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Original Paper

MiR-1246 Promotes LPS-Induced Inflammatory Injury in Chondrogenic Cells ATDC5 by Targeting HNF4γ

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Key Words
miR-1246 • Inflammation • HNF4γ • ATDC5 cells • Osteoarthritis

Abstract:
Background/Aims: Osteoarthritis (OA) is a common inflammatory joint disease. miRNAs are associated with OA and functionally implicated in the pathogenesis of the disease. In the present study, we investigated the role of miR-1246 in the lipopolysaccharide (LPS)-induced inflammatory injury of ATDC5 cells. ***Methods:*** ATDC5 cells were cultured and treated with LPS in a series of concentration (0, 1, 5, and 10 μg/ml) for 5 h. The cells were transfected with miR-1246-mimic, inhibitor, si-HNF4γ or negative control, then were assessed for cell viability using CCK8 assay, apoptosis by flow-cytometry and expressions of miR-1246 and pro-inflammatory cytokines by qRT-PCR and western blot analysis. ***Results:*** Cell viability was significantly reduced and cell apoptosis was added in ATDC5 cells injured with LPS at the dosage of 5 and 10 μg/ml. Relative mRNA expressions of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) were significantly increased. miR-1246 was up-regulated in ATDC5 cells treated with LPS. Moreover, miR-1246 overexpression aggravated LPS-induced decrease in cell viability, increase in apoptosis and overproduction of pro-inflammatory factors. mRNA and protein expressions of HNF4γ were significantly suppressed in cells transfected with miR-124-mimic. Further, miR-1246 knockdown alleviated LPS-induced inflammatory injury by up-regulating the expression of HNF4γ and activation of PI3K/AKT and JAK/STAT pathways. ***Conclusions:*** Suppression of miR-1246 alleviated LPS-induced inflammatory injury in chondrogenic ADTC5 cells by up-regulation of HNF4γ and activation of PI3K/AKT and JAK/STAT pathways. The findings of this study will provide a novel viewpoint regarding miR-1246 target for clinical.

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Introduction

Osteoarthritis (OA) is the most common degenerative joint disease, including the degradation of articular cartilage and joint inflammation, which is characterized by inflammation. For example, the levels of pro-inflammatory cytokines were increased in the

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synovial fluid to indicate synovitis in early OA [1]. In OA, the balance of expression and response to anabolic and catabolic factors appears improperly skewed toward catabolism, leading to gradual loss of cartilage extracellular matrix. Nuclear receptors are known to be important intracellular transcriptional regulators, and their irregular expression in OA can provide clues to the pathophysiology and therapeutic intervention of the diseases [2]. Nuclear receptors constitute an important group of candidate pharmacological targets, because they modulate diverse cellular functions associated with various diseases, such as cancer, osteoporosis, diabetes, etc [3-9]. Hepatocyte Nuclear Factor 4 (HNF4) is one of nuclear receptor proteins, which are usually expressed in the liver, gut, kidney and pancreatic beta-cells, and is important for liver development. In humans, there are two isoforms of HNF4, HNF4α and HNF4γ, encoded by two separate genes HNF4A and HNF4G, respectively [10]. In order to verify the role of nuclear receptors, we had evaluated the role of HNF4γ in OA in this study.

MicroRNAs (miRNAs) are a class of small single-stranded and non-coding RNAs of around 22 bases that bind to the complementary sites in 3'untranslated regions (3'-UTR) of target genes to induce translational repression or degradation [11-15]. Recent studies showed that miRNAs play vital roles in regulating individual development, cell proliferation, metabolism, differentiation, invasion and migration in different disease [16-22]. A number of studies dispalyed that different miRNAs could increase or decrease cell apoptosis in OA, such as miR-149 and miR-30b [23, 24]. miR-1246 is associated with inflammation, which is a positive regulator of the immune response of leukocytes, and is also involved in many cellular processes, such as tumor cell proliferation [25]. However, the mechanism of miR-1246 in OA has not been studied. Therefore, it is very important to understand role of miRNAs in OA, which will help to provide new therapeutic targets. In the present study, we had investigated the effect of miR-1246 in the lipopolysaccharide (LPS)-induced 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 cultured in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM/F-12) (Thermo Fisher 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 CO_2 (5%) incubator. The cells were split at 75% confluence using 0.25% trypsin (Ameresco, Framingham, MA, USA), and were treated by different concentrations (0, 1, 5, and 10 μg/ml) of lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, USA) for 5 h. Further, 5 μg/ml LPS was selected for use in the subsequent experiments.

Cell transfection

miR-1246 mimic, inhibitor, their negative controls (NCs) and small interfering RNA (siRNA) HNF4γ (si-HNF4γ) were synthesized (GenePharma Co., Shanghai, China) and transfected into ATDC5 cells in the study. According to the manufacturer's protocol, all cell transfections were conducted using Lipofectamine 3000 Reagent (Life Technologies Corporation, Carlsbad, CA, USA). After 48 h of transfection, the stably transfected cells were selected for approximately 4 weeks by the culture medium containing 0.5 mg/ml G418 (Sigma-Aldrich, St Louis, MO, USA). G418-resistant cells were directly collected for use in subsequent experiments.

