Original Paper

MiR-146a Aggravates LPS-Induced Inflammatory Injury by Targeting CXCR4 in the Articular Chondrocytes

Taitao Sun^a Xianjun Li^b Hua Song^c Fei Gao^a Guannan Zhou^d Lei Chen^e Zhentong Chen^a Lei Chen^e

^aDepartment of Orthopaedics, Jining No. 1 People's Hospital, Jining, ^bDepartment of Orthopaedics, Heze Municipal Hospital, Heze, ^cDepartment of Orthopaedics, Tengzhou Municipal People's Hospital, Tengzhou, ^dDepartment of Emergency, Jining No. 1 People's Hospital, Jining, ^eDepartment of Orthopaedics, Affiliated Hospital of Jining Medical University, Jining, China

Key Words

Inflammatory injury • MicroRNA-146a • CXCR4 • Chemokine receptor type 4 • PI3K/AKT • Wnt/β-catenin

Abstract

Background/Aims: Osteoarthritis (OA) as a degenerative disease is a major problem in ageing populations. To better understand the molecular mechanisms in the pathogenesis of OA, this study explored the role of microRNA (miR)-146a in the articular chondrocytes. **Methods:** The articular chondrocyte line ATDC5 was used to simulate inflammatory injury by LPS administration *in vitro*. Cell viability, apoptosis, mRNA expressions and productions of inflammatory factors were assessed, respectively. *Mir-146a* and *Cxcr4* mRNA expressions were measured by qPCR. Targeting effect of miR-146a on *Cxcr4* 3'UTR was assessed by luciferase activity analysis. Protein expression levels of CXCR4 and main factors in PI3K/AKT, Wnt/β-catenin signal pathways were measured by western blotting. **Results:** LPS exposure suppressed cell viability, prompted apoptosis of ATDC5 cells, and stimulated expression and release of inflammatory factors. MiR-146a was upregulated in LPS-induced cells. Overexpression of miR-146a further aggravated LPS-induced inflammatory injury, while it was reduced after miR-146a was knocked down. CXCR4 expression was negatively regulated by miR-146a. CXCR4 was a direct target of miR-146a and thus involved in regulatory effect of miR-146a on the injured chondrocytes, which was also related with phosphorylation levels of PI3K/AKT and expressions of Wnt/β-catenin signal factors. **Conclusion:** miR-146a promoted inflammatory response of articular chondrocytes via targeting CXCR4 and suppressing CXCR4 expression. Overexpression of CXCR4 could attenuate the inflammatory injury. Our findings provided novel evidence which might be useful for further studies exploring therapeutic approaches for OA via targeting miR-146a.

© 2017 The Author(s)
Published by S. Karger AG, Basel

T. Sun and X. Li share the first authorship.

Lei Chen

Department of Orthopedics, Affiliated Hospital of Jining Medical University
No. 89, Guhuai Road, Jining, (China)
E-Mail chenlei4922@126.com

Introduction

Osteoarthritis (OA) is one of the most common joint disorders with no significant ethnic and regional differences [1]. OA is characterized by a progressive degradation of articular leading to loss of joint mobility and function accompanied by chronic pain [2]. The incidence of OA increases with age, and thus becomes a major problem in ageing populations [3]. For decades, OA has been regarded as a pathological condition resulting from the degeneration or destruction of the articular cartilage tissue, in which the extracellular matrix (ECM) serves as primary target of articular cartilage [4]. However, this view ignored the involvement of articular chondrocytes, as potential instigator of OA. The articular chondrocytes, which only comprise 2-3% of total cartilage volume, could regulate the fate of articular cartilage by synthesize the structural components of ECM as well as matrix-degrading proteins [5]. Owing to the pivotal role of cartilage cells in OA disease process, the cartilage chondrocytes became the preferred cells in OA researches, and the study of chondrocytes in inflammatory injury is of great significance in understanding the pathogenesis and clinical treatment of OA [6].

MicroRNAs (miRNAs), a class of small non-coding RNAs (about 22 nucleotides), act as an important regulator in gene expression, especially in the transcriptional level [7]. A growing number of evidence indicated that miRNAs are involved in the control of various basic cell functions [8]. Studies have indicated that miRNAs play an important role in the process of chondrogenesis and cartilage remodeling [9]. The aberrant miRNA expression profiles have been demonstrated to be associated with OA [10, 11], and miRNAs acted as important regulators in OA development, as well as participated in inflammatory response [12, 13]. Previous studies have demonstrated that many miRNAs were involved in the regulation of OA, such as miR-320, miR-21, and miR-146a [14-16]. The miR-146a, as a negative regulator of leukocyte immune response, involved in many other cell processes including cancer cell proliferation [17, 18]. The expression pattern of miR-146a in cartilage from patients with OA has been identified that miR-146a was intensely expressed in low-grade OA cartilage, suggesting the potential role of miR-146a in OA cartilage pathogenesis [19]. Another investigation indicated that miR-146a could protect cartilage from degeneration in OA, as miR-146a overexpression could inhibit hypoxia-induced autophagy in chondrocytes [20]. However the exact role of miR-146a in OA is still unclearly.

