrated that miR-138 significantly suppressed the relative luciferase activity of pmirGLO-p65-3'UTR. More importantly, treatment with TNFα significantly enhanced the protein levels of p65, COX-2 and IL6. However, overexpression of miR-138 could partially abolish such effects.

CONCLUSIONS: We demonstrated that reduced miR-138 expression enhanced the destruction of the cartilage tissues among OA patients, mainly through targeting p65.

Key Words:
miR-138, Osteoarthritis, p65, Inflammatory responses.

Introduction

Osteoarthritis (OA) is one of the most prevalent degenerative joint diseases, characterized by pain, tenderness, limitation of movement, and joint inflammation[1,2]. Multiple factors are found to be involved in the pathogenesis of OA, including genetic predisposition, altered mechanical loading and abnormal expression of genes in the articular chondrocytes[3,4].

MicroRNAs (miRNAs) are a group of small non-coding RNAs that are suggested to participate in the progression of OA[5,6]. Studies have shown that miRNAs exert their roles through regulating the main risk factors for OA, including modulating the expression of genes related to aging and inflammation[7]. For instance, miR-146 and miR-155 are reported to be upregulated, thereby suppressing the expression of MMP-3 in synovial fibroblasts, which then participate in the development of rheumatoid arthritis (RA)[6]. Additionally, miR-210 is shown to suppress DR6, thereby inhibiting NF-κB signaling in OA[8]. Thus, further study between miRNAs and the pathogenesis of OA may shed light on novel therapy methods for clinicians.

Upregulation of pro-inflammatory cytokines in the synovial fluid (SF) is reported to be an early hallmark of OA, and the nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) signaling plays a key role in the immune response[9-11]. Aberrant activation of NF-κB has been widely reported in the development of OA[9-11]. However, the specific micro-environmental factors that affect NF-κB signaling have not been fully understood. Recently, miRNAs have been reported to negatively regulate NF-κB activation and the subsequent secretion of pro-inflammatory cytokines[12,13]. In the current paper, we explored the expression of miR-138 in the tissues of OA patients. Our data showed that the NF-κB p65 subunit was a target gene of miR-138. Through targeting p65, decreased miR-138 in the chondrocytes enhanced NF-κB activation, triggering the inflammatory response.

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Patients and Methods

Patients

Human tissue was obtained from the Sir Run Hospital Nanjing Medical University. OA cartilage samples were collected from 22 patients (mean ± SD age 64.12 ± 5.75 years) who underwent total joint arthroplasty at Sir Run Hospital Nanjing Medical University. OA was diagnosed according to the Chinese College of Rheumatology criteria (28,29). Normal cartilage samples were obtained from trauma patients with no known history of OA or RA (n = 3). This investigation was approved by the Human Ethics Committee of Sir Run Hospital Nanjing Medical University. Each donor signed an informed consent.

Preparation of Chondrocytes

The cartilage and subchondral bone specimens were washed with sterile phosphate-buffered saline (PBS). Macroscopic cartilage degeneration was examined by India ink staining[14]. To prepare chondrocytes, cartilage specimens with smooth articular surfaces were selected and enzymatic digestion performed as previously described [15]. Primary OA chondrocytes at 80% confluence were applied for this study.

Primary chondrocytes and human chondrogenic SW1533 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) supplemented with streptomycin (100 μg/mL) and penicillin (100 U/mL) (Life Technologies, Rockville, MD, USA).

Chondrocyte Treatment and Preparation of microRNA

Before TNFα (20 nM) treatment, OA chondrocytes were serum starved overnight, and total RNA was prepared using TRIzol reagent (Invitrogen). To purify microRNAs, the mirVana Kit (Applied Biosystems) was used according to the manufacturer's instructions. MicroRNAs were isolated from normal and OA cartilage samples. In brief, cartilage was ground to a fine powder in liquid nitrogen, and then the microRNA was purified as described above.

