

MicroRNA-23a-5p regulates cell proliferation, migration and inflammation of TNF- α -stimulated human fibroblast-like MH7A synoviocytes by targeting TLR4 in rheumatoid arthritis

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Abstract. Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial joint inflammation. RA synovial fibroblasts (RASFs) constitute a major cell subset of the RA synovia. MicroRNAs (miRNAs/miRs) have been reported to serve a role in the activation and proliferation of RASFs. The present study aimed to investigate the effects and underlying mechanisms of miR-23a-5p on RA progression. Peripheral blood was collected from patients with RA (n=20) to analyze the expression levels of miR-23a-5p. The effects of miR-23a-5p on cell apoptosis, proliferation and migration in MH7A cells were determined in TNF- α -treated human fibroblast-like synoviocytes (MH7A cells) by flow cytometry, colony formation assay and Transwell assay, respectively. The cell cycle distribution was evaluated using flow cytometry. The binding relationship between miR-23a-5p and toll-like receptor (TLR) 4 was analyzed using a dual luciferase reporter gene assay. ELISA and reverse transcription-quantitative PCR assays were used to detect the levels of the inflammatory factors IL-6, IL-1 β and IL-10. The expression levels of apoptosis- and migration-related proteins were analyzed using western blotting. The results of the present study revealed that the expression levels of miR-23a-5p were significantly downregulated in the plasma of patients with RA and in MH7A cells. In addition, the TNF- α -induced increase in the cell proliferative and migratory rates and the production of IL-6 and IL-1 β were markedly inhibited following miR-23a-5p overexpression. The TNF- α -induced decreases in MH7A cell apoptosis were also reversed following miR-23a-5p overexpression. Additionally,

transfection with miR-23a-5p mimics significantly inhibited the activation of the TLR4/NF- κ B signaling pathway in TNF- α -treated MH7A cells by targeting TLR4. Notably, TLR4 overexpression weakened the effects of miR-23a-5p mimic on cell proliferation, apoptosis, migration, inflammation and the TLR4/NF- κ B signaling pathway in TNF- α -induced MH7A cells. In conclusion, the findings of the present study indicated that the miR-23a-5p/TLR4/NF- κ B axis may serve as a promising target for RA diagnosis and treatment.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by pannus formation and the progressive destruction of articular cartilage and bone (1,2). RA has high morbidity and disability rates and is often accompanied by cardiovascular and other systemic complications, which seriously affect the quality of life of patients (3,4). It was previously reported that the abnormal proliferation and apoptosis of RA synovial fibroblasts (RASFs) served a key role in RA progression (5). RASFs generally affect the biological activity of synovial cells, eventually leading to synovitis and progressive bone destruction (6). Therefore, studying the molecular mechanisms that affect the proliferation of RASFs may provide a novel target for RA treatment.

MicroRNAs (miRNAs/miRs) are a class of non-coding RNAs of ~22 nucleotides in length that are ubiquitous in almost all species. miRNAs inhibit gene translation by binding to the target gene 3'-untranslated region (3'-UTR), thereby regulating gene expression at the post-transcriptional level (7). miRNAs are involved in the regulation of numerous cell biological activities, including cell proliferation, apoptosis, cell division and the immune response (8). miRNAs were also discovered to serve important roles in numerous types of diseases, including cancer, diabetes, cardiovascular diseases and cognitive disorders (9). In addition, as negative regulators of gene regulation, miRNAs were demonstrated to affect the progression of various types of orthopedic disease, such as ankylosing spondylitis, osteoarthritis, osteoporosis and RA (10). High-throughput sequencing technology previously identified 50 upregulated and 35 downregulated miRNAs in RASFs (11,12). Related

