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Original Article

Suppression of miR-301a alleviates LPS-induced inflammatory injury in ATDC5 chondrogenic cells by targeting Sirt1

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Abstract: Osteoarthritis is one of the most common joint diseases and is characterized by joint inflammation. MicroRNAs (miRNA) play an important role in osteoarthritis. In this study, we examined the role of miR-301a in lipopolysaccharide (LPS)-treated murine chondrogenic ATDC5 cells. LPS at 10 μg/mL concentration was used to induce inflammatory injury in chondrogenic cells. Cell Counting Kit-8 assay was used to measure cell viability and flow cytometry was used to measure cell apoptosis. Effect of miR-301a on concentrations of inflammatory cytokines was measured using ELISA, and on mRNA expressions was measured using qRT-PCR. The miR-301 target was identified by luciferase reporter assay. Western blot analysis was used to measure the expressions of apoptotic proteins, Sirt1, and PI3K/AKT and NF-κB pathways proteins. Treatment with LPS decreased cell viability, and increased apoptosis, inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) level, and miR-301a expression. Overexpression of miR-301a aggravated the effects of LPS on the chondrogenic cells and the inflammatory cytokines by negatively regulating Sirt1 expression. Sirt1 was identified as a target of miR-301. Suppression of miR-301a showed the opposite effects. Western blot showed that suppression of miR-301a increased the expression of PI3K/AKT and NF-κB pathways proteins. Suppression of miR-301a expression alleviated LPS-induced chondrogenic cell injury by upregulating Sirt1 and activating the PI3K/AKT and NF-κB signal pathways.

Keywords: MicroRNA-301a, osteoarthritis, lipopolysaccharide, inflammation, chondrogenic cell injury, Sirt1

Introduction

Osteoarthritis is the most common adult joint disease, involving degeneration of articular cartilage, bone remodeling and joint inflammation [1, 2]. Degeneration of articular cartilages of joints such as the hip and knee is hallmark of osteoarthritis. These degenerations lead to complete loss of the cartilage surface and exposure of the subchondral bones [3]. Mechanical and enzymatic factors are believed to impair chondrocyte function and damage the matrix [2]. A variety of enzymatic and mechanical factors stimulate cartilage cells leading to the secretion of cytokines, cartilage degeneration protease, and inflammatory mediators, which in turn cause cartilage degeneration [4]. The clinical manifestations of osteoarthritis include joint pain, tenderness, stiffness, joint swelling, limitation of motion, and joint deformity [2, 5]. Treatments used for osteoarthritis ranges from drugs, orthopedic aids, physiotherapy to surgery [2]. Osteoarthritis is not yet a curable disease, and its pathogenesis is yet to be understood fully. Therefore, it is very important to study the underlying molecular mechanism of inflammatory injury of the cartilage cells for better management of osteoarthritis.

MicroRNAs (miRNAs) are small, endogenous non-coding RNAs that regulate the expression of human protein encoding genes at the post-transcriptional level. MiRNAs are involved in several physiological and pathological processes, including cell differentiation and development, metabolism, immunity, and inflammation [6-8]. Several studies indicated that miRNAs play crucial roles in the regulation of osteogenic

and chondrogenic differentiation and proliferation, thereby influencing the catabolism and anabolism of bone and cartilage [9-11]. It has been suggested that miRNAs have important diagnostic and therapeutic potential, and can act as a novel therapeutic option for treating osteoarthritis [12].

MiR-301a, a newly discovered miRNA, is known to be a positive regulator of the immune response of leukocytes and is therefore associated with inflammation [13]. MiR-301a is also involved in many cellular processes, such as tumor cell proliferation [14]. However, the role of miR-301a in osteoarthritis has not yet been studied. In the present study, we investigated the role of miR-301a in LPS-treated murine chondrogenic ATDC5 cells.

Materials and methods

Cell culture and treatment

The murine chondrogenic ATDC5 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and was cultured in DMEM (Nutrient Mixture F-12 [DMEM/F-12], Thermo Scientific, Rockford, IL, USA), supplemented with 2 mM glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 10% (for cell growth) or 2% (for cell maintaining) fetal bovine serum (FBS; HyClone, Logan, UT, USA) at 37°C in a humidified 5% CO_2 incubator. Cells with more than 75% confluence were split in 1:2 using 0.25% trypsin (Ameresco, Framingham, MA, USA). Cells were treated with lipopolysaccharide (LPS) in a series of concentration (1, 5, and 10 μg/mL) for 5 hrs.