Cell viability assay

Cell viability was assessed by Cell Counting Kit-8 (CCK8, Dojindo Molecular Technologies, Gaithersburg, MD, USA). ATDC5 cells were seeded in 96-well plate with 5×10^3 cells/well, treated by LPS for 12 h. After stimulation, the CCK8 solution (10 μl/well) was added to the culture medium. Then, the cells were incubated for 1 h at 37°C in humidified 95% air and 5% CO_2. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Apoptosis assay

Flow cytometry analysis was performed to identify and quantify the apoptotic cells by using Annexin V-FITC/PI apoptosis Detection Kit (Beijing Biosea Biotechnology, Beijing, China). LPS-treated cells (1×10^5 cells/well) were seeded in 6 well-plate, washed twice with cold PBS and resuspended in 200 µl binding buffer including 10 µl FITC-Annexin V in the presence of 50 µg/ml RNase A (Sigma-Aldrich, St. Louis, MO, USA), then incubated at room temperature for 1 h in the dark. The adherent and floating cells were combined and treated according to the manufacturer's instruction and measured with flow cytometer (Beckman Coulter, Fullerton, USA) to differentiate apoptotic cells (Annexin-V positive).

Enzyme-linked immunosorbent assay (ELISA)

Culture supernatant was collected from 24-well plates and concentrations of inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) were measured by ELISA (R&D Systems, Abingdon, UK) following instruction supplied by the manufacturer, and were normalized to cell protein concentrations.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells and tissues using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II were used for detecting mRNA level of miR-1246 (both from Applied Biosystems, Foster City, CA, USA). The GAPDH was used in this study for normalizing miR-1246 levels. Fold changes were calculated by relative quantification ($2^{-\Delta\Delta Ct}$) method (23).

Dual luciferase activity assay

The 3'UTR target site and the luciferase reporter constructs with the HNF4γ 3'UTR carrying a putative miR-1246-binding site into pMiR-report vector were amplified by PCR. Cells were co-transfected with the reporter construct, control vector and miR-1246 or scramble using Lipofectamine 3000 regent (Life Technologies Corporation, Carlsbad, CA, USA). Reporter assays were done using the Dual-Luciferase® Reporter Assay System Protocol (Promega, Madison, WI, USA) according to the manufacturer's information.

Western blot assay

The proteins used for western blot were extracted using RIPA Lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China), and were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. The primary antibody of Bcl-2 (ab32124), Bax (ab32503), pro-caspase-3 (ab32150), cleaved-caspase-3 (ab2302), pro-caspase-9 (ab138412), cleaved-caspase-9 (ab2324), HNF4G (ab182192), p-P13K (ab182651), PI3K (ab182651), p-AKT (ab38449), AKT (ab8805), JAK1 (ab47435), p-STAT1 (ab30645), STAT1 (ab31369), p-STAT3 (ab76315) and STAT3 (ab68153) were obtained from Abcam (Shanghai, China). The antibody of p-JAK1 (SAB4300123) and GAPDH (G2267) was purchased from Sigma-Aldrich (St. Louis, MO, USA). They were prepared in 5% blocking buffer at a dilution of 1:1, 000, incubated with the membrane at 4°C overnight and followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the Polyvinylidene Difluoride (PVDF) membrane carried blots and antibodies were transferred into the 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 deviation (SD). Statistical analyses were performed using SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA). P-values were calculated using a one-way analysis of variance (ANOVA). A P-value of < 0.05 was considered to indicate a statistically significant result.

Results

LPS induced inflammatory injury in ATDC5 cells

Cell viability evaluated by CCK8 assay was significantly decreased (Fig. 1A) and cell apoptosis was significantly increased by flow cytometry in ATDC5 cells treated with LPS at the concentrations of 5 and 10 μg/ml ($P < 0.01$ or $P < 0.001$, Fig. 1B). The apoptosis-related proteins were measured using western blot analysis. Then, Fig. 1C showed that Bcl-2 expression was obviously down-regulated and the expressions of Bax, cleaved-Caspase-3 and cleaved-Caspase-9 were up-regulated after LPS stimulated. According to above data, LPS and ATDC5 cell damage in a dose-dependent manner. Thus, 5 μg/ml was selected as a LPS-stimulating condition for use in the subsequent experiments. Considering the importance of cytokine in inflammatory disorders, we assessed the mRNA expression levels of related pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α. The results revealed that mRNA levels were significantly up-regulated in ATDC5 cells injured with LPS as compared to the control ($P < 0.05$, $P < 0.01$ or $P < 0.001$, Fig. 1D). Further, the concentrations of these four cytokines in culture supernatant were apparent enhanced in LPS group by using ELISA assay (all $P < 0.001$, Fig. 1E-1H).