The present study aimed to investigate the role of miR-146a in the articular chondrocytes cells which were subjected to LPS to induce inflammatory lesions, and the effect of altered miR-146a expression on the cells proliferation and apoptosis *in vitro*. In addition, miRNAs negatively regulate gene expression by controlling complementary target mRNAs and prohibiting their translation into functional proteins [21]. Therefore, the present study also investigated the target gene of miR-146a in cartilage chondrocytes cells, and the potential regulatory mechanism of miR-146a in OA pathogenesis.

Materials and Methods

Cell culture and administration of LPS

The murine articular chondrocyte line ATDC5 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified eagle medium: Nutrient Mixture F-12 (DMEM/F-12, Thermo Scientific, Rockford, IL, USA) supplemented with 2 mM Glutamine (Sigma-Aldrich, St. Louis, MO, USA) and fetal bovine serum (FBS, HyClone, Logan, UT, USA) which is 10% (v/v) for cell growth; or 2% (v/v) for cell maintaining. The culture medium was changed every 3 days until confluence reached. Stable cells were cultured in a humidified CO₂ (5%) incubator at 37°C.

Stable cultured cells with more than 75% confluence were split at 1:2 by using 0.25% trypsin (Ameresco, Framingham, MA, USA) and been cultured to 80% confluence. The culture medium was replaced with serum-free DMEM/F-12 medium and cells were cultured for another 24 h in a humidified atmosphere with 5% CO₂ at 37°C. The ATDC5 cells were cultured with LPS (Sigma-Aldrich, St. Louis, Mo, USA) in a

series of concentrations (0, 1, 5, and 10 $\mu\text{g/ml}$) for 6 h and then been harvested and processed for analyses. Treatment by DMEM/F-12 culture medium without LPS was performed as a control regimen.

Cell viability assay

Cell viability was analyzed by using Cell counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc., Kumamoto, Japan). In brief, ATDC5 cells after LPS treatment were seeded at a density of 5×10^3 cells per well in six-well plates and been cultured for 24 h. Following incubation, 20 μL CCK-8 solution (5 g/L) in phosphate-buffered saline (PBS, Sigma-Aldrich) was added. The plates were incubated for additional 4 h at 37°C. The absorbance at 450 nm was measured by using a plate reader (Bio-Rad Laboratories, Tokyo, Japan). Three independent experiments each in triplicate were performed.

Apoptotic assay

Apoptosis analysis was performed to identify and quantify the apoptotic cells by using Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) followed by flow cytometry. Briefly, after LPS treatment as described above, cells were cultured for 48 h and harvested, 1×10^5 cells were stained by 5 μL Annexin V-FITC (BD Biosciences) for 20 min at room temperature in the dark. Then 10 μL PI (5 $\mu\text{g/mL}$) in $1 \times$ binding buffer was added to each sample for 15 min in the dark. Subsequently, apoptosis in the cells was determined by using FCM (FacSCalibur; Becton-Dickinson, Franklin Lakes, NJ, USA) and Cell Quest software (Becton-Dickinson) to differentiate apoptotic cells (Annexin-V positive and PI negative) from necrotic cells (Annexin-V and PI-positive). The apoptotic assay was performed in three independent experiments.

Enzyme-linked immunosorbent assay (ELISA) assay

Following treatment of LPS for 48 h as described above, the culture supernatant was collected from 24-well plates and concentrations of inflammatory cytokines, including interleukin (IL)-1 β , IL-6, IL-8 and tumor necrosis factor (TNF)- α , were measured by using ELISA (R&D Systems, Abingdon, UK) according to the manufacturers protocols. Data were normalized and calculated on the basis of linear calibration curves generated by standard solutions.

Real-time quantitative PCR (qRT-PCR)

Total RNA from administrated cells was extracted using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions and been purified by RNeasy Mini kit (Qiagen, Germany). The extracted RNA samples were reversed by TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) and TaqMan MicroRNA Assay supplemented with the TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) were used for qRT-PCR analysis of miR-146a. For the mRNA measurement, reverse transcription kit (Applied Biosystems, CA, USA) supplemented with random hexanucleotide primers (dT) and Fast START Universal SYBR Green Master (ROX) (Roche, USA) were used for qRT-PCR on the ABI PRISM 7500 Real-time PCR System (Applied Biosystems, Foster City, CA). All primers were obtained from the NCBI GeneBank database and were synthesized by GenePharma (Shanghai, China). The reactions were performed in triplicate for each sample at least three independent runs. Data were analyzed according to the classic $2^{-\Delta\Delta Ct}$ method, and normalized to *U6* snRNA or *Gapdh* expression in each sample. The primers used were provided in Table 1.

Cell transfection

Cells were seeded into six-well plates at 5×10^4 per well and incubated for 24 h at 37°C. After reaching to 70% confluence, cells were respectively transfected with miR-146a mimic, miR-146a inhibitor, siRNA directly against CXCR4 (siCXCR4), or negative control, that were synthesized by GenePharma Co. (Shanghai, China) for 48 h by using Lipofectamine 3000 reagents (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The stably transfected cells were selected by G418 selection with the culture medium containing 0.5 mg/mL G418 (Sigma-Aldrich, St Louis, MO, USA) for 4 to 5 weeks, and stable cultured clones were isolated and selected [22]. The sequences for mmu-miR-146a mimic and inhibitor were as follows: mimic sense, UGAGAACUGAAUCCAUGGGUU, mimic anti-sense, CCCAUGGAAUUCAGUUCUAUU; inhibitor, AACCCAUGGAAUUCAGUUCUA.