Cell Counting Kit-8 (CCK-8) assay

Cell proliferation was assessed by a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). Cells were seeded in a 96-well plate, with 5000 cells/well. After stimulation, the CCK-8 solution was added to the culture medium, and the cultures were incubated for 1 hr at 37°C in humidified 95% air and 5% CO_2. Absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

Apoptosis assay

Flow cytometry analysis was done to identify and quantify the apoptotic cells using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). The cells (1,00,000 cells/well) were seeded in a 6 well-plate. Treated cells were washed twice with cold phosphate buffered saline and re-suspended in buffer. The adherent and floating cells were combined and treated according to the manufacturer's instruction and measured with flow cytometer (Beckman Coulter, USA) to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

Enzyme-linked immunosorbent assay (ELISA)

Culture supernatant was collected from 24-well plates and concentrations of inflammatory cytokines were measured by ELISA (R&D Systems, Abingdon, UK) as per the manufacturer's protocols and normalized to cell protein concentrations.

Cell transfection

MiR-301a mimic, miR-301a inhibitor (si-miR-301a), si-Sirt1, and negative control (NC) were synthesized by GenePharma Co. (Shanghai, China). Cells were transfected using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's protocol.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. For the measurement of mRNA expression level of miR-301a, TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal Master Mix II were used; U6 (Applied Biosystems, Foster City, CA, USA) was used as internal control.

Dual luciferase activity assay

The 3'UTR target site was generated by PCR and the luciferase reporter constructs with the Sirt1 3'UTR carrying a putative miR-301a-binding site into pMiR-report vector were amplified by PCR. Cells were co-transfected with the reporter construct, control vector and miR-301a or scramble using Lipofectamine 3000 (Life Technologies, USA). Reporter assays were done using the dual-luciferase assay system (Promega) as per the manufacturer's instructions.

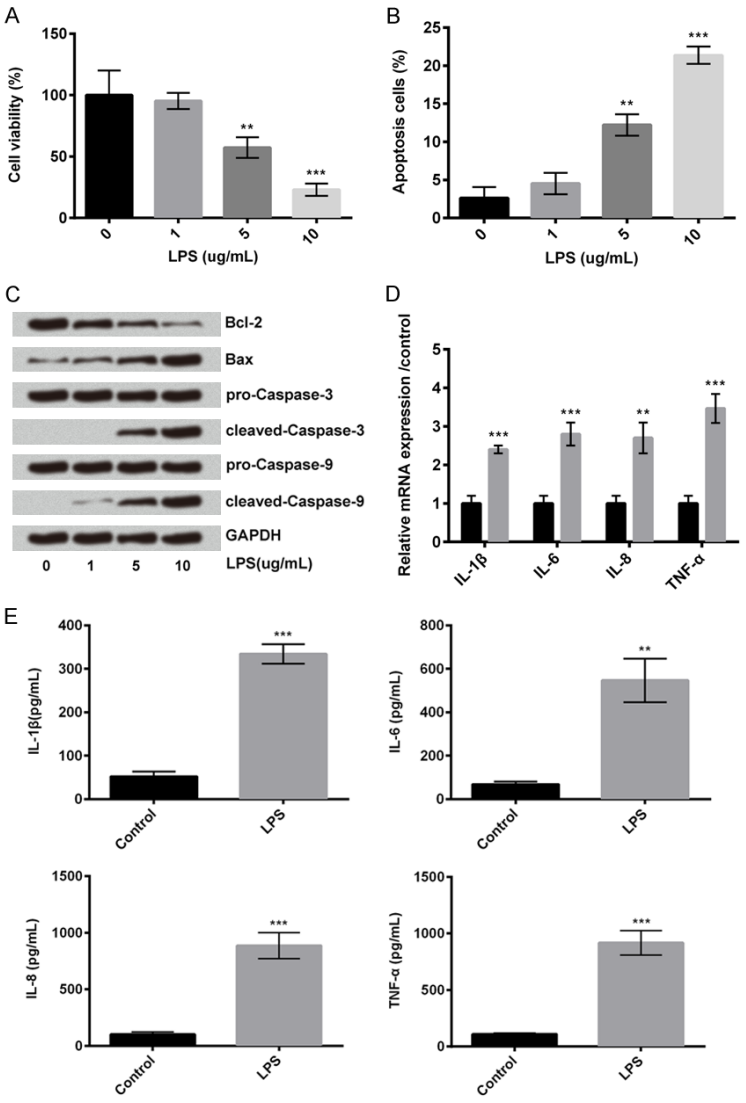

Figure 1. LPS induces cell injury and increases expression of inflammatory cytokines in chondrogenic cells. ATDC5 chondrogenic cells were treated with LPS (1, 5, or 10 μg/mL). (A) Cell viability. (B) Cell apoptosis. (C) Western blot was used to measure the protein expression of Bcl-2, Bax, cleaved caspase-3, and cleaved caspase-9. (D) RT-PCR was used to measure the mRNA expression and (E) ELISA was used to measure the concentration of IL-1β, IL-6, IL-8, and TNF-α in LPS-treated and control cells. **P<0.01, ***P<0.001. ELISA: enzyme-linked immunosorbent assay; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IL: interleukin; LPS: lipopolysaccharide; RT-PCR: reverse transcription polymerase chain reaction; TNF-α: tumor necrosis factor-α.