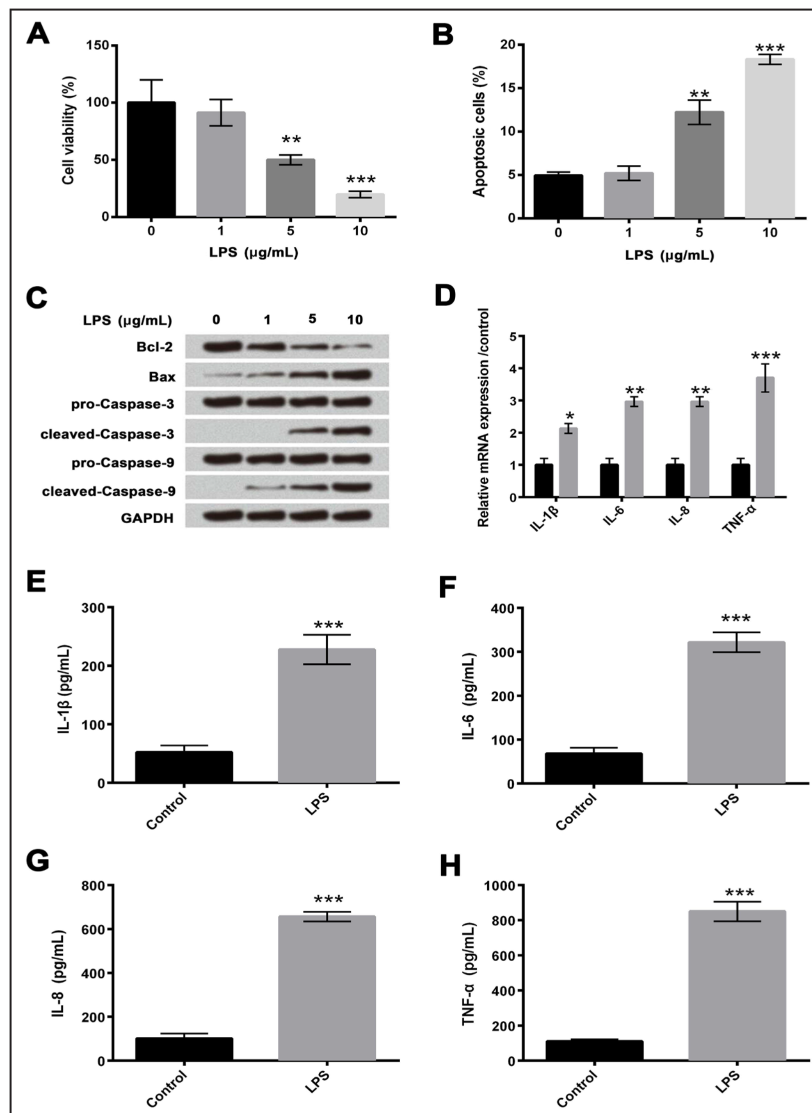

Fig. 1. LPS-induced inflammation injury in ADTC5 cells. Different concentrations of LPS (0, 1, 5, and 10 μg/ml) were used to treat cells for 5 h. (A) Cell viability, (B) Cell apoptosis and (C) The expression of apoptosis-related proteins were detected by CCK8 assay, flow cytometry and western blot. After 5 μg/ml LPS stimulated cells, (D) mRNA levels of inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α were tested by qRT-PCR. The concentrations of (E) IL-1β, (F) IL-6 , (G) IL-8, (H) TNF-α in the culture supernatant were measured by ELISA. **: P<0.01, ***: P<0.001.

LPS up-regulated the expression of miR-1246 in ATDC5 cells

The mRNA expression of miR-1246 in ADTC5 cells was detected using qRT-PCR analysis. As shown in Fig. 2A, obvious increase of miR-1246 expression was observed in the LPS-stimulated cells (P < 0.05). In Fig. 2B, the results showed that miR-1246 expression was clearly up-regulated by transfection with miR-1246 mimic compared to scramble (P < 0.001), and were down-regulated in cells transfected with miR-1246 inhibitor (P <0.01).

miR-1246 increased inflammatory injury in ATDC5 cells

The effect of miR-1246 mimic or inhibitor was measured in ATDC5 cells. The cell viability was reduced and cell apoptosis was markedly added when miR-1246 overexpression, while miR-1246 inhibitor showed the adverse consequences, that cell viability was increased and cell apoptosis was deceased (P < 0.05 or P < 0.01, Fig. 3A and 3B). Western blot analytical results displayed that miR-1246 overexpression clearly down-regulated Bcl-2, and up-regulated Bax, cleaved caspase-3 and caspase-9, meanwhile, the result of miR-1246 suppression was the opposite (Fig. 3C). In addition, the effects of miR-1246 on pro-inflammatory factor productions were analyzed. As shown in Fig. 3D-3H, the levels of IL-1β, IL-6, IL-8 and TNF-α were enhanced in miR-1246 group (P < 0.01 or P <0.001). By contrast, the results of miR-1246 knockdown exhibited the opposite impacts (P < 0.05 or P < 0.01).