Transfection of miRNAs

Human chondrocytes were transfected with the mature type of hsa-miR-138 or the antisense inhibitor, anti-miR-138, at a final concentration of 50 nM using the calcium phosphate precipitation method. SW1353 cells were seeded in 12- or 24-well plates. The following day, cells were transfected with miR-138 or anti-miR-138 at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For the following experiments, cells were used 48 h after transfection. Nonspecific control miR (miR-Control, Dharmacon Inc., Lafayett, CO, USA) was used as a control for off-target effects.

Reverse Transcription—PCR (RT-PCR) Analysis

TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) were used to analyze the expression of miR-138. U6 snRNA was used as a loading control[16]. Real-time PCR for miRNAs was performed with the SYBR Green PCR mixture (Invitrogen, Carlsbad, CA, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Western Blotting

Total proteins were isolated from tissues using a total protein extraction kit (Keygen, Nanjing, China). A total of 20 μg protein was separated using SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes and blocked with 5% fat-free milk at room temperature for 2 h. The immunoblot was incubated with primary antibody detecting p65 (1:1000 dilution; CST) and GAPDH (1:1000 dilution; CST) was used as a control. The signals were detected using a Super ECL Plus Kit (Keygen, China) and determined by quantitative analysis using UVP software (UVP, LLC, Upland, CA, USA).

Luciferase Reporter Assay

HEK293T cells were seeded in 24-well plates at a density of 5×10^4 cells/well. After 24 h, wild-type or mutated p65 3'-UTR luciferase reporter vector and miR-138 mimic or inhibitor were transfected into the cells with Vigofect transfection reagent, used according to the manufacturer's instructions. After transfection for 48 h, the Dual-luciferase reporter assay system (Promega, Madison, WI, USA) was applied to determine the change in relative luciferase units (RLU). Renilla activity was used as the internal control.

Quantitative Analysis of Inflammatory Cytokines

Enzyme-linked immunosorbent assay (ELISA) kits for IL-6, IL-8, and TNFα were purchased from

Peprotech (Rocky Hill, NJ, USA), and the assays were carried out according to the manufacturer's instructions. Samples were run in duplicate, and each experiment was repeated independently at least three times.

Statistical Analysis

Comparisons were performed using the Origin 6.1 software package (1 paired 2-tailed t-test with one-way analysis of variance and Tukey's post hoc analysis). P values less than 0.05 were considered significant, and *p* values less than 0.001 were considered highly significant.

Results

The Expression of miR-138 and TNFα in Normal and OA Cartilage

We evaluated the expression of miR-138 and TNFα in normal and OA cartilage tissues. As shown in Figure 1A, the level of miR-138 was markedly reduced in the OA cartilage tissues compared with those of normal controls. Furthermore, the level of TNFα was found to be significantly enhanced in the OA cartilage compared with the control (Figure 1B).

The Reverse Correlation Between TNFα-regulated NF-κB and miR-138 Expression in normal and OA Chondrocytes

To investigate whether miR-138 was involved in TNFα-induced NF-kB activation, the normal chondrocytes and OA chondrocytes were treated with 10 nM TNFα. Real-time PCR analysis demonstrated that the level of miR-138 was markedly decreased after TNFα treatment for 3, 6, and 12 h in the normal chondrocytes and OA chondrocytes (Figure 2A and 2B). We also measured the protein level of p65 after TNFα treatment. As shown in Figures 2C and 2D, the protein expression of p65 was increased after TNFα treatment for 3, 6, and 12 h in a time-dependent manner in both the normal and OA chondrocytes. These data indicated a reverse correlation between TNFα-regulated NF-κB and miR-138 expression in normal and OA chondrocytes.

Negative Regulation of Pro-inflammatory Factors Produced by miR-138 in Human Chondrocytes

We explored the effects of miR-138 on NF-κB signaling activation in human OA chondrocytes in the presence of 10 nM TNFα. Western blot analysis demonstrated that overexpression of miR-138 markedly suppressed the protein levels of p65, COX-2 and IL6 in human OA chondrocytes (Figure 3A). In contrast, the protein levels of p65, COX-2 and IL6 were significantly increased in human OA chondrocytes transfected with miR-138 inhibitors (Figure 3B). We also collected the supernatant from the culture. As shown in Figure 3C, overexpression of miR-138 significantly decreased the production of pro-inflammatory factors, including TNFα, IL-6 and IL-8, in human OA chondrocytes. In contrast, inhibition of miR-138 enhanced the levels of TNFα, IL-6 and IL-8 in human OA chondrocytes (Figure 3D). These data show the anti-inflammatory effects of miR-138 in human OA chondrocytes.