Western blot analysis

The proteins used for western blotting were extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. GAPDH antibody was purchased from Sigma. Primary antibodies were prepared in 5% blocking buffer at adilution of 1:1000. Primary antibody was incubated with the membrane at 4°C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 hour at room temperature. After rinsing, the polyvinylidene difluoride membrane-carried blots and antibodies were transferred into Bio-Rad ChemiDoc™ XRS system, and then 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as mean ± standard error. Statistical analyses were performed using SPSS 19.0 statistical software. *P*-values were calculated using one-way analysis of variance. *P*-value of <0.05 was considered to indicate a statistically significant result.

Results

LPS induces cell injury and increases expression of inflammatory cytokines in chondrogenic cells

To measure the effects of LPS on cell viability and apoptosis in ATDC5 chondrogenic cells, the cells were treated with LPS at different con-

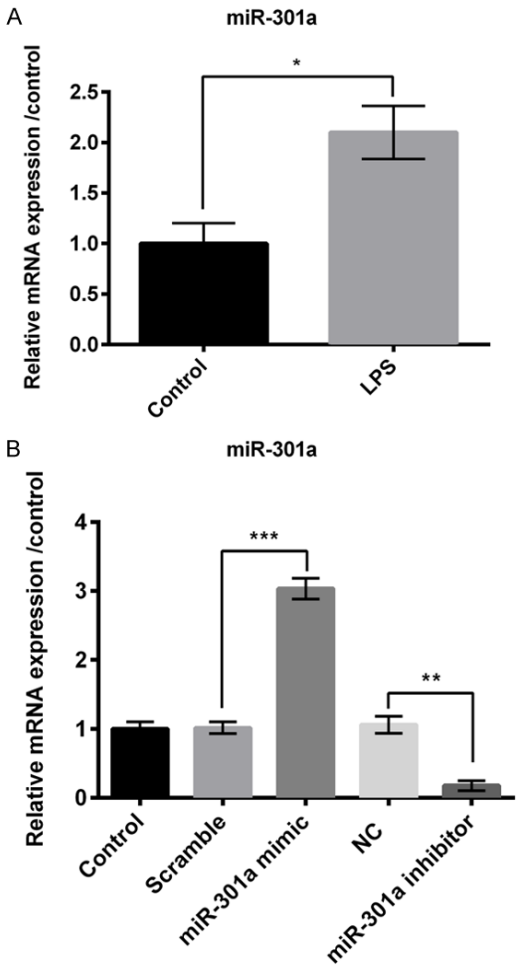

Figure 2. LPS increases the expression of miR-301a. RT-PCR was used to measure the relative mRNA expression of miR-301a in (A) LPS-treated or control cells; and in (B) cells transfected with scramble, miR-301a mimic, NC, or miR-301a inhibitor and control cells. *P<0.05, **P<0.01, ***P<0.001. LPS: lipopolysaccharide; NC: negative control; RT-PCR: reverse transcription polymerase chain reaction.

centrations (1, 5, and 10 μg/mL) for 5 hrs. Compared to the control group of cells, LPS significantly decreased cell viability at 5 μg/mL (P<0.01) and 10 μg/mL (P<0.01; **Figure 1A**); and increased apoptosis at 5 μg/mL (P<0.01) and 10 μg/mL (P<0.001; **Figure 1B**). Western blot analysis for apoptosis showed that LPS decreased the protein expression of Bcl-2 (anti-apoptotic protein) and increased the expression of Bax, cleaved caspase-3, and cleaved caspase-9 (pro-apoptotic proteins) in chondrogenic cells at 5 μg/mL and 10 μg/mL compared to the control group of cells (**Figure 1C**). In the further experiments, LPS was used at 5 μg/mL concentration.

As osteoarthritis is associated with synovial inflammation, we measured the effects of LPS on inflammatory cytokines, interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-alpha (TNF-α). The relative mRNA expressions of these pro-inflammatory cytokines were measured by RT-PCR and the concentration levels of these cytokines were measured by ELISA. Compared to the control group of cells, LPS increased the mRNA expressions (**Figure 1D**) and concentrations (**Figure 1E**) of these inflammatory cytokines.

These results indicated that LPS induced cell injury and increased expression of inflammatory cytokines in chondrogenic cells.

LPS increases the expression of miR-301a

We then measured effects of LPS on expression of miR-301a in chondrogenic cells using RT-PCR. The results showed that LPS significantly increased the relative mRNA expression of miR-301a in chondrogenic cells compared to the control group (P<0.05; **Figure 2A**).