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studies reported that several miRNAs were abnormally expressed during RA development, such as miR-155 (13), miR-21 (14), miR-150-5p (15) and miR-23b (16). Notably, the expression levels of miR-23a-5p were reported to be down-regulated in RASFs (12). To date, miR-23a-5p was found to be abnormally expressed in pancreatic ductal adenocarcinoma (PDAC) cells (17), acute myeloid leukemia cells (18) and renal cell carcinoma (RCC) cells and tissues (19). Accumulating evidence has suggested that the miR-23a-5p/ATP-binding cassette transporter A1/G1 axis may promote plaque stability and macrophage-derived foam cell formation, which eventually inhibits atherosclerosis progression (20). Furthermore, osteoclast-derived miR-23a-5p-containing exosomes efficiently suppressed osteogenic differentiation, indicating that miR-23a-5p in exosomes may serve a critical role in the process of bone remodeling (21). However, the biological effects and underlying mechanisms of miR-23a-5p in RA pathogenesis remain to be investigated.

The present study first investigated the expression levels of miR-23a-5p in the plasma of patients with RA. Furthermore, the biological functions and underlying mechanism of miR-23a-5p on the proliferation, migration, apoptosis and inflammatory cytokine secretion of RASFs were determined. Specifically, the regulatory effect of miR-23a-5p on toll-like receptor (TLR) 4, which is important for immune and inflammatory responses (22,23), was investigated.

Materials and methods

Patient studies. Patients with RA (n=20, 9 males and 11 females; mean age, 48.95±15.23 years) were clinically diagnosed at Southwest Medical University Affiliated Hospital (Luzhou, China) from March 2018 to March 2019. All enrolled patients met the RA classification diagnostic criteria established by the American College of Rheumatology/European League Against Rheumatism (24). A standard score of ≥6 points can be diagnosed as RA. All patients were diagnosed with RA for the first time and had not previously received any RA-related treatment. The exclusion criteria for the RA patients were as follows: i) The patients were suffering from autoimmune liver disease, ankylosing spondylitis, autoimmune hemolytic anemia, Sjogren's syndrome and other autoimmune diseases; the patient is suffering from acute coronary syndrome, hypertensive disease, cerebral infarction and other cardiovascular and cerebrovascular diseases; ii) the patients were suffering from endocrine diseases, such as diabetes mellitus and hyperthyroidism; iii) the patients were suffering from infectious diseases, such as hepatitis B and epidemic encephalitis; iv) the patients had cancer or other chronic diseases; v) the patients had abnormal liver and kidney function; vi) the patients had a history of smoking and/or alcoholism; and vii) the patients had recently taken anticoagulant drugs or immunosuppressive agents. A total of 5 ml venous blood was collected from each subject in the morning following fasting. After leaving to stand for 30 min, blood samples were centrifuged at 4°C at 1,000 × g for 10 min. Subsequently, 200 µl serum (non-hemolytic state) was added to a tube and preserved at -80°C until further analysis. The present study was approved by the Ethics Committee of Southwest Medical University Affiliated Hospital (Luzhou, China) and written

informed consent was obtained from all participants prior to the study.

Cell culture. Human fibroblast-like synoviocytes (MH7A cells) and normal synovial fibroblasts (HFLS) were purchased from the American Type Culture Collection. The cells were cultured in high-glucose DMEM (HyClone; Cytiva) supplemented with 10% FBS (HyClone; Cytiva) and 1% penicillin/streptomycin (Beijing Solarbio Science & Technology Co., Ltd.), and maintained in a humidified incubator with 5% CO₂ and 37°C. Where indicated, MH7A cells were treated with human TNF-α (R&D Systems, Inc.) at 20 ng/ml for 12 h at 37°C.