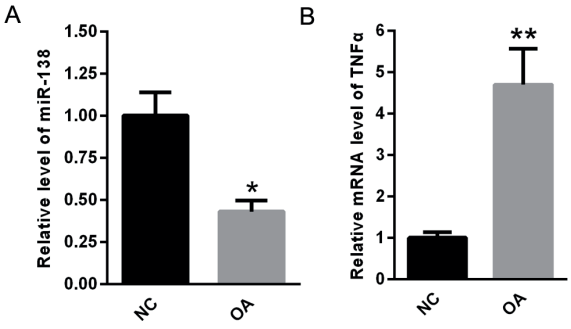

Figure 1. Decreased miR-138 level and enhanced TNFα in the OA cartilage tissues compared with those of normal controls. ***(A)*** Real-time PCR analysis of miR-138 level. ***(B)*** The level of TNFα analyzed by ELISA. $^{*}p<0.05$, $^{**}p<0.01$, *vs.* control.

P65 was a Target Gene of miR-138

We analyzed the possible target gene of miR-138. According to TargetScan, a conserved binding site of miR-138 in the 3' untranslated region (3'UTR) of p65 was identified (Figure 4A). The 3'UTR of p65 containing the binding site was cloned into the luciferase reporter vector, pmirGLO plasmid, creating pmirGLO-p65-3'UTR. A dual-luciferase reporter assay demonstrated that miR-138 significantly suppressed the relative luciferase activity of pmirGLO-p65-3'UTR. However, when the possible binding sites were muted, no changes in luciferase activity were identified (Figure 4B).

Overexpression of miR-138 Could Partially Abolish TNFα-induced Inflammation Responses

The effect of miR-138 on TNFα-induced NF-κB activation was analyzed. As shown in Figure 5A,