We transfected the chondrogenic cells with miR-301a mimic to increase the expression of miR-301a and si-miR-301a to decrease its expression, and measured their efficiency using RT-PCR. As shown in **Figure 2B**, transfection with miR-301a mimic significantly increased the mRNA expression of miR-301a (P<0.001) as compared to the scramble; and si-miR-301a significantly decreased the mRNA expression of miR-301a (P<0.01) as compared to the negative control.

Overexpression of miR-301a aggravates LPS-induced cell injury and increases expression of inflammatory cytokines

As LPS increased the expression of miR-301a, we measured the effects of altered expression of miR-301a on cell viability, apoptosis, and inflammatory cytokines. For these tests, chondrogenic cells were transfected with LPS, LPS+ scramble, LPS+miR-301a mimic, LPS+NC, or LPS+si-miR-301a; untransfected cells served as control. The results obtained from miR-301a mimic group were compared with those of LPS+ scramble, whereas those obtained from si-miR-301a group were compared with those of LPS+NC.

Overexpression of miR-301a significantly decreased cell viability (**Figure 3A**) and increased

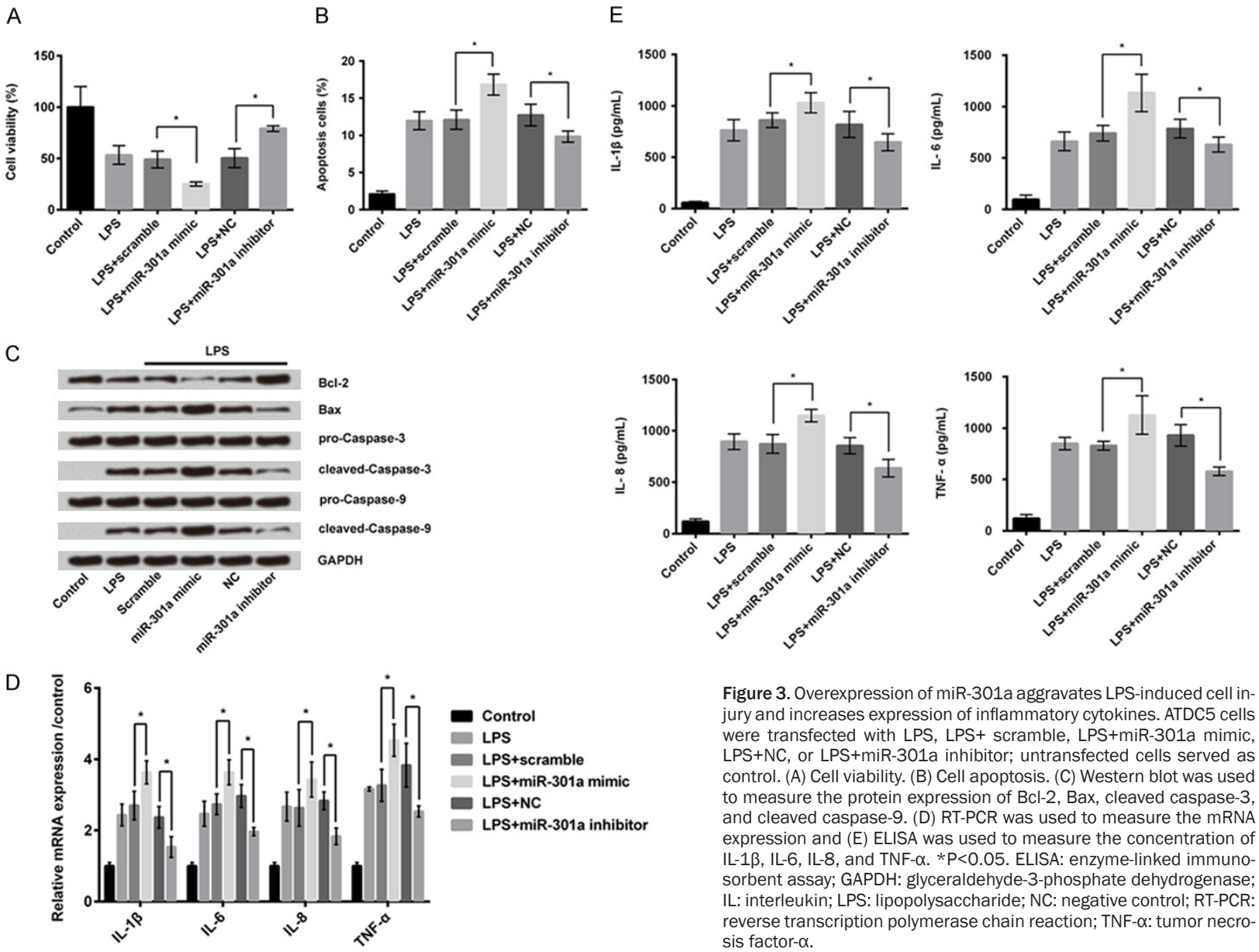

Figure 3. Overexpression of miR-301a aggravates LPS-induced cell injury and increases expression of inflammatory cytokines. ATDC5 cells were transfected with LPS, LPS+ scramble, LPS+miR-301a mimic, LPS+NC, or LPS+miR-301a inhibitor; untransfected cells served as control. (A) Cell viability. (B) Cell apoptosis. (C) Western blot was used to measure the protein expression of Bcl-2, Bax, cleaved caspase-3, and cleaved