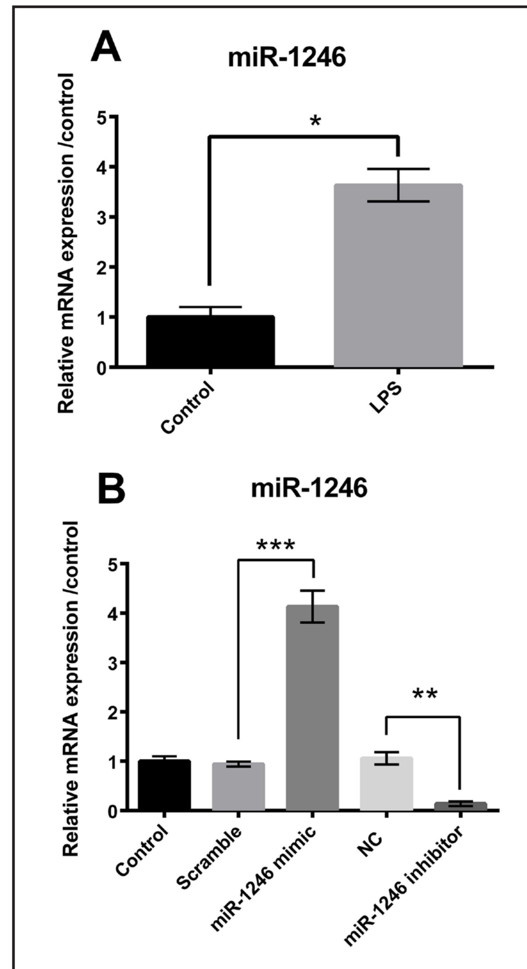

Fig. 2. LPS up-regulated the expression of miR-1246 in ATDC5 cells. (A) mRNA level of miR-1246 in cells treated with LPS, or (B) transfected with miR-1246-mimic and miR-1246-inhibitor were evaluated by qRT-PCR. *: P<0.05, **: P<0.01, ***: P<0.001.

miR-1246 overexpression alleviated LPS-induced inflammatory injury in ATDC5 cells

After transfection with miR-1246-mimic in ATDC5 cells, LPS-induced cell viability was decreased and LPS-stimulated cell apoptosis was boosted (both P < 0.05, Fig. 4A and 4B). The up-regulation of Bcl-2 and down-regulations of Bax, cleaved caspase-3 and cleaved caspase-9 were enhanced in LPS + miR-1246 mimic group (Fig. 4C). As expected, mRNA expression and productions of the pro-inflammatory cytokines in culture supernatant were significantly increased by transfection with miR-1246-mimic (P < 0.05) after LPS exposure (Fig. 4D-4H).

miR-1246 negatively regulated HNF4γ

The mRNA and protein expressions of HNF4γ were down-regulated in miR-1246-mimic group compared with scramble group (P < 0.05) and significantly up-regulated when miR-1246 knocked down (P < 0.01, Fig. 5A and 5B). Then, the relative luciferase activity in ATDC5 cells which were co-transfected with HNF4G-promotor and miR-1246 mimic was lower than the cells co-transfected with HNF4G-promotor and control (P < 0.01, Fig. 5C).

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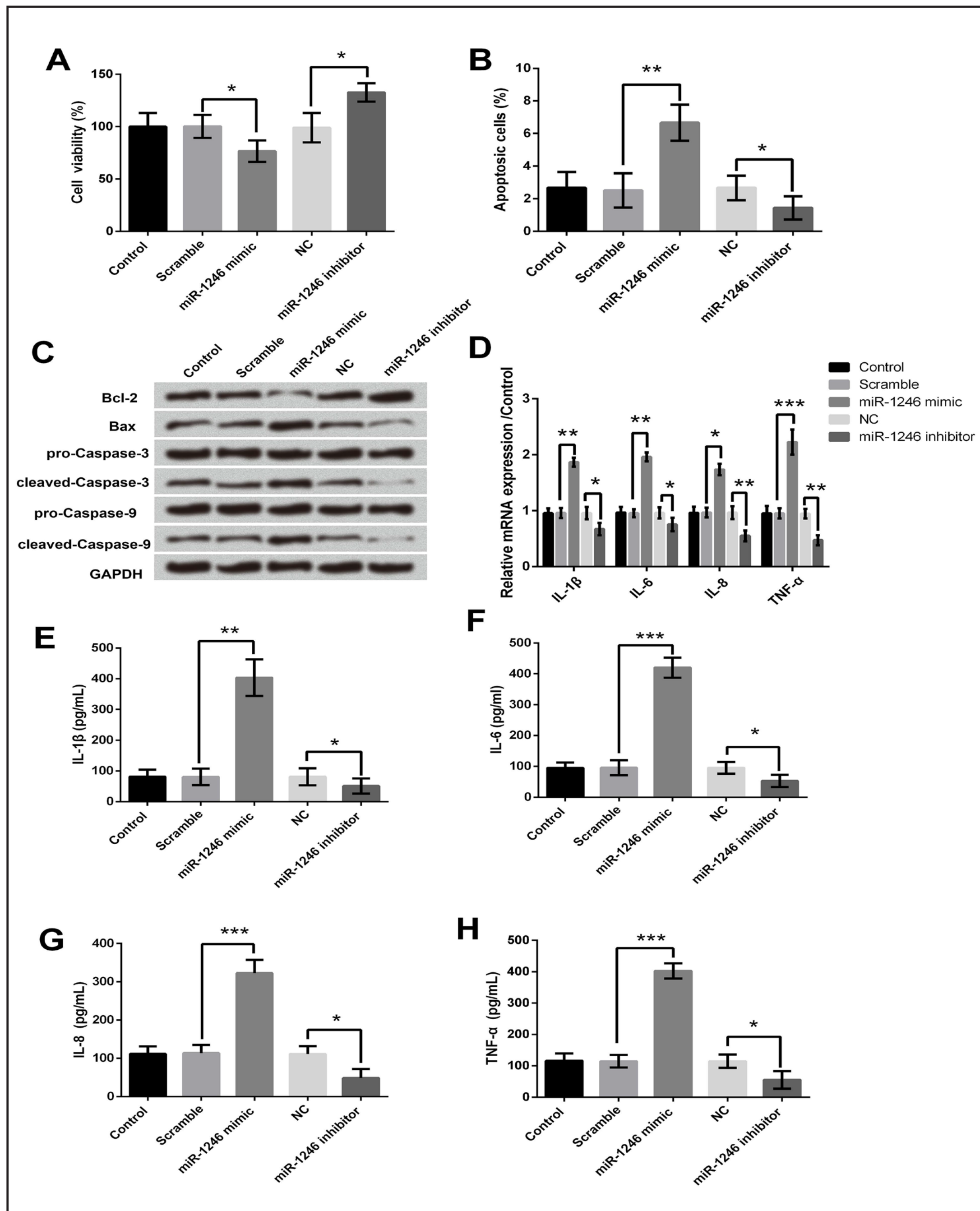