Cell transfection. The transient transfection of pcDNA-TLR4 and pcDNA-negative control (NC) plasmids (both from Shanghai GenePharma Co., Ltd.), miR-23a-5p mimic and NC mimic (both from Guangzhou RiboBio Co., Ltd.) or small interfering RNA (siRNA/si)-TLR4 and si-NC (both from Guangzhou RiboBio Co., Ltd.) was performed in 6-well plates using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Briefly, MH7A cells were seeded in a 6-well plate at a concentration of 2×10⁵ cells/well, and siRNA transfection was performed when the cells grew to 40-60% confluence. All molecules were transfected at a concentration of 50 nM. After transfection, the culture plate was placed in a constant temperature incubator at 37°C and 5% CO₂ for 48 h. The sequences of transfected RNA oligonucleotides were as follows: miR-23a-5p mimic forward, 5'-GGGGUUCUGGG GAUGGGAUUU-3' and reverse, 5'-AAAUCCCAUCCCCAG GAACCCC-3'; NC mimic forward, 5'-UUCUCCGAACGU GUCACGUdTdT-3' and reverse, 5'-ACGUGACACGUUCGG AGAAdTdT-3'; si-TLR4 forward, 5'-GGGCUUAGAACA ACUAGAATT-3' and reverse, 5'-UUCUAGUUGUUCUAA GCCCTT-3'; si-NC forward, 5'-CCCUUGUCGUGAAUU UACUTT-3' and reverse, 5'-AGUAAAUUCACGACAAGG GTT-3'.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from MH7A cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was subsequently performed using a SYBR Premix Ex Taq™ II kit (Takara Bio, Inc.) according to the manufacturer's protocol. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 10 min; followed by 40 cycles of 95°C for 5 sec, 60°C for 30 sec and 70°C for 60 sec. The following primer pairs were used for the qPCR (Takara Bio, Inc.): IL-6 forward, 5'-CCTGACCCA ACCACAAATGC-3' and reverse, 5'-ATCTGAGGTGCC CATGCTAC-3'; IL-1β forward, 5'-CCTGTCCTGCGTGTT GAAAGA-3' and reverse, 5'-GGGAAGTGGGACAGACTCA AA-3'; IL-10 forward, 5'-GAGATGCCTTCAGCAGAG TGAAGA-3' and reverse, 5'-AGGCTTGGCAACCCAGGT AAC-3'; TLR4 forward, 5'-AGAAGCTGGACCTGAGCTTTA ATC-3' and reverse, 5'-GAGGTGGCTTAGGCTCTGATA TG-3' and β-actin forward, 5'-CATGTACGTTGCTATCCA GGC-3' and reverse, 5'-CTCCTTAATGTCACGCCACGA T-3'. The relative gene expression levels were determined using the 2^{-ΔΔC_q} method (25) on ABI 7500 PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Western blotting. Whole MH7A cell lysates were prepared using RIPA buffer (1% Triton X-100, 150 mmol/l NaCl, 1 mmol/l EGTA, 50 mmol/l Tris-HCl, 0.1% SDS, 1% sodium deoxycholate and PMSF; Cell Signaling Technology, Inc.). The concentration of protein was determined by a BCA kit (Sigma-Aldrich; Merck KGaA). Total protein (30 μ g/sample) was separated via 10% SDS-PAGE. The separated proteins were subsequently transferred onto nitrocellulose membranes (EMD Millipore) and blocked with 5% non-fat milk at room temperature for 1 h. Subsequently, the membranes were incubated with the following primary antibodies: Anti-Bax (1:500; cat. no. ab53154; Abcam), anti-Bcl-2 (1:500; cat. no. ab196495; Abcam), anti-caspase-3 (1:500; cat. no. ab4051; Abcam), anti-cleaved-caspase-3 (1:50; cat. no. ab2302; Abcam), anti-matrix metalloproteinase (MMP) 2 (1:500; cat. no. ab97779; Abcam), anti-MMP9 (1:1,000; cat. no. ab38898; Abcam), anti-proliferating cell nuclear antigen (PCNA; 1:500; cat. no. ab18197; Abcam), anti-NF- κ Bp65 (1:500; cat. no. ab16502; Abcam), anti-phosphorylated (p)-NF- κ Bp65 (1:2,000; cat. no. ab86299; Abcam), anti-TLR4 (1:500; cat. no. ab13556; Abcam), anti-VEGFB (1:1,000; cat. no. 2463; Cell Signaling Technology, Inc.) and anti- β -actin (1:1,000; cat. no. ab8227; Abcam). Following primary antibody incubation, the membranes were incubated with an HRP-conjugated goat anti-rabbit IgG secondary antibody (1:5,000; cat. no. BA1054; Boster Biological Technology). Protein bands were visualized using ECL reagent (Affinity Biosciences) on a gel imaging system (Bio-Rad Laboratories, Inc.) and analyzed using Image Pro Plus 6.0 software (Media Cybernetics, Inc.). β -actin was used as the internal loading control.