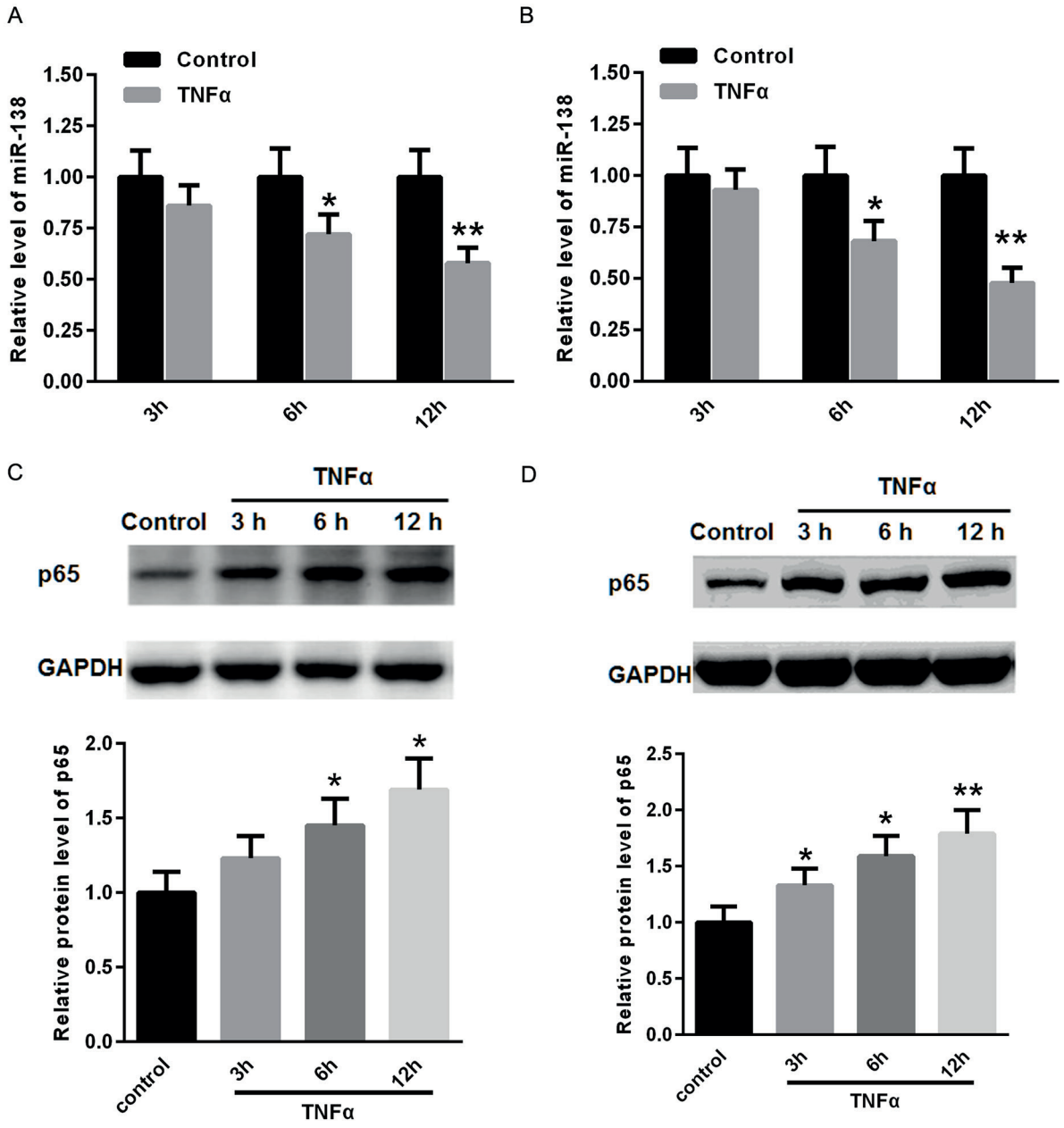

Figure 2. The reverse correlation between TNFα-regulated NF-κB and miR-138 expression in normal and OA chondrocytes. Real-time PCR analysis of miR-138 in normal chondrocytes ***(A)*** and OA chondrocytes ***(B)***. The protein expression of p65 was increased after TNFα treatment for 3, 6, and 12 h in normal chondrocytes (C) and OA chondrocytes (D). $^{*}p<0.05$, $^{**}p<0.01$, *vs.* control.

treatment with TNFα significantly enhanced the protein levels of p65, COX-2 and IL6. However, overexpression of miR-138 could partially abolish such effects. We also evaluated the inflammatory factors. In line with the protein expression changes, TNFα significantly increased the levels of inflammatory factors, while miR-138 overexpression reversed the production of TNFα, IL-6 and IL-8 induced by TNFα (Figures 5B, 5C and 5D).

Discussion

OA is a common, chronic joint disease. Increasing evidence has suggested the important role of miRNAs in the progression of OA[17]. For instance, miR-140 was reported to prompt age-related OA-like changes[18]. Furthermore, upregulation of miR-21 suppressed the chondrogenesis process in OA patients[13]. In the current study, we mainly