Fig. 3. miR-1246 expression was increased inflammatory injury in ATDC5 cells. (A) Cell viability, (B) Cell apoptosis and (C) The expression of apoptosis-related proteins were detected by CCK8 assay, flow cytometry and western blot. (D) mRNA levels of inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α were tested by qRT-PCR. The concentrations of (E) IL-1β, (F) IL-6 , (G) IL-8, (H) TNFα in the culture supernatant were measured by ELISA. *P<0.05, **: P<0.01, ***: P<0.001.

Suppression of miR-1246 reduced LPS-induced inflammatory injury in ATDC5 cells by overexpression of HNF4γ

The effects of HNF4G expression under LPS stimulation were further examined in ATDC5 cells. The significant increase of cell viability was observed in cells transfected with miR-

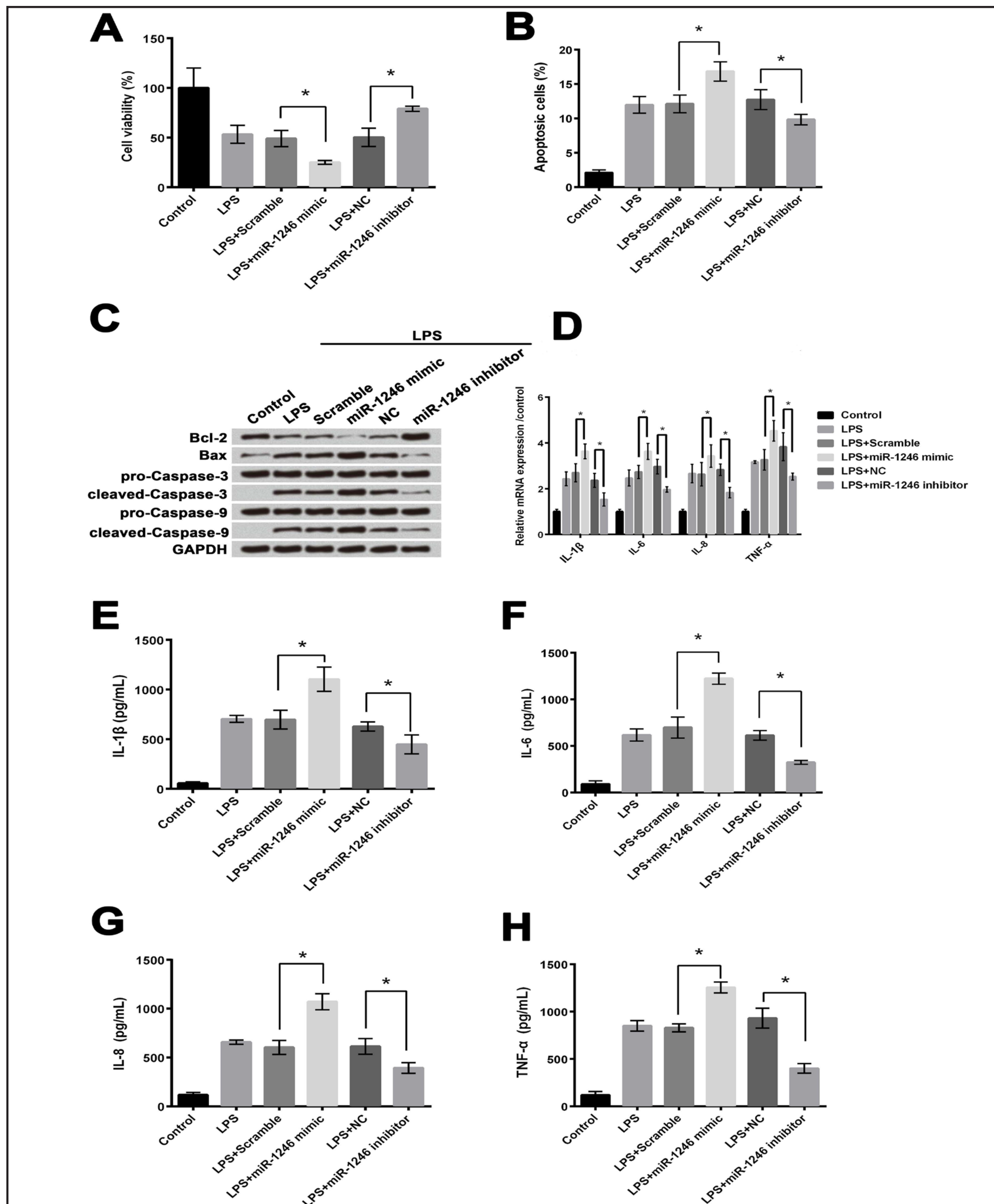

Fig. 4. miR-1246 overexpression alleviated LPS-induced inflammatory injury in ATDC5 cells (A) Cell viability, (B) Cell apoptosis and (C) The expression of apoptosis-related proteins were assessed by CCK8 assay, flow cytometryand western blot. (D) mRNA levels of inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α were obtained from qRT-PCR. The concentrations of (E) IL-1β, (F) IL-6 , (G) IL-8, (H) TNFα in the culture supernatant were evaluated using ELISA assay. *: $P<0.05$.