Dual luciferase activity assay

The constructed pMiR-luciferase report vector (Promega, Madison, WI, USA) with the mmu-CXCR4 3'UTR carrying the putative mmu-miR-146a-binding sites (UUCAGUU) were amplified by PCR referred as CXCR4-wt. To mutate the putative binding site of mmu-miR-146a in the mmu-CXCR4 3'UTR, the sequence of putative

binding site was replaced to GGGGCGG, and referred as CXCR4-mut (CXCR4-mt). Cells were co-transfected with the reporter constructs or control vectors, and 14 h later cells were harvested using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Reporter analyses were performed using the dual-luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instruction.

Western blot assay

The total protein in treated cells was extracted by RNeasy lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitor (Roche, Shanghai, China), and then been quantified using BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The Western blot system was established by using a Bio-Rad Bis-Tris Gel system and polyvinylidene difluoride (PVDF) membranes according to the manufacture's instruction. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1000 and incubated with the membranes at 4°C overnight, followed by incubation with secondary antibodies marked by horseradish peroxidase (HRP, ab150668 and ab1575332, Abcam, USA) for 1 h at room temperature. The densitometry of the protein bands was analyzed by using Quantity One software (Bio-Rad Laboratories, Inc.) with values expressed relative to GAPDH as the control for the loading and transfer. Western blot analysis was performed using monoclonal primary antibodies: B cell lymphoma 2 (Bcl-2, sc509), Bax (sc20067), pro-Caspase 3 (sc7272), pro-Caspase 9 (sc17784), C-X-C chemokine receptor type 4 (CXCR4, sc6279), Wnt3a (sc80457), p-ERK1/2 (sc365370), (Santa Cruz, USA); Cleaved Caspase 3 (ab2302), Cleaved Caspase 9 (ab2324), pro-Caspase 9 (ab2324), p-ERK1/2 (ab54752), p-AKT (ab38449), phosphatidylinositol 3-kinases (PI3K, ab86714), p-PI3K (ab38264), β-catenin (ab22656), and GAPDH (ab8245) purchased from Abcam.

Statistical analysis

All data from independent experiments were expressed as mean ± standard deviation (SD). Statistical analyses were performed using Graphpad Prism 6.0 statistical software (GraphPad Software Inc., La Jolla, USA). *P* values were calculated by using a two-way analysis of variance (ANOVA) followed by Bonferroni (more than two groups) or student's *t*-test for two groups. *P*-value of < 0.05 was considered to indicate statistically significant result.

Results

LPS induced inflammatory response of ATDC5 cells

After administration with different concentrations of LPS (0, 1, 5 and 10 µg/mL), ATDC5 cells viability was assessed and results in Fig. 1A showed that 5 and 10 µg/mL LPS exposure significantly decreased ATDC5 cells viability compare with control groups without LPS administration ($P < 0.01$ or $P < 0.001$). Flow cytometry assay results in Fig. 1B showed that the relative apoptotic cell number in LPS treated groups was significantly increased compared

Table 1. The primers used in real-time quantitative PCR (qRT-PCR)

Gene	Forward primer	Reverse primer
IL-1β	5'-AAGATGAAGGGCTGCTTCCAAACC-3'	5'-ATACTGCCTGCCTGAAGCTCTTGT-3'
IL-6	5'-CATCCAGTTGCCTTCTTGGGA-3'	5'-CTGAAGGACTCTGGCTTGTC-3'
IL-8	5'-CATCTTCGTCGCCCTCTGTG-3'	5'-GCCAACAGTAGCCTTCACCCA-3'
Tnf-α	5'-CGTCAGCCGATTGCTATCT-3'	5'-CGGACTCCGCAAAGTCTAAG-3'
mir-146a	5'-GGGTGAGAACTGAATTCCA-3'	5'-CAGTGCCTGTGAGAGT-3'
Cxcr4	5'-TCAGTGGCTGACCTCCTCT-3'	5'-CTTGGCCTTGGACTCTCTCT-3'
U6	5'-CTCGCTTCGGCAGCACATATACT-3'	5'-ACGCTTCAAGGCGGAG-3'
Gapdh	5'-GGTGAAGGTCGGTGTGAACG-3'	5'-CTCGCTCCTGATGCTGT-3'

Fig. 1. LPS induced chondrocyte inflammatory injury in ATDC5 cells. Chondrocyte line ATDC5 was administrated by different contents of LPS (1, 5, and 10 $\mu\text{g/mL}$) to simulate inflammatory lesions. Cells without LPS administration (0 $\mu\text{g/mL}$) was used as control. (A) CCK-8 was performed to assess cell viability. (B) Relative apoptotic cells were measured by flow cytometry. (C) The protein immunoblots of apoptosis-related factors were measured by western blotting. GAPDH acted as an internal control. (D) The mRNA expression levels of pro-inflammatory cytokines (IL-1 β , IL-6, IL-8, and TNF- α) were measured by qRT-PCR. ELISA was performed to measure the productions of IL-1 β (E) IL-6 (F), IL-8 (G), and TNF- α (H). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with control.