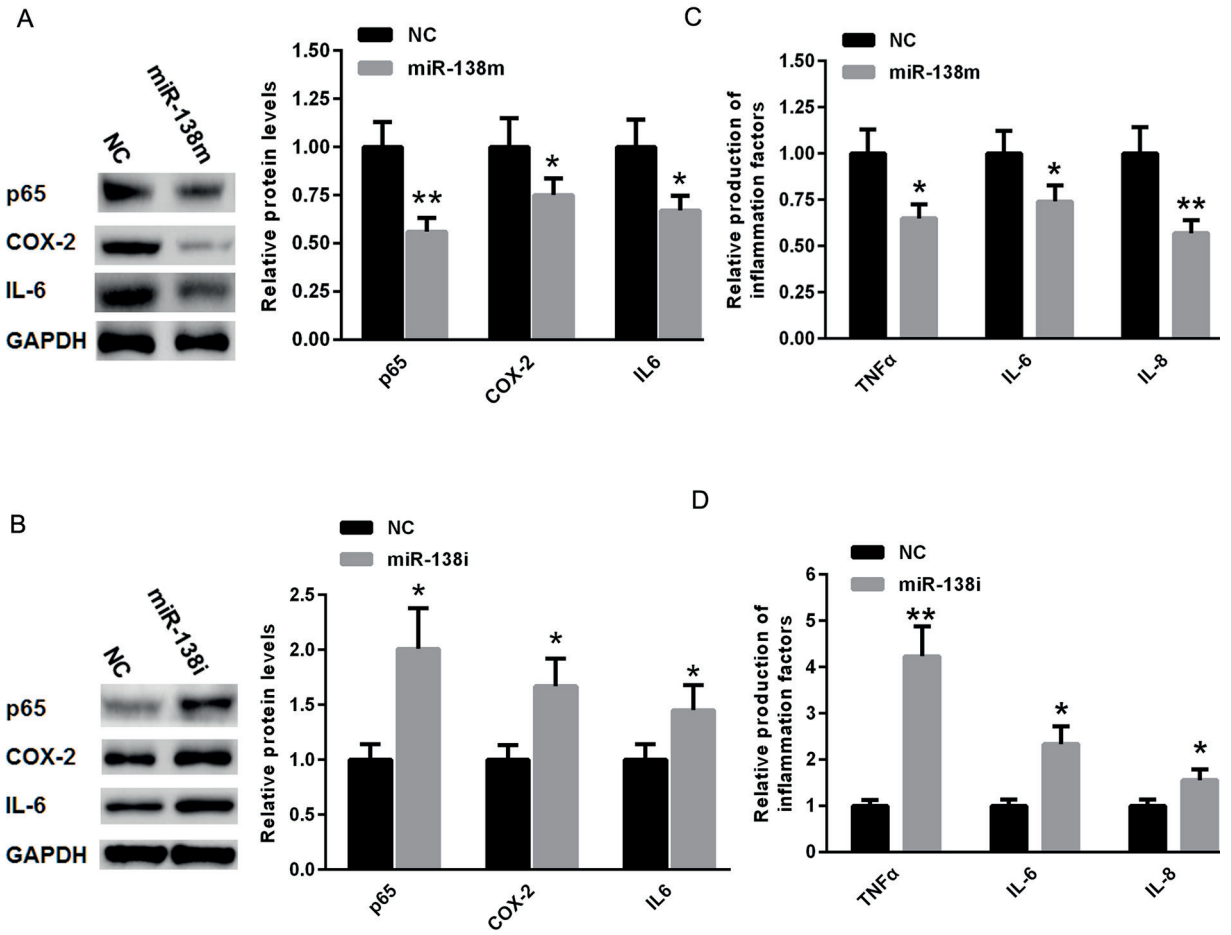

Figure 3. Negative regulation of pro-inflammatory factors produced by miR-138 in human chondrocytes. ***(A)*** Overexpression of miR-138 suppressed protein levels of p65, COX-2 and IL6 in human OA chondrocytes. ***(B)*** Protein levels of p65, COX-2 and IL6 were significantly increased in human OA chondrocytes transfected with miR-138 inhibitors. ***(C)*** Overexpression of miR-138 significantly decreased the production of pro-inflammatory factors in human OA chondrocytes. ***(D)*** Inhibition of miR-138 enhanced the levels of TNFα, IL-6 and IL-8 in human OA chondrocytes. $^{*}p<0.05$, $^{**}p<0.01$, *vs.* control.