1246 inhibitor and si-HNF4γ ($P < 0.01$) even when injured with LPS (Fig. 6A). Then, HNF4γ overexpression alleviated the decrease of cell apoptosis by LPS exposure plus miR-1246 suppression ($P < 0.01$, Fig. 6B). Western blot analytical results showed that the expression of Bcl-2 was down-regulated and Bax, cleaved caspase-3 and caspase-9 were up-regulated when miR-1246 was knocked down and HNF4γ was overexpressed under LPS-treated conditions (Fig. 6C). The levels of pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α was

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improved in LPS + miR-1246 inhibitor + si-HNF4γ group than LPS + miR-1246 inhibitor group (all P < 0.05, Fig. 6D-6H).

Suppression of miR-1246 reduced LPS-induced inflammatory injury by activating PI3K/AKT and JAK/STAT signaling pathways

Further, inhibition of miR-1246 alleviated LPS-induced inflammatory injury in ATDC5 cells by activation of PI3K/AKT and JAK/STAT signaling pathways was observed in Fig. 7A and 7B.

The results of western blot demonstrated that the protein levels of HNF4γ, p-PI3K and p-AKT were all significantly down-regulated after LPS stimulation plus miR-1246 overexpression, while were up-regulated following miR-1246 knockdown in ATDC5 cells under LPS stimulation. The expressions of p-JAK1, p-STAT1 and p-STAT3 were contrary to the above results.

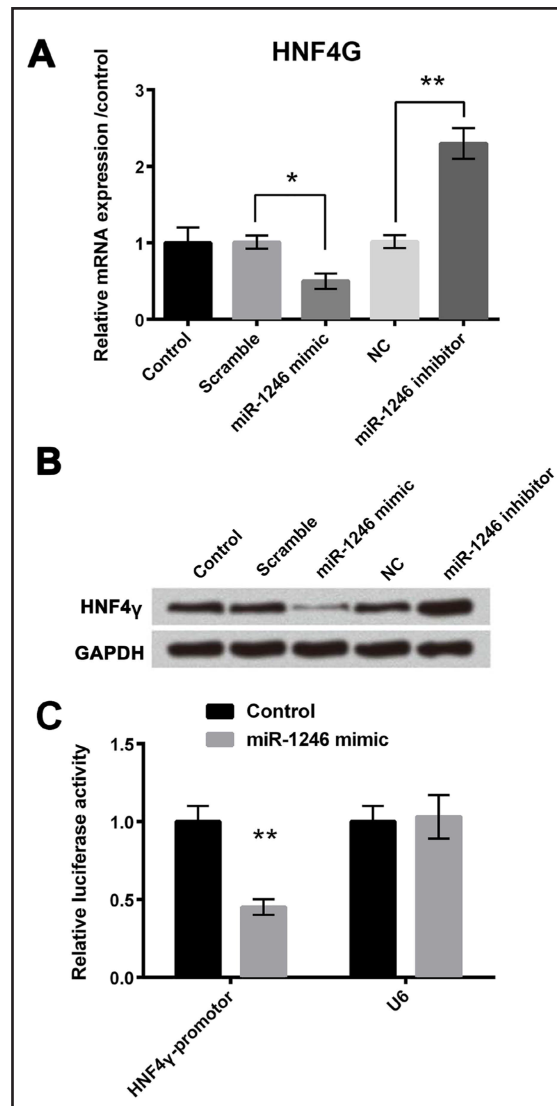

Fig. 5. miR-1246 negatively regulated HNF4γ. (A) mRNA and (B) protein levels of HNF4γ expression in ADTC5 cells transfected with miR-1246-mimic and miR-1246-inhibitor were evaluated using qRT-PCR and western blot. (C) HNF4γ could bind to of miR-1246 was observed by dual luciferase assay. *: P<0.05, **: P<0.01.