that in control group ($P < 0.05$ for 1 $\mu\text{g/mL}$ LPS administration group, $P < 0.01$ for 5 $\mu\text{g/mL}$ LPS administration group, and $P < 0.001$ for 10 $\mu\text{g/mL}$ LPS administration group). Western blot assay in Fig. 1C suggested that in LPS-induced ATDC5 cells, Bcl-2 expression was decreased, while expressions of Bax, cleaved Caspase 3 and cleaved Caspase 9 were all elevated compare with groups without LPS treatment. It suggested that LPS administration inhibited cell viability and promoted apoptosis of ATDC5 cells *in vitro*. According to the effect of LPS on cell viability, 5 $\mu\text{g/mL}$ was selected as LPS-stimulating concentration used in the following experiments.

In addition, qRT-PCR analysis results showed that mRNA expression levels of *Il-1 β* , *Il-6*, *Il-8* and *Tnf- α* were all increased in LPS administrated cells compare with control group

Fig. 2. miR-146a was upregulated in LPS-administrated ATDC5 cells. ATDC5 cells were administrated by LPS (5 μ g/mL) to simulate inflammatory response. Cells without LPS administration were used as control. (A) Relative miR-146a expression was measured by qRT-PCR. (B) ATDC5 cells were transfected with miR-146a mimic, scramble control, miR-146a inhibitor, or inhibitor control. Relative miR-146a expressions were measured by qRT-PCR. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with corresponding negative control.

($P < 0.01$, Fig. 1D). At the same time, ELISA assay results showed that in LPS administrated groups, the production of inflammatory factors IL-1 β (Fig. 1E), IL-6 (Fig. 1F), IL-8 (Fig. 1G) and TNF- α (Fig. 1H) were all significantly increased compared with control group ($P < 0.001$), suggesting that LPS effectively induced the upregulation of inflammatory factors and the inflammatory response of ATDC5 cells.

miR-146a promoted LPS-induced inflammatory injury in ATDC5 cells

After LPS administration, qRT-PCR analytical results in Fig. 2A showed that expression of miR-146a was elevated in ATDC5 cells compared with control ($P < 0.05$). ATDC5 cells were then transfected with miR-146a to assess the effect of miR-146a on LPS-induced inflammatory damage. qRT-PCR results in Fig. 2B showed that miR-146a mimic transfection significantly increased miR-146a expression level compare with scramble control ($P < 0.001$), while miR-146a inhibitor transfection decreased miR-146a expression compare with the inhibitor control group ($P < 0.01$), suggesting that miR-146a mimic and miR-146a inhibitor transfections could effectively alter the expression of miR-146a in ATDC5 cells *in vitro*.

Flow cytometry results in Fig. 3A showed that LPS administration decreased cell viability of ATDC5 cells. After that, cell viability was decreased in miR-146a mimic transfection group compare with LPS-induced control ($P < 0.05$), while miR-146a inhibitor transfection increased cell viability compare with inhibitor control ($P < 0.05$). In Fig. 3B, after that LPS promoted cell apoptosis, miR-146a mimic further increased the number of apoptotic cells ($P < 0.05$), while miR-146a inhibitor transfection decreased cell apoptosis compared with negative control ($P < 0.05$). In addition, decreased expression of Bcl-2 by LPS was reduced after miR-146a mimic transfection, and it was increased by miR-146a inhibitor transfection. Expression of Bax, cleaved Caspase 3 and cleaved Caspase 9 were all further increased after miR-146a mimic transfection in LPS-induced cells, while they were all reduced in miR-146a inhibitor transfected cells (Fig. 3C). Meanwhile, mRNA expression levels (Fig. 3D) and productions (Fig. 3E-3H) of inflammatory factors were all further increased by miR-146a mimic transfection compared with the increased expression and production by LPS ($P < 0.05$). While the increased inflammatory factors expression and productions of these inflammatory factors induced by LPS administration were suppressed by miR-146a inhibitor

Fig. 3. miR-146a promoted LPS-induced inflammatory injury. ATDC5 cells were administered by LPS (5 μ g/mL), and then been transfected with miR-146a mimic, scramble control, miR-146a inhibitor, or inhibitor control, respectively. (A) CCK-8 was performed to assess cell viability. (B) Relative apoptotic cells were measured by flow cytometry. (C) The protein immunoblots of apoptosis-related factors were measured by western blotting. GAPDH acted as an internal control. (D) The mRNA expression levels of pro-inflammatory cytokines (IL-1 β , IL-6, IL-8, and Tnf- α) were measured by qRT-PCR. ELISA was performed to measure the productions of IL-1 β (E), IL-6 (F), IL-8 (G), and Tnf- α (H). * indicates $P < 0.05$ compared with corresponding LPS control.