caspase-9. (D) RT-PCR was used to measure the mRNA expression and (E) ELISA was used to measure the concentration of IL-1β, IL-6, IL-8, and TNF-α. *P<0.05. ELISA: enzyme-linked immunosorbent assay; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IL: interleukin; LPS: lipopolysaccharide; NC: negative control; RT-PCR: reverse transcription polymerase chain reaction; TNF-α: tumor necrosis factor-α.

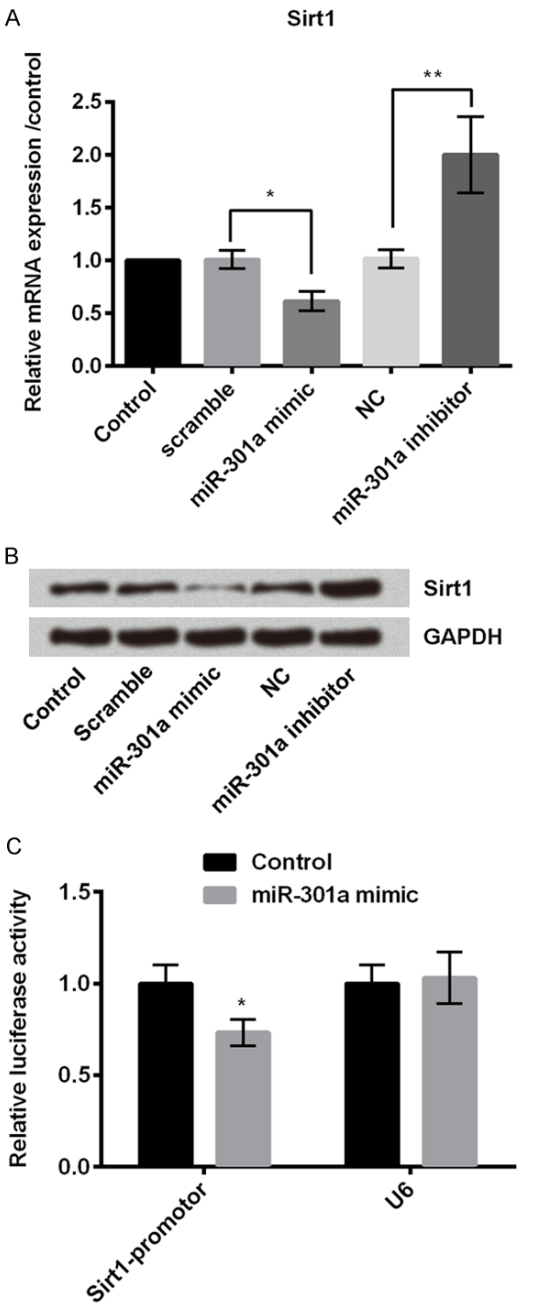

Figure 4. miR-301a negatively regulates Sirt1. (A) Quantitative RT-PCR was used to measure relative mRNA expression and (B) western blot was used to measure the protein expression of Sirt1 in ATDC5 cells transfected with scramble, miR-301a mimic, NC, or miR-301a inhibitor; untransfected cells served as control. (C) Relative luciferase activity of Sirt1 was measured in ATDC5 cells transfected with control or miR-301a mimic. *P<0.05 **P<0.01. NC: negative control; RT-PCR: reverse transcription polymerase chain reaction.

apoptosis (**Figure 3B**), while suppression of miR-301a showed opposite results (P<0.05 for all). Western blot analysis for apoptosis showed that overexpression of miR-301a decreased the protein expression of Bcl-2 and increased the expression of Bax, cleaved caspase-3, and cleaved caspase-9 in chondrogenic cells, whereas suppression of miR-301a showed opposite results (**Figure 3C**). Moreover, overexpression of miR-301a significantly increased the mRNA expression (**Figure 3D**) and concentration (**Figure 3E**) of the inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α), while suppression of miR-301a showed opposite results (P<0.05 for all).