Discussion

OA is a complex, multifactorial inflammatory disease of the joint [26]. Studies showed that more than 25 miRNAs are functionally associated with the pathogenesis of OA [27]. Hence, our study aimed to investigate the regulation mechanism of miR-1246 in LPS-induced inflammatory injury of cartilage cells. In this study, we found that the expression of miR-1246 was up-regulated in ATDC5 cells treated with LPS. miR-1246 overexpression aggravated LPS-induced the decrease in cell viability, the increase in apoptosis, and the overproduction of pro-inflammatory factors. Then, further study suggested that miR-1246 negatively regulated HNF4γ expression. miR-1246 knockdown alleviated cellular inflammatory injury by up-regulating the expression of HNF4γ. We also found that HNF4γ overexpression attenuated the inflammatory injury of ATDC5 cells via activating PI3K/AKT and JAK/SATA signaling pathways.

In a study by Bott et al., miR-1246 was identified as critical regulator of NF-κB signaling pathway and increased pro-inflammatory responses in mesenchymal stem cells to affect different cell types. Meanwhile, this study also demonstrated that miR-1246 induced secretion of key pro-inflammatory cytokines and chemokines (IL-6, CCL2 and CCL5) in MSCs was mediated via NF-κB signaling pathway [25]. At the functional level, miR-1246 had been shown to induce the proliferation, invasion and migration and enhance cell apoptosis of cervical or hepatocellular carcinoma cells [28, 29]. In our study, we found that our results