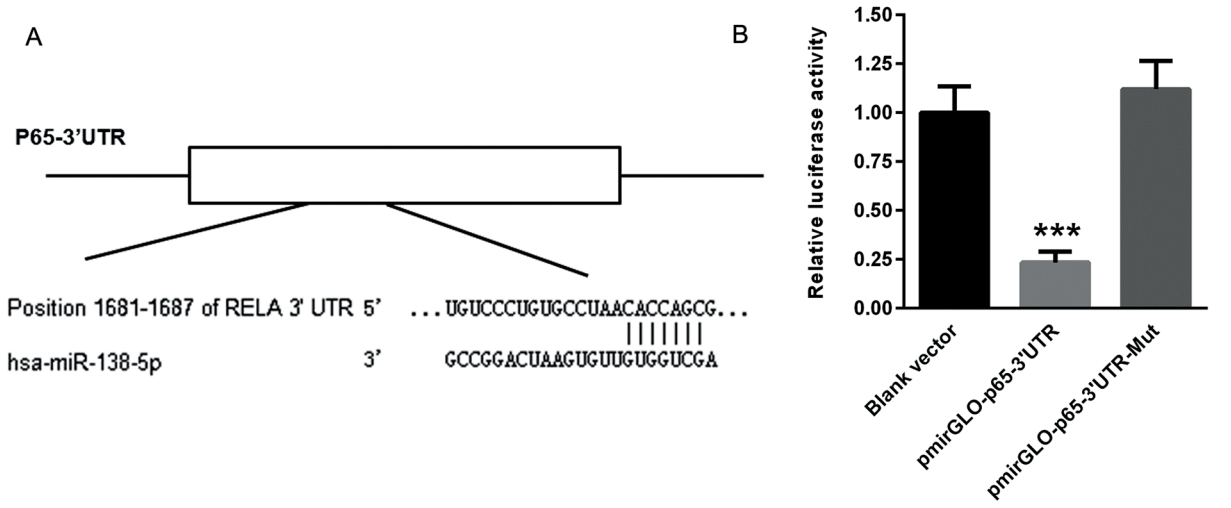

Figure 4. p65 was a target gene of miR-138. ***(A)*** One conserved binding site was identified in the 3'UTR of p65. ***(B)*** Dual-luciferase reporter assay demonstrated that miR-138 significantly suppressed the relative luciferase activity of pmirGLO-p65-3'UTR. $^{*}p<0.05$, $^{**}p<0.01$, *vs.* control.