ELISA. A MH7A cell suspension was prepared and centrifuged for 10 min at 800 \times g at 4°C, then stored at -20°C until subsequent analysis. The secretory levels of IL-6 (cat. no. ZC-32466), IL-1 β (cat. no. ZC-32420) and IL-10 (cat. no. ZC-32403) were analyzed using their corresponding ELISA kits (Shanghai Zhuo Cai Biological Technology Co., Ltd; <http://www.zcibio.com/>) according to the manufacturers' instructions.

Dual luciferase reporter assay. The binding site of miR-23a-5p and TLR4 mRNA 3'-UTR was predicted using bioinformatics online software, including TargetScan version 7.1 (<http://www.targetscan.org/>) and miRDB version 6.0 (<http://mirdb.org/>). The fragment of the TLR4 3'-UTR containing the predicted miR-23a-5p binding site was generated by PCR using specific primers. The resulting PCR amplicon was cloned into a pmirGLO Dual-Luciferase miRNA target expression vector (Promega Corporation) containing the luciferase coding sequence. Cells were seeded into 96-well plates, cultured to 50-70% confluence/well and co-transfected with the TLR4 3'-UTR-wild-type (wt) 1 or TLR4 3'-UTR-wt2 construct and miR-23a-5p mimic or NC mimic. Cells were transfected using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following 24 h of transfection, the relative luciferase activity was measured using a Dual Luciferase Reporter Assay system (Promega Corporation) according to the manufacturer's protocol. Firefly luciferase activity was normalized to that of Renilla luciferase.

Cell viability assay. MH7A cell viability was monitored using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) assay according to the manufacturer's protocol. CCK-8 solution was added to each well and incubated at 37°C for 1.5 h. Cell viability was measured using an ELISA microplate reader at a wavelength of 450 nm.

Colony formation assay. MH7A cells were seeded into 3.5-cm cell culture dishes (1 \times 10³ cells/dish) and incubated at 37°C with 5% CO₂ for 2 weeks. Following incubation, MH7A cells were fixed with 20% methanol for 10 min at room temperature and then stained with 0.1% crystal violet for 5 min at room temperature. The number of colonies (>50 cells) were counted using an inverted microscope (Olympus Corporation).

Flow cytometric analysis of apoptosis. The apoptosis of MH7A cells was analyzed using an Annexin V-FITC/propidium iodide (PI) flow cytometry kit (Becton, Dickinson and Company) according to the manufacturer's protocol. Briefly, transfected MH7A cells in 6-well plates were washed with PBS (Invitrogen; Thermo Fisher Scientific, Inc.) and adjusted to a density of 1 \times 10⁶ cells/ml. The cells were subsequently resuspended in 100 μ l binding buffer, 5 μ l Annexin V-FITC and 5 μ l PI and incubated in the dark at 4°C for 15 min. Apoptotic cells were then analyzed by a BD FACSCelesta[™] flow cytometer (Becton, Dickinson and Company) and FlowJo software version 7.6.1 (FlowJo LLC).

Transwell assay. A total of 1 \times 10⁵ MH7A cells/ml were resuspended in DMEM and 200 μ l cell suspension/well was plated into the upper chambers of 24-well Transwell plates. The lower chambers were filled with 600 μ l DMEM supplemented with 10% FBS (HyClone; Cytiva). Following incubation at 37°C for 48 h, MH7A cells were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 15 min (both at room temperature). An inverted microscope (Olympus Corporation) was used to observe the number of cells in five randomly selected fields of view (magnification, \times 100).