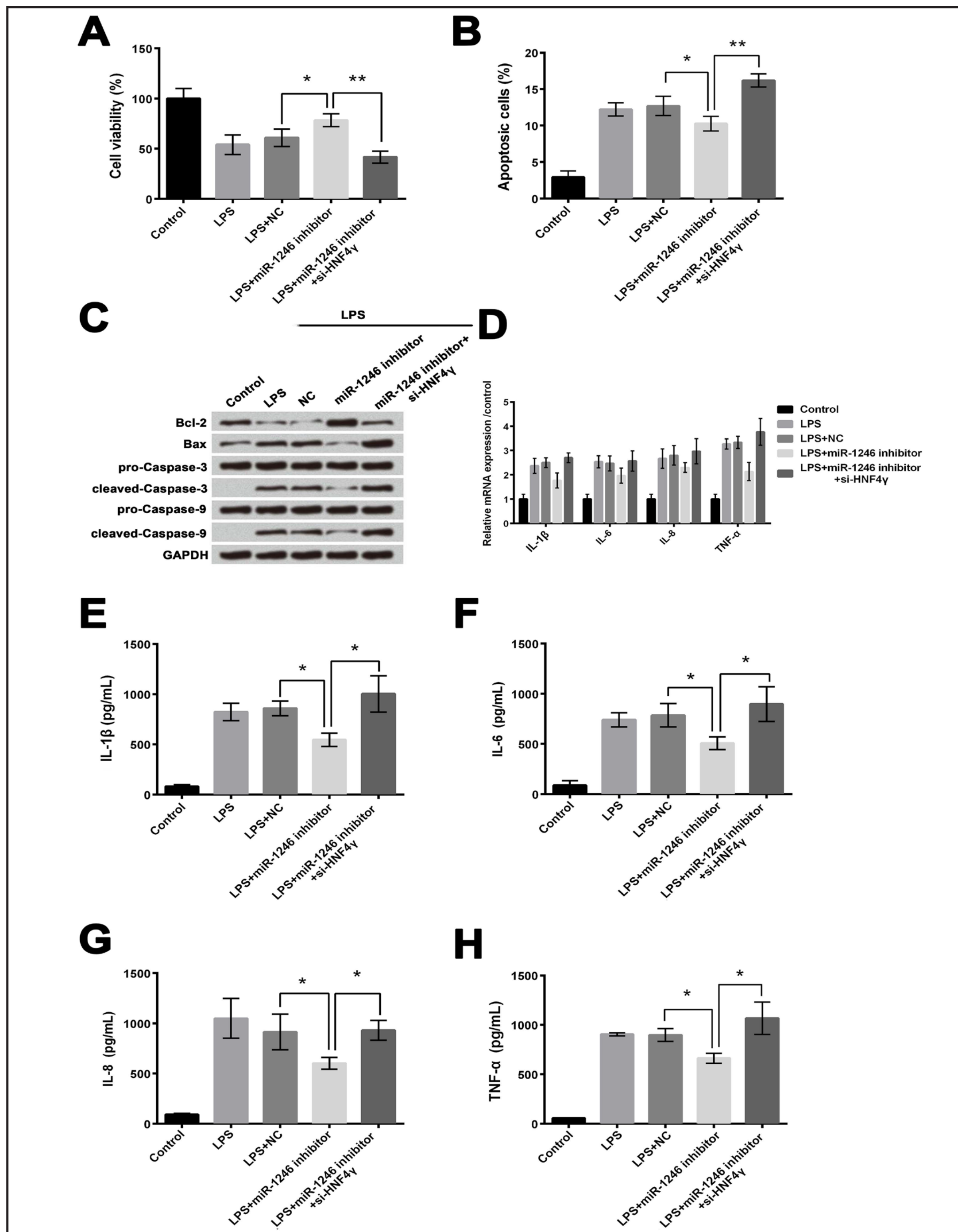

Fig. 6. Suppression of miR-1246 reduced LPS-induced inflammatory injury in ATDC5 cells by overexpression of HNF4γ. ATDC5 cells were transfected with miR-203 inhibitor or si-HNF4γ. (A) Cell viability, (B) Cell apoptosis and (C) The expression of apoptosis-related proteins were detected using CCK8 assay, flow cytometry and western blot. (D) mRNA levels of inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α were measured using qRT-PCR. The concentrations of (E) IL-1β, (F) IL-6, (G) IL-8 and (H) TNF-α in the culture supernatant were examined by ELISA. *: P<0.05, **: P<0.01.

were consistent with previous studies, that overexpression of miR-1246 strengthened the decrease in cell viability, the increase in apoptosis, and the overproduction of pro-

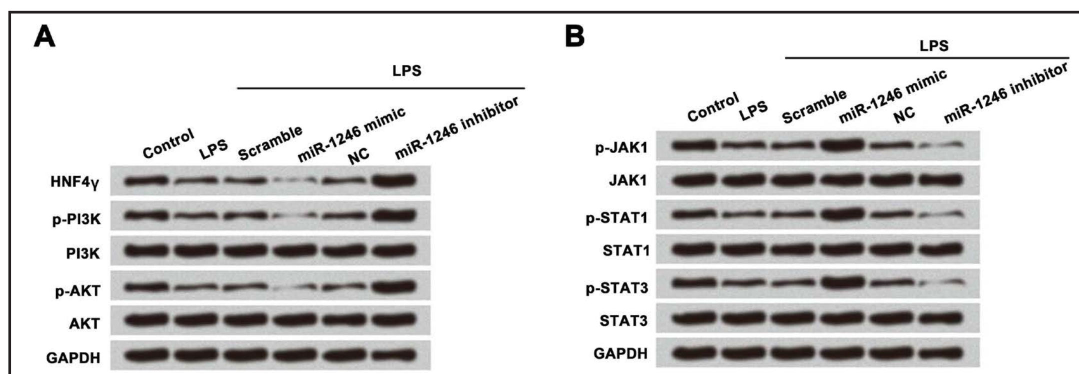

Fig. 7. Suppression of miR-1246 alleviated LPS-induced ATDC5 inflammatory injury by activation of PI3K/AKT and JAK/STAT pathways. Cells were transfected with miR-1246-mimic or miR-1246 inhibitor. The protein expressions of main factors in (A) PI3K/AKT and (B) JAK/STAT signaling pathways were measured.

inflammatory factors after LPS exposure. Sum up, we conjectured that miR-1246 might be involved in the regulation of inflammatory injury in OA.

HNF4γ is one of the two isoforms of human HNF4 and the other isoform is HNF4α. Numbers of studies had evaluated the function of HNF4α, for instance, its role in liver development and metabolism [30], and cancers [31, 32]. On the contrary, very few studies have focused on HNF4γ in OA. From the study of Sousa et al., HNF4γ acted as a target gene of miR-30a in gastric adenocarcinoma, that miR-30a overexpression was down-regulated HNF4γ expression [33]. Thus, we hypothesized that miR-1246 could regulate the expression of HNF4γ in OA. Our study had shown the evidence that miR-1246 silence reduced the inflammatory injury by up-regulation of HNF4γ. Further, miR-1246 targeted HNF4γ and negatively regulated the expression of HNF4γ.

According to a relevant research, the PI3K/AKT signaling pathway had been demonstrated to involve in the degradation of extracellular matrix and the death of chondrocytes in OA. And MMP productions by chondrocytes were added via activating the PI3K/AKT signaling pathway [34]. Another study suggested that JAK/STAT signaling pathway down-regulated steady state mRNA levels of key cellular components associated with cartilage degradation. In order to explore the mechanism(s) of which miR-1246 expression affected LPS-induced inflammatory injury in ATDC5 cells, we focused on PI3K/AKT and JAK/STAT signaling pathways. The western blot results from our study displayed that miR-1246 silence alleviated the inflammatory damage of ATDC5 cells by activation of PI3K/AKT and JAK/STAT signaling pathways.

In conclusion, we demonstrated that miR-1246 overexpression strengthened LPS-induced inflammatory injury in ATDC5 cells. Further studies indicated that HNF4γ is a target of miR-1246. miR-1246 silence mitigated inflammatory injury via up-regulation of HNF4γ and activation of PI3K/AKT and JAK/STAT signaling pathways. The findings of this study will provide a novel viewpoint regarding miR-1246 target for clinical.

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Disclosure Statement

There is no potential conflict of interests.

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