transfection ($P < 0.05$). Taken together, these data suggested that miR-146a upregulation promoted LPS-induced inflammatory response of ATDC5 cells.

miR-146a directly targeted CXCR4

In ATDC5 cells, after miR-146a transfection, we found that CXCR4 expression was negatively related with miR-146a expression. As shown in Fig. 4A, mRNA expression level of *Cxcr4* was significantly decreased in miR-146a mimic transfected group compare with negative control ($P < 0.05$), while it was increased in miR-146a inhibitor transfected group ($P < 0.01$). Western blotting results in Fig. 4B showed that miR-146a mimic transfection

Fig. 4. miR-146a negatively regulated expression of CXCR4 and CXCR4 was a direct target of miR-146a. ATDC5 cells were transfected with miR-146a mimic, scramble control, miR-146a inhibitor, or inhibitor control, respectively. (A) The mRNA expression level of CXCR4 was measured by qRT-PCR. (B) Protein expression of CXCR4 was measured by western blotting. GAPDH acted as an internal control. (C) Cells were co-transfected with miR-146a mimic and CXCR4-wild type or recombinant vector. The CXCR4-mutant type (mt) recombinant vector was used as positive control. The effect of miR-146a on the 3'UTR of CXCR4 was measured by Dual-luciferase activity assay. *, $P < 0.05$; **, $P < 0.01$ compared with corresponding negative control.

decreased protein expression of CXCR4, but miR-146a inhibitor transfection increased CXCR4 expression. Dual luciferase activity assay was performed to determine the binding effect of miR-146a on 3'UTR of CXCR4. Results in Fig. 4C showed that co-transfected with miR-146a mimic significantly reduced the luciferase activity of CXCR4-wt group compared with negative control group ($P < 0.05$). These results suggested that CXCR4 might be a direct target of miR146a in ATDC5 cells and miR-146a negatively regulated the expression of CXCR4.

miR-146a Inhibition reduced LPS-induced inflammatory injury via upregulating CXCR4

ATDC5 cells were pre-administrated with LPS and then were transfected with miR-146a inhibitor and/or CXCR4. Cell viability was assessed and results in Fig. 5A showed that miR-146a inhibitor transfection increased cell viability compare with LPS-induced group ($P < 0.05$) and co-transfected with siCXCR4 inhibited cell viability which was increased in miR-146a inhibitor transfected group ($P < 0.01$). In Fig. 5B, the relative apoptotic cells were decreased after LPS exposure, and miR-146a inhibitor transfection decreased it ($P < 0.05$). In miR-146a inhibitor + siCXCR4 group, the relative number of apoptotic cells was significantly increased compare with miR-146a inhibitor group ($P < 0.01$). Consistent results was found in Western blot assay as shown in Fig. 5C. In LPS-induced groups, siCXCR4 transfection decreased the expression of Bcl-2, which was decreased by miR-146a inhibitor transfection, and transfection with siCXCR4 increased the expressions of Bax, cleaved Caspase 3 and cleaved Caspase 9, that was decreased after miR-146a inhibitor transfection.

In addition, the mRNA expression levels of inflammatory factors were all increased after siCXCR4 co-transfection compared with miR-146a inhibitor alone ($P < 0.05$) as shown in Fig. 5D. And also Fig. 5E-5H showed that the productions of inflammatory factors in siCXCR4 transfected group were all significantly increased compared with that was decreased in miR-146a inhibitor transfected groups ($P < 0.05$, or $P < 0.01$). Taken together, these results suggested that miR-146a downregulation reduced inflammatory response of ATDC5 cells via upregulating the expression of CXCR4.

Fig. 5. miR-146a regulated inflammatory injury in ATDC5 cells via targeting CXCR4. ATDC5 cells were administrated by LPS (5 μ g/mL), and then were co-transfected with miR-146a inhibitor, and CXCR4 siRNA (siCXCR4). (A) CCK-8 was performed to assess cell viability. (B) Relative apoptotic cells were measured by flow cytometry. (C) The protein immunoblots of apoptosis-related factors were measured by western blotting. GAPDH acted as an internal control. (D) The mRNA expression levels of pro-inflammatory cytokines (IL-1 β , IL-6, IL-8, and Tnf- α) were measured by qRT-PCR. ELISA was performed to measure the productions of IL-1 β (E), IL-6 (F), IL-8 (G), and Tnf- α (H). *P < 0.05 compared with LPS group, **P < 0.01 compared with LPS group, and ***P < 0.001 compared with LPS group.