Statistical analysis. Data are presented as the mean \pm SD. Statistical analysis was performed using SPSS 20.0 (IBM Corp.). Statistical differences between groups were determined using one-way ANOVA with Tukey's post hoc test of means and unpaired Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of miR-23a-5p are downregulated in the plasma from patients with RA and MH7A cells. The expression levels of miR-23a-5p in the plasma of patients with RA and MH7A cells were investigated. The results revealed that miR-23a-5p expression levels were significantly downregulated in the plasma of patients with RA compared with normal controls (Fig. 1A). Similarly, the expression levels of miR-23a-5p were significantly downregulated in MH7A cells compared with HFLS (Fig. 1B).

Pro-apoptotic effects of miR-23a-5p on TNF- α -stimulated MH7A cells. The effects of miR-23a-5p on TNF- α -induced

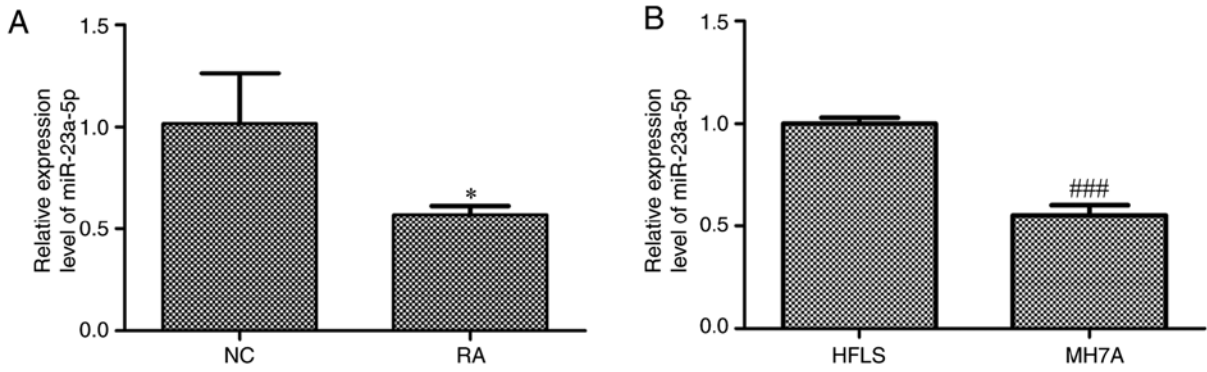


Figure 1. miR-23a-5p expression levels are downregulated in the plasma of patients with RA and MH7A cells. (A and B) Expression levels of miR-23a-5p were analyzed using reverse transcription-quantitative PCR. * $P < 0.05$ vs. NC; ### $P < 0.001$ vs. HFLS. miR, microRNA; NC, negative control; RA, rheumatoid arthritis.