These results indicated that overexpression of miR-301a aggravated LPS-induced cell injury and increases expression of inflammatory cytokines, while suppression of miR-301a reversed these effects.

miR-301a negatively regulates Sirt1 expression

We then measured the effects of miR-301a on Sirt1 expression, which is shown to be involved in cartilage biology [15]. RT-PCR was used to measure the mRNA expression of Sirt1 and western blot was used to measure the protein expression of Sirt1 in chondrogenic cells. For these assays, chondrogenic cells were transfected with scramble, miR-301a mimic, NC, or si-miR-301a; untransfected cells served as control. As shown in **Figure 4A** and **4B**, overexpression of miR-301a significantly decreased the mRNA and protein expression of Sirt1 compared to the scramble group (P<0.05), while suppression of miR-301a significantly increased the mRNA and protein expression of Sirt1 as compared to the NC group (P<0.01). We also measured the relative luciferase activity of Sirt1 in chondrogenic cells, and found that miR-301a mimic significantly decreased the luciferase activity of Sirt1 promotor in chondrogenic cells compared to the control (P<0.05; **Figure 4C**).

These results indicated that miR-301a negatively regulated Sirt1, which was a target of miR-301a.

Suppression of miR-301a reduces cell injury by upregulating Sirt1

As Sirt1 was identified as a target of miR-301a in chondrogenic cells, we measured cell viability, cell apoptosis, and expressions of inflamma-