Expression of miR-146a was negatively related with the activation of PI3K/AKT and Wnt/ β -Catenin signaling pathways

After LPS administration, ATDC5 cells were transfected with miR-146a mimic, miR-146a inhibitor or corresponding negative control, respectively. Western blotting was performed to measure the expressions of mainly factors in PI3K/ATK and Wnt/ β -Catenin pathways. As shown in Fig. 6A, in miR-146a mimic transfected group, expressions of CXCR4, phosphorylated PI3K and phosphorylated AKT were all decreased, while they were increased

Fig. 6. miR-146a was related with activation of PI3K/AKT and Wnt/β-Catenin signaling pathways via negatively regulating CXCR4 expression. ATDC5 cells were administered by LPS (5 and 10 μg/mL), and then were transfected with miR-146a mimic, scramble control, miR-146a inhibitor, miR-146a inhibitor + LPS, or control, respectively. Expression of CXCR4, phosphorylated (p-) PI3K, PI3K, p-AKT, AKT (A) and expressions of Wnt3a, Wnt5a, β-Catenin (B) were measured by western blotting. GAPDH acted as an internal control.

in miR-146a inhibitor transfected group, suggesting phosphorylation levels of PI3K and AKT were negatively related with miR-146a expression. In panel B, it showed that the expressions of Wnt3a, Wnt5a and β-Catenin were all decreased in miR-146a mimic transfected group, as well as CXCR4 expression. Meanwhile, in miR-146a inhibitor transfected group, expressions of these factors were all increased, suggesting that the activation of Wnt/β-Catenin signaling pathway and expression of CXCR4 were positively related with miR-146a expression in ATDC5 cells *in vitro*.

Discussion

Over the last decade, there have been great strides in genomic analyses of OA owing to the study of adequately powered patient cohorts, the detailed analysis of candidate genes, and the application of genome-wide approaches [23]. These studies have led to unexpected and exciting discoveries, implicating some potential pathways and factors in this complex arthritis. Based on the prior observations that the OA disease is associated with inflammation, and the inflammatory gene is the OA risk factor that alteration in the inflammation is highly relevant to the disease process [24, 25]. Our present studies indicated the role of miR-146a in the inflammatory injury of chondrocytes, which were induced by LPS to simulate inflammatory model *in vitro*, with regulatory effect on the activation of PI3K/AKT and Wnt/β-catenin signaling pathways which was mediated by CXCR4. We found that administration of LPS (5 and 10 μg/mL) effectively caused cell inflammatory response, cell viability suppression and apoptosis promotion, suggesting that LPS could effectively induce an *in vitro* model of articular chondrocyte inflammatory injury.

MiRNAs act as important regulators in modulating and maintaining normal physiological conditions [26]. The levels of miRNA have been proved to be changed in response to pathological disorders, resulting in the pathogenesis of degenerative diseases, such as OA [27, 28]. Therefore, it is important to determine the regulation of miRNA expressions in OA. In this present study, we found that miR-146a was significantly increased in the chondrocytes after LPS exposure. Previous studies have suggested the negatively regulatory effect of miR-146a on immune response [29]. It was proved that miR-146a acted as an immunoregulatory

miRNA in the innate response in virus infection [30]. The present study hypothesized that miR-146a may be another mediator of inflammatory response in chondrocytes. Transfection of synthetic miR-146a mimic *in vitro* was found to elevate LPS-induced suppression of ATDC5 cell viability and the increased apoptosis, exacerbated the inflammatory response of chondrocytes including the increased expressions and products of IL-1 β , IL-6, IL-8 and TNF α . Our study suggested that miR-146a overexpression might promote inflammatory response of chondrocytes.

In the study of Liang et al., it was found that miRNA against CXCR4 to block the invasion and metastasis of breast cancer cells [31]. In the present studies, we found that expression of CXCR4 was negatively regulated by miR-146a. The regulatory effect of miR-146a on expression of CXCR4 was also been investigated. Besides, dual-luciferase activity assay was performed to investigate the underlying mechanisms of miR-146a and CXCR4. CXCR4 and CXCR4 was identified as a potential target gene of miR-146a. CXCR4 is an adhesion-chemokine receptor specific for stromal-derived-factor-1 (SDF-1 also called CXCL12), a molecule endowed with potent chemotactic activity for lymphocytes. It has been reported that CXCR4 signaling regulated the expression of CD20 on B cells [32]. Evidence in animal model suggested the ubiquitin was the natural ligand of CXCR4 and it was an anti-inflammatory immune modulator and endogenous opponent of pro-inflammatory damage associated molecular pattern molecules [33], suggesting that CXCR4 was involved in the immune response.

In this present study, we found that CXCR4 was negatively regulated by miR-146a and thus regulated cell inflammatory damages. Western blotting analysis was then used to predict several signaling pathways that possibly contribute to the effect of CXCR4 which was regulated by miR-146a on chondrocytes. Results suggested that CXCR4 as the target of miR-146a was positively related with the activation of PI3K/AKT and Wnt/ β -catenin signaling pathways in chondrocytes *in vitro*. It is known that PI3K/AKT signaling plays a critical role in modulating cell survival and apoptosis [34], and it has been found over-activated in many cancers reducing apoptosis and promoting proliferation [35]. Wnt/ β -catenin signaling pathway is involved in proliferation and differentiation [36]. The activation of PI3K/AKT can activate the Wnt signaling through the phosphorylation of GSK-3 β by the phosphorylated Akt1/2, leading to the formation of β -catenin destroying complexes [37]. Thus we deduced that miR-146a effect on cell viability and apoptosis of inflammatory chondrocytes might be via regulating CXCR4 expression and PI3K/AKT and Wnt/ β -catenin signaling pathways.