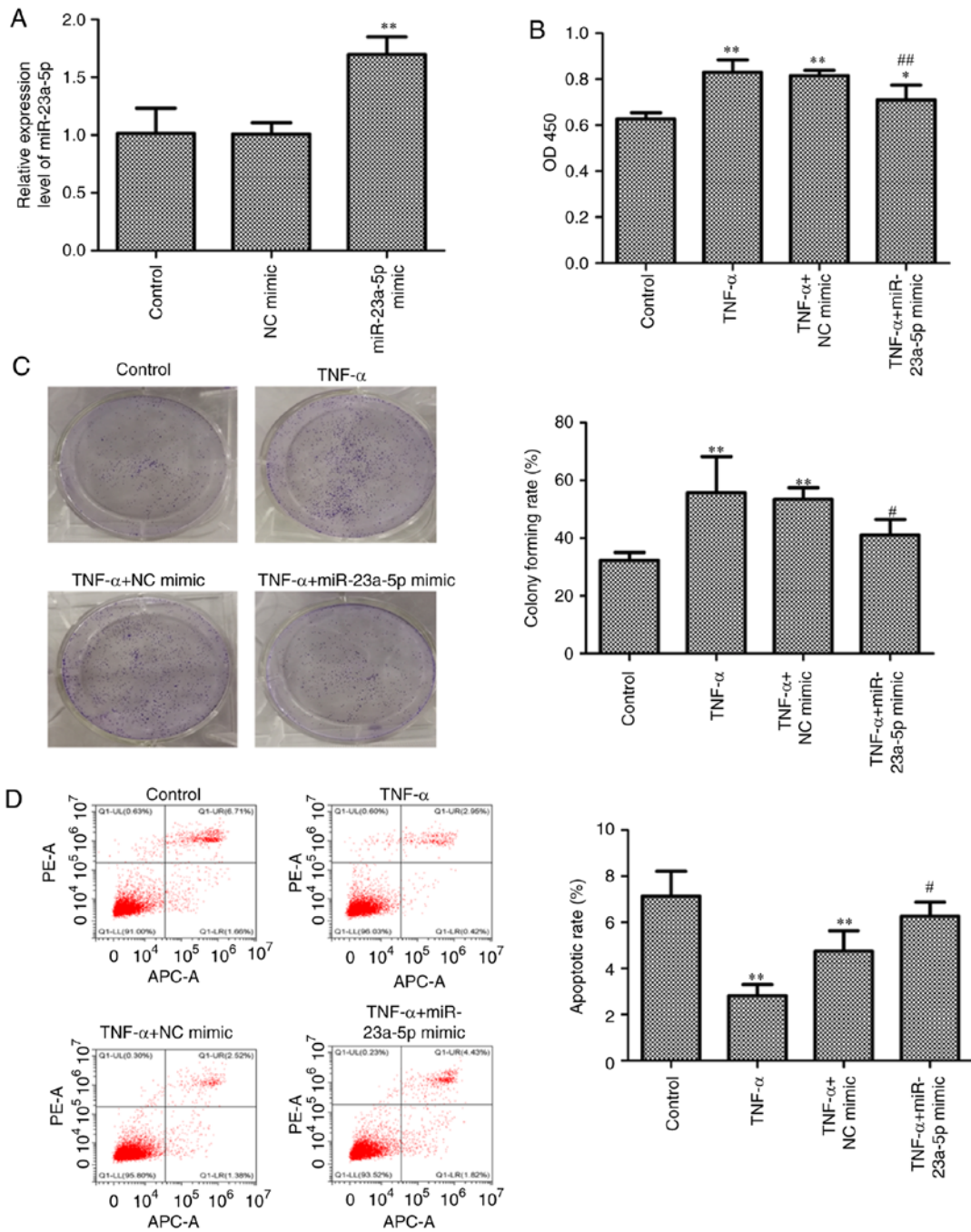


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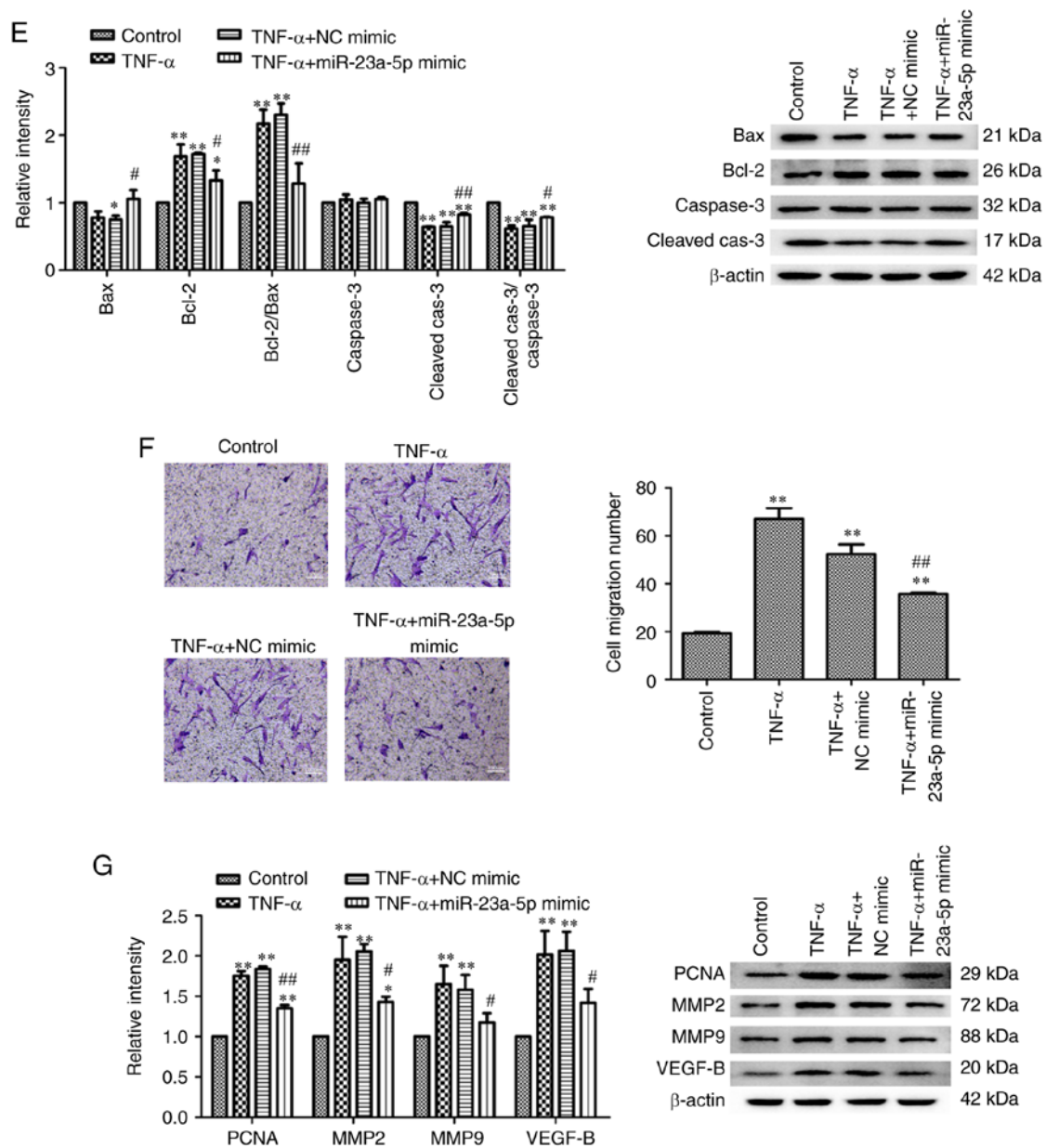


Figure 2. Pro-apoptotic effects of miR-23a-5p on TNF- α -stimulated MH7A cells. (A) miR-23a-5p expression levels were analyzed using reverse transcription-quantitative PCR. Proliferation of MH7A cells was evaluated using (B) Cell Counting Kit-8 and (C) colony formation assays. (D) Apoptosis of MH7A cells was assessed using Annexin V-FITC/PI staining flow cytometry. (E) Bax, Bcl-2, caspase3 and cleaved caspase-3 expression levels were analyzed using western blotting. β -actin was used as the loading control. (F) Migratory ability of MH7A cells was evaluated using a Transwell assay (magnification, $\times 100$). (G) PCNA, MMP2, MMP9 and VEGF-B expression levels were analyzed using western blotting. β -actin was used as the loading control. * $P < 0.05$ and ** $P < 0.01$ vs. control. # $P < 0.05$ and ## $P < 0.01$ vs. TNF- α + NC mimic. miR, microRNA; PI, propidium iodide; PCNA, proliferating cell nuclear antigen; MMP, matrix metalloproteinase; NC, negative control; Cleaved-cas3, cleaved caspase-3.

MH7A cell development was subsequently determined using miR-23a-5p mimics (Fig. 2A). MH7A cells were transfected with miR-23a-5p mimic or NC mimic and then stimulated with TNF- α . As shown in Fig. 2B, MH7A cell viability was significantly increased by TNF- α compared with the untreated control group, while transfection with miR-23a-5p mimic significantly inhibited TNF- α -induced increases in MH7A cell viability (Fig. 2B). In addition, colony formation assay results revealed that miR-23a-5p overexpression significantly blocked the increase in MH7A proliferation induced by TNF- α (Fig. 2C). By contrast, the apoptotic rate was gradually decreased following TNF- α stimulation, while transfection with miR-23a-5p mimic significantly reversed the apoptosis