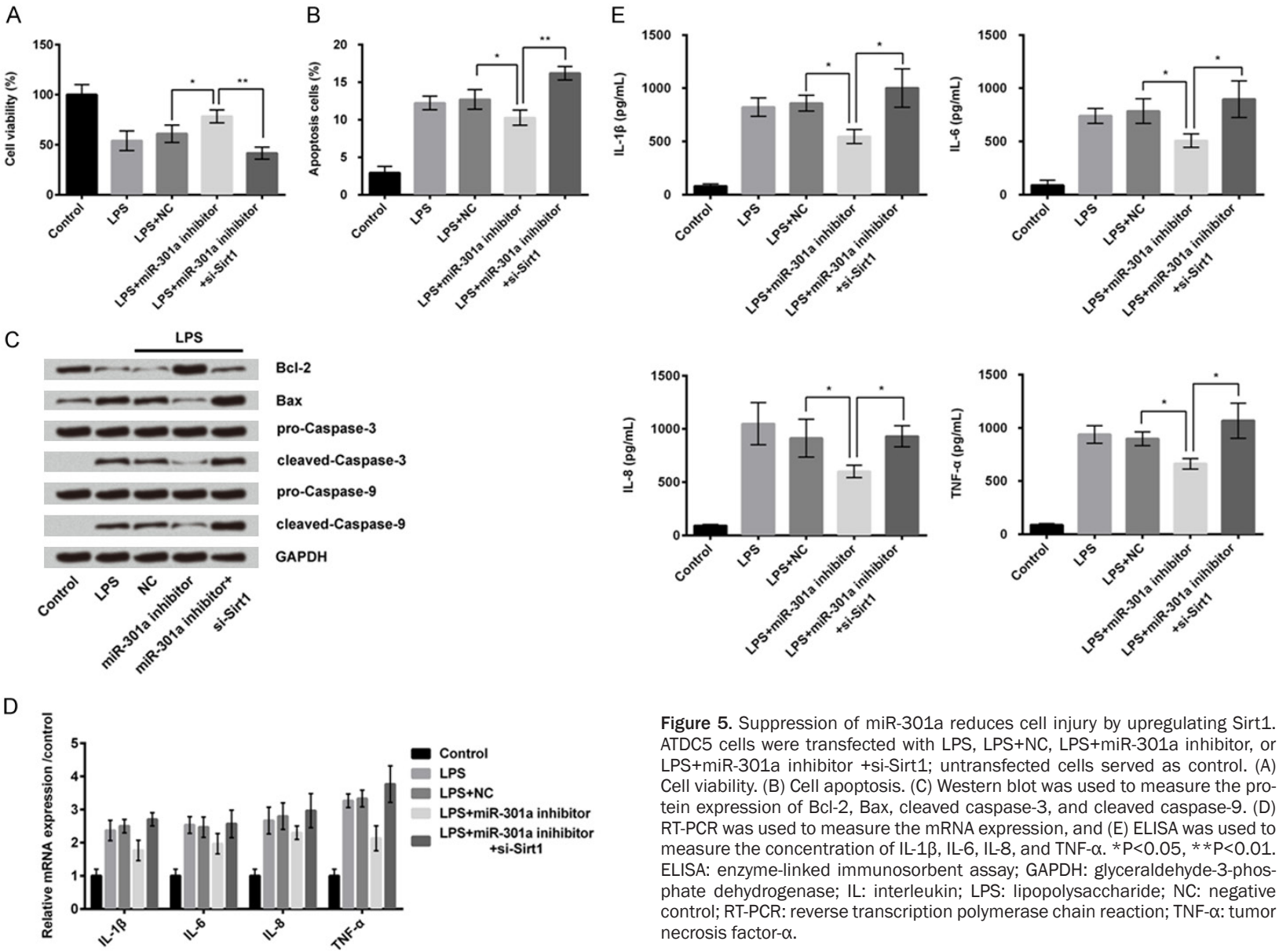

Figure 5. Suppression of miR-301a reduces cell injury by upregulating Sirt1. ATDC5 cells were transfected with LPS, LPS+NC, LPS+miR-301a inhibitor, or LPS+miR-301a inhibitor +si-Sirt1; untransfected cells served as control. (A) Cell viability. (B) Cell apoptosis. (C) Western blot was used to measure the protein expression of Bcl-2, Bax, cleaved caspase-3, and cleaved caspase-9. (D) RT-PCR was used to measure the mRNA expression, and (E) ELISA was used to measure the concentration of IL-1β, IL-6, IL-8, and TNF-α. *P<0.05, **P<0.01. ELISA: enzyme-linked immunosorbent assay; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IL: interleukin; LPS: lipopolysaccharide; NC: negative control; RT-PCR: reverse transcription polymerase chain reaction; TNF-α: tumor necrosis factor-α.

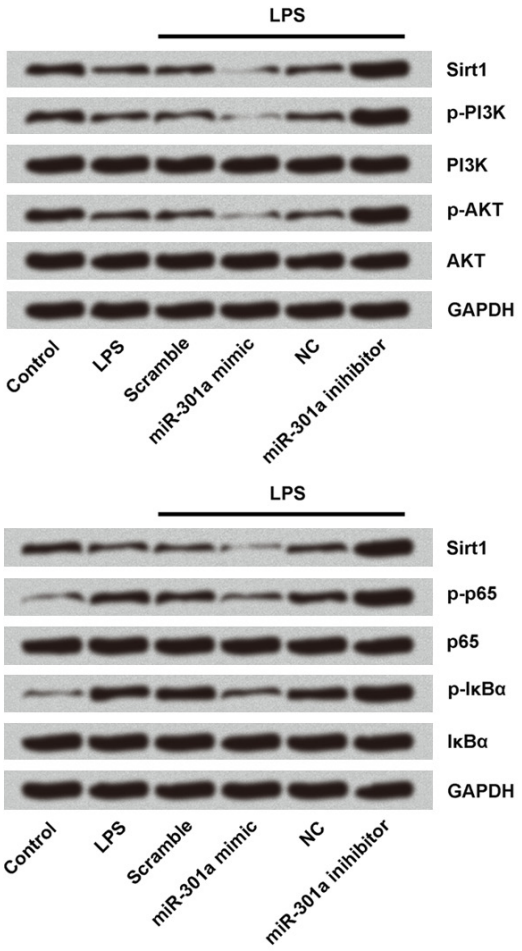

Figure 6. Suppression of miR-301a activates PI3K/AKT and NF-κB pathways. Western blot analysis was used to measure the protein expressions of Sirt1, PI3K, AKT, p65, IκBα, and GAPDH in the cells transfected with LPS, LPS+ scramble, LPS+miR-301a mimic, LPS+NC, or LPS+miR-301a inhibitor; untransfected cells served as control. AKT: serine-threonine kinase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IκBα: inhibitory κB proteins alpha; LPS: lipopolysaccharide; NC: negative control; NF-κB: nuclear factor-kappaB; PI3K: phosphoinositide 3-kinase.

tory cytokines in chondrogenic cells with knockdown of miR-301a and Sirt1 expressions. For these tests, chondrogenic cells were transfected with LPS, LPS+NC, LPS+si-miR-301a, or LPS+si-miR-301a+si-Sirt1; untransfected cells served as control.

As discussed earlier, suppression of miR-301a expression increased cell viability, decreased apoptosis, and also decreased the concentrations of the inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) in chondrogenic cells compared to the negative control group ($P<0.05$ for all; **Figure 5A-E**). As compared to these effect of miR-301a suppression, down-regulation of both miR-301a and Sirt1 significantly decreased cell viability ($P<0.01$; **Figure 5A**), increased apoptosis ($P<0.01$; **Figure 5B**), and increased mRNA expression (**Figure 5D**), and concentration level ($P<0.05$ for all; **Figure 5E**) of the inflammatory cytokines in chondrogenic cells. Additionally, western blot analysis for apoptosis showed that down-regulation of both miR-301a and Sirt1 decreased the protein expression of Bcl-2 and increased the expression of Bax, cleaved caspase-3, and cleaved caspase-9 (**Figure 5C**).