Conclusion

The present study demonstrated that miR-146a promoted inflammatory response of articular chondrocytes might be via targeting CXCR4 and deactivating PI3K/AKT and Wnt/ β -catenin pathways. According to these results, we might in fact be close to demonstration at a basic level of OA pathogenesis, that this study preliminarily explored the role of miR-146a in the pathogenesis of OA. It must be admitted that more *in vivo* or animal researches will be helpful to understand the pathogenesis of OA and make miRNAs contribute to clinical treatment of OA. These results might have implication for the design of trials of novel OA interventions, and might also guide the intelligent repurposing of anti-inflammatory therapies.

Acknowledgements

The work received no funding support from any agency.

Disclosure Statement

There is no conflict of interests.

References

- Colen S, Bekerom MPJVD, Mulier M: Intra-articular viscosupplementation in patients with hip osteoarthritis. *Eur Musculoskelet Rev* 2011;6:079-082.
- Wang XD, Kou XX, He DQ, Zeng MM, Meng Z, Bi RY, Liu Y, Zhang JN, Gan YH, Zhou YH: Progression of Cartilage Degradation, Bone Resorption and Pain in Rat Temporomandibular Joint Osteoarthritis Induced by Injection of Iodoacetate. *PLoS One* 2012;7:e45036.
- Dreier R: Hypertrophic differentiation of chondrocytes in osteoarthritis: the developmental aspect of degenerative joint disorders. *Arthritis Res Ther* 2010;12:216.
- Zeng G, Cui X, Liu Z, Zhao H, Zheng X, Zhang B, Xia C: Disruption of phosphoinositide-specific phospholipases $\text{C}\alpha 1$ contributes to extracellular matrix synthesis of human osteoarthritis chondrocytes. *J Mol Sci* 2014;15:13236.
- Aigner T, Soder S, Gebhard PM, McAlinden A, Haag J: Mechanisms of disease: role of chondrocytes in pathogenesis of osteoarthritis--structure, chaos and senescence. *Nat Clin Pract Rheumatol* 2007;3:391-399.
- Goldring MB, Otero M, Plumb DA, Dragomir C, Favero M, El HK, Hashimoto K, Rasmussen J, Olivetto E, Borzi RM: Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis. *Eur Cell Mater* 2012;22:1-12.
- Lee S, Vasudevan S: Post-transcriptional Stimulation of Gene Expression by miRNAs. *Adv Exp Med Biol* 2013;768:97-126.
- Hong E, Reddi AH: MicroRNAs in chondrogenesis, articular cartilage, and osteoarthritis: implications for tissue engineering. *Tissue Eng Part B Rev* 2012;18:407-417.
- Dong S, Yang B, Guo H, Kang F: MicroRNAs regulate osteoarthritis and chondrogenesis. *Biochem Biophys Res Commun* 2012;418:587-591.
- Kirilov N, Dimov S, Dalgalarondo M, Ignatova T, Kamenov S, Ivanovski S, Danova S, Iliev I, Haertlé T, Chobert JM: Characterization of microRNA expression profiles in normal and osteoarthritic human chondrocytes. *Osteoarthritis Cartilage* 2011;19:1012-1022.
- Hou C, Meng F, Zhang Z, Kang Y, Chen W, Wang Y, Fan Y, Sheng P, Zhang Z, Liao W: The Role of MicroRNA-381 in Chondrogenesis and Inflammation in Interleukin-1 β Induced Chondrocyte Responses. *Cell Physiol Biochem* 2015;36:1753-1766.
- Swingler TE, Wheeler G, Carrozzini M, Barter MJ, MA-E, STD, Boot-Handford RP, Hajihosseini MK, Münsterberg A: The expression and function of microRNAs in chondrogenesis and osteoarthritis. *Arthritis Rheum* 2012;64:1009.
- Le LT, Swingler TE, Clark IM: The role of microRNAs in osteoarthritis and chondrogenesis. *Arthritis Rheumatol* 2016;63:1971.
- Zhang Z, Meng F, Zhang Z, Kang Y, Sheng P, Liao W: Regulation of matrix metalloproteinase-13 by MIRNA-140 in chondrogenesis and osteoarthritis. *Osteoarthr Cartilage* 2016;24:S381.
- Zhang Z, Meng F, Zhang Z, Ye S, Tian H: MicroRNA-21 controls the development of osteoarthritis by targeting p38 α and p38 β in chondrocytes. *Exp Mol Med* 2014;46:e79.
- Carlsson L, Guza GAD, Ødegaard B, Engebretsen L, Brinchmann JE: microRNA-140 Inhibits Inflammation and Stimulates Chondrogenesis in a Model of Interleukin 1 β -induced Osteoarthritis. *Mol Ther Nucleic Acids* 2016;5:e373.
- Perry MM, Moschos SA, Williams AE, Shepherd NJ, Larnersvensson HM, Lindsay MA: Rapid Changes in MicroRNA-146a Expression Negatively Regulate the IL-1 β -Induced Inflammatory Response in Human Lung Alveolar Epithelial Cells. *J Immunol* 2008;180:5689-5698.
- Lee HM, Choi Y, Cho S, Lin LL, Kang S, Aly K, Xiao C, Crotty S, Lu LF: miR-146a as a negative regulator in controlling humoral immunity (IRM10P.612). *J Immunol* 2015;194.
- Yamasaki K, Nakasa T, Miyaki S, Ishikawa M, Deie M, Adachi N, Yasunaga Y, Asahara H, Ochi M: Expression of MicroRNA-146a in osteoarthritis cartilage. *Arthritis Rheumatol* 2009;60:1035-1041.
- Zhang F, Wang J, Chu J, Yang C, Xiao H, Zhao C, Sun Z, Gao X, Chen G, Han Z, Zou W, Liu T: MicroRNA-146a Induced by Hypoxia Promotes Chondrocyte Autophagy through Bcl-2. *Cell Physiol Biochem* 2015;37:1442-1453.
- Behmamsant I, Rehwinkel J, Izaurralde E: MicroRNAs Silence Gene Expression by Repressing Protein Expression and/or by Promoting mRNA Decay. *Cold Spring Harb Symp Quant Biol* 2006;71:523.