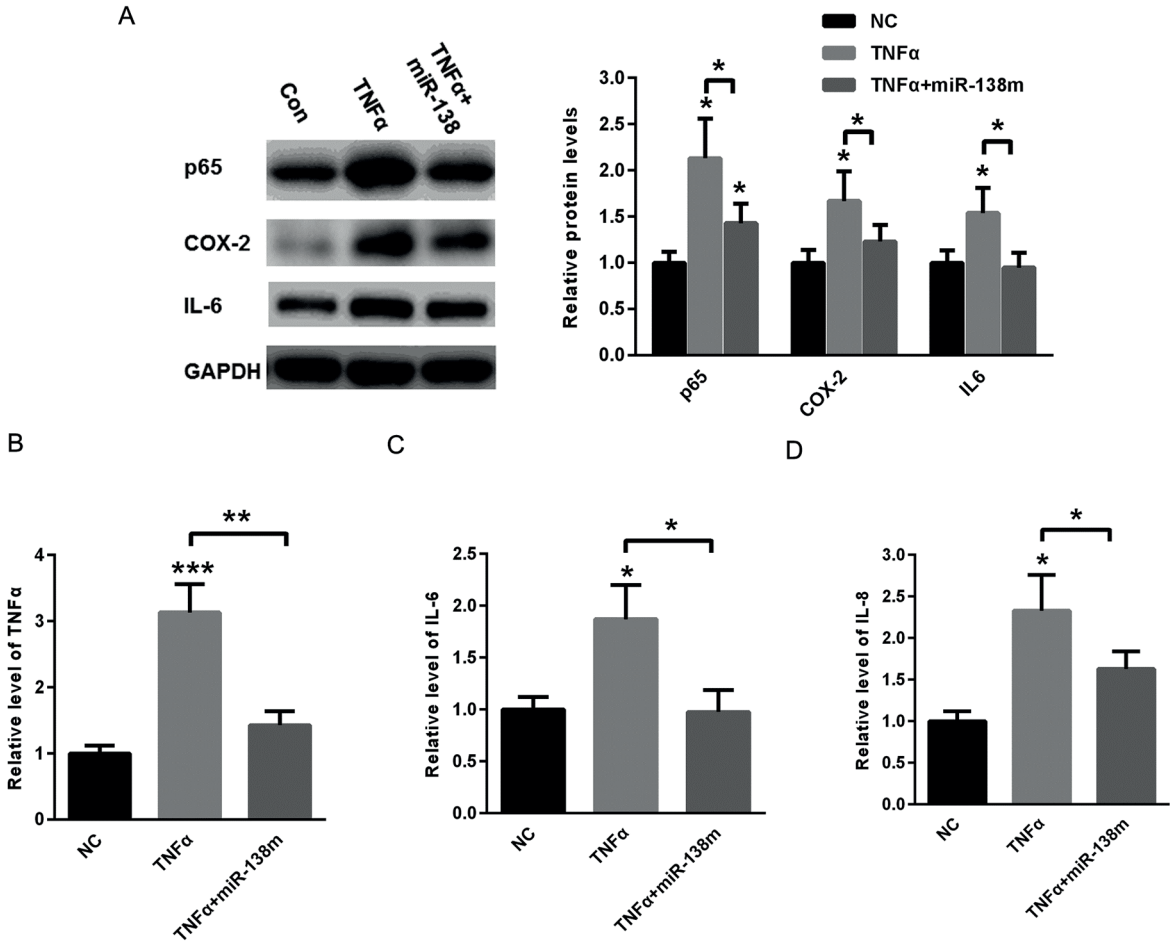

Figure 5. Overexpression of miR-138 could partially abolish TNFα-induced inflammatory responses. ***(A)*** Overexpression of miR-138 could partially abolish TNFα-induced upregulation of p65, COX-2 and IL6 protein levels. miR-138 overexpression could reverse the production of TNFα ***(B)***, IL-6 ***(C)*** and IL-8 ***(D)*** induced by TNFα. $^{*}p<0.05$, $^{**}p<0.01$, *vs.* control.

focused on miR-138, which has never been explored in OA patients. Our data show that the level of miR-138 was significantly decreased in cartilage tissues of OA patients compared with those of normal controls. Further study demonstrated that suppressed miR-138 levels contributed to the inflammatory responses in chondrocytes, indicating an anti-inflammatory role of miR-138 in the progression of OA.

NF-κB participates in the regulation of multiple cellular processes, including cell growth, apoptosis, and inflammatory and immune responses[19, 20]. Abnormal activation of NF-κB is reported to cause numerous auto-immune diseases, including arthritis[21]. NF-κB-dependent gene expression is found to play a key role in the activity of many cytokines, including IL-1, IL-2, IL-6, IL-8, IL-12, TNFα, and monocyte chemotactic protein factor (MCP)-1[22, 23]. However, the regulatory mechanism of NF-κB activation is not fully understood. Currently, increasing evidence has suggested that miRNAs are involved in the regulation of NF-κB signaling. For example, miR-210 has been found to reduce inflammation in the articular cavity in OA rats mainly by suppressing the protein level of DR6[8]. Here, we first demonstrated that p65, an important NF-κB subunit, was a target gene of miR-138. By suppressing the expression of p65, reduced miR-138 expression contributed to the progression of inflammatory responses in chondrocytes.

Under normal conditions, NF-κB is found in the cytoplasm and is inhibited by an inhibitor of NF-κB(IκB)[24, 25]. After exposure to external stimuli, IκB was phosphorylated and then degraded. Then, NF-κB translocated into the nucleus, initiating the

transcription of pro-inflammatory cytokines, including TNFα, IL-6 and IL-8[19]. It is well-accepted that NF-κB plays a critical role in the progression of OA; therefore, it is the transcription factor that is an enticing target for drug candidates. We suggest that overexpression of miR-138 significantly suppressed the production of TNFα, IL-6 and IL-8. In contrast, inhibition of miR-138 markedly enhanced the levels of TNFα, IL-6 and IL-8.

Conclusions

Our data demonstrate miR-138 expression was decreased in the cartilage tissues of OA patients. Downregulation of miR-138 induced inflammatory responses in the chondrocytes. We also found that p65, an important NF-κB subunit, was a target gene of miR-138, suggesting that miR-138 might be a medical target for the treatment of OA.

Conflict of interest

The authors declare no conflicts of interest.

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