These findings indicated that suppression of miR-301a alleviated cell injury and decreased inflammatory cytokines by increasing the expression of Sirt1.

Suppression of miR-301a alleviated LPS-induced cell injury by activation of PI3K/AKT and NF-κB pathways

Finally, we explored the mechanism underlying the effects of miR-301a in chondrogenic cells by measuring the expressions of the proteins associated with PI3K/AKT and NF-κB pathways using western blot. For this analysis, chondrogenic cells were transfected with LPS, LPS+ scramble, LPS+miR-301a mimic, LPS+NC, or LPS+si-miR-301a; untransfected cells served as control. As shown in **Figure 6**, overexpression of miR-301a mimic decreased the expressions of Sirt1, p-PI3K, p-AKT, p-65, and p-IκBα in chondrogenic cells, while suppression of miR-301a showed opposite results.

These results indicated that suppression of miR-301a alleviates LPS-induced cell injury and decreased inflammation by activation of PI3K/AKT and NF-κB pathways.

Discussion

In this study, we investigated the effects of miR-301a in the LPS-induced ATDC5 cell injury and the possible underlying mechanism. First, we induced inflammatory injury to the chondrogenic cells by treating them with LPS, and then measured viability and apoptosis of the injured cells, and expressions of inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) and miR-301a in the injured cells. Next, we examined

the effect of miR-301a and Sirt1 on the LPS-injured cells and inflammatory cytokines. Lastly, we studied the mechanism underlying these effects by measuring the expressions of the proteins associated with PI3K/AKT and NF-κB pathways in the injured cells.

Clinical osteoarthritis is characterized by inflammation of the joints. Chronic inflammation is a key driver of progressive cartilage degeneration in the joints. Lots of biomechanical factors are involved in chronic inflammation [16]. Pro-inflammatory cytokines-IL-1β, IL-6, IL-8, and TNF-α are biomechanical factors which are involved in the development of chronic inflammation. Studies showed that IL-6 and IL-8 levels are increased in osteoarthritic serum and synovial fluid [17, 18]. In our study, LPS increased the concentration of these inflammatory cytokines in the chondrogenic cells.

In our study, overexpression of miR-301a aggravated the LPS-induced cell injury and increased the expression of inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α), while suppression of miR-301a reversed these effects. Effects of miR-301a on LPS-injured chondrogenic cells have not been studied yet; however, its effect on inflammatory cytokines has been studied. Overexpression of miR-301a activates NF-κB, which produces IL-8, interferon-β, nitric oxide synthase 2A and cytochrome oxidase subunit 2 [13]. He et al showed that miR-301a promotes intestinal mucosal inflammation through induction of IL-17A and TNF-α in inflammatory bowel disease [19].

In further experiments, we showed that suppression of miR-301a expression reduces cell injury and decreases the expressions of inflammatory cytokines by increasing the expression of Sirt1. Sirt1 is a member of the sirtuin family, which plays a wide variety of biological functions, including cell development and survival, and inflammation [20]. In human chondrocytes, Sirt1 promotes cell survival, even under pro-inflammatory stress [15]. Therefore, Sirt1 could serve as a novel drug target in treating osteoarthritis. No study has examined the association between miR-301a and Sirt1, except the present study. Our findings indicate that miR-301a exerts its effect on the chondrogenic cells by targeting Sirt1.

Lastly, we investigated the molecular mechanism underlying the effects of miR-301a by measuring the expression of PI3K/AKT and NF-κB signal pathway associated proteins. The PI3K/AKT signal pathway is an important pathway for cell survival, proliferation, and migration [21]. The PI3K/AKT pathway is involved in the degradation of extracellular matrix and the death of chondrocytes in osteoarthritis [22]. The nuclear factor-kappaB (NF-κB) proteins are a family of transcription factors that play an important role in most immune and inflammatory processes. NF-κB proteins have prominent role in cartilage degradation, cell proliferation, angiogenesis, and pannus formation. Inhibitory κB proteins alpha (IκBα) and p65 belong to the NF-κB family. NF-κB signaling pathways mediate important events of inflammatory response by chondrocytes, which led to progressive extracellular matrix damage, and cartilage destruction [23]. MiR-301a down-regulates the expression of NF-κB pathway proteins in immune cells, thereby increasing the production of IL-1β and TNF-α [13]. In our study, suppression of miR-301a increased the expression of PI3K/AKT and NF-κB signal pathway proteins.

In conclusion, suppression of miR-301a expression alleviates LPS-induced chondrogenic cell injury by upregulating Sirt1 and activating the PI3K/AKT and NF-κB signal pathways. Results of the current study indicated that miR-301a could serve as a potential novel therapeutic target for osteoarthritis.

Acknowledgements

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Disclosure of conflict of interest

None.

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