- 22 Lin-Cereghino J, Hashimoto MD, Moy A, Castelo J, Orazem CC, Kuo P, Xiong S, Gandhi V, Hatae CT, Chan A: Direct selection of *Pichia pastoris* expression strains using new G418 resistance vectors. *Yeast* 2008;25:293-299.
- 23 Rogers EL, Reynard LN, Loughlin J: The role of inflammation-related genes in osteoarthritis. *Osteoarthritis Cartilage* 2015;23:1933.
- 24 Ray A, Ray BK: An inflammation-responsive transcription factor in the pathophysiology of osteoarthritis. *Biorheology* 2008;45:399-409.
- 25 Zhang M, Wang J: Epigenetic regulation of gene expression in osteoarthritis. *Genes & Diseases* 2015;2:68-75.
- 26 Bhaskaran M, Mohan M: MicroRNAs: history, biogenesis, and their evolving role in animal model of disease. *Vet Pathol* 2014;51:759.
- 27 Kutty RK, Nagineni CN, Samuel W, Vijayasathya C, Hooks JJ, Redmond TM: Inflammatory cytokines regulate microRNA-155 expression in human retinal pigment epithelial cells by activating JAK/STAT pathway. *Biochem Biophys Res Commun* 2010;402:390.
- 28 Shumnalieva R, Kachakova D, Monov S, Kaneva R, Kolarov ZI, Rashkov R: A8.01 Systemic and local miRNA expression in patients with osteoarthritis. *Ann Rheum Dis* 2016;75:A65.61-A65.62.
- 29 Meisgen F, Xu LN, Wang A, Réthi B, Bouez C, Zuccolo M, Gueniche A, Gille M, Sonkole M, Breton L: MiR-146a negatively regulates TLR2-induced inflammatory response in keratinocytes. *J Invest Dermatol* 2014;134:1931.
- 30 Bellinghausen C, Beuken EV, Wouters EFM, Rohde GGU, Stassen FR: miR-146a is an immunoregulatory microRNA miR-146a in the innate immune response to rhinovirus infection. *Eur Respir J* 2013;42.
- 31 Liang Z, Wu H, Reddy S, Zhu A, Wang S, Blevins D, Yoon J, Zhang Y, Shim J: Blockade of invasion and metastasis of breast cancer cells via targeting CXCR4 with an artificial microRNA. *Biochem Biophys Res Commun* 2007;363:542-546.
- 32 Pavlasova G, Borsky M, Seda V, Cerna K, Osickova J, Jirasek M, Koller J, Calogero R, Trbusek M, Pospisilova S: Ibrutinib inhibits CD20 upregulation on CLL B cells mediated by the CXCR4/SDF-1 axis. *Blood* 2016;128:1609.
- 33 Majetschak M: Extracellular ubiquitin: its role as a modulator and endogenous opponent of damage-associated molecular pattern responses. *J Intern Med* 2011;89:205-219.
- 34 Xie D, Gore C, Zhou J, Pong RC, Chen H, Yu L, Tessella RL, Min W, Hsieh JT: DAB2IP coordinates both PI3K-Akt and ASK1 pathways for cell survival and apoptosis. *Proc Natl Acad Sci U S A* 2009;106:19878-19883.
- 35 Wang C, Zeng L, Tao Z, Li J, Wang W: IL-1β Inhibitor Prevents IL-1β-induced Inflammation in Human Osteoarthritis Chondrocytes by Suppressing PI3K/AKT/NF-κB Signaling Pathway. *Inflammation* 2016;39:807-812.
- 36 Novo MC, Osorio R, Reis VC, Longomaugéri IM, Mariano M, Popi AF: Blockage of Wnt/β-catenin signaling by Wnt inhibitors increases survival and proliferation of B-1 cells *in vitro*. *Immunobiology* 2015;220:60-67.
- 37 Baranovskiy N, Svendsen U, Ekstrand S, Chen CS, Kogner P, Johnsen JI: Small-molecule inhibitors of phosphatidylinositol 3-kinase/Akt signaling inhibit Wnt/beta-catenin pathway cross-talk and suppress medullary thymoma growth. *Cancer Res* 2010;70:266.