of TNF- α -induced MH7A cells (Fig. 2D). miR-23a-5p overexpression-induced apoptosis was also confirmed using western blotting. TNF- α stimulation also significantly downregulated Bax and cleaved-caspase-3 expression levels while significantly upregulating the expression levels of Bcl-2 in MH7A cells; these findings were reversed following miR-23a-5p overexpression (Fig. 2E). Furthermore, transfection with miR-23a-5p mimic significantly inhibited TNF- α -induced cell migration (Fig. 2F). The expression levels of four cell growth-related proteins (PCNA, MMP2, MMP9 and VEGFB) were also significantly upregulated in response to TNF- α stimulation. Meanwhile, transfection with miR-23a-5p mimic significantly weakened this trend (Fig. 2G).

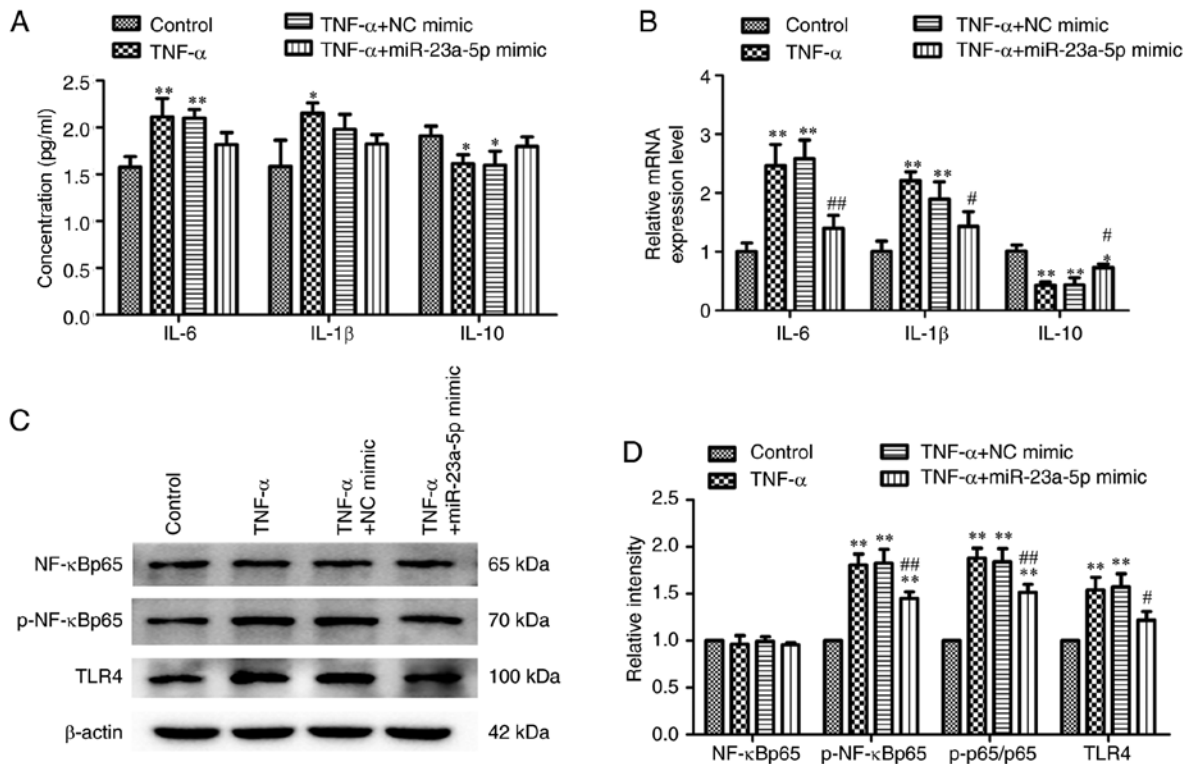


Figure 3. miR-23a-5p overexpression reverses TNF- α -induced inflammatory injury and TLR4/NF- κ B signaling activation in MH7A cells. (A) IL-6, IL-1 β and IL-10 concentrations were measured using ELISA. (B) mRNA expression levels of IL-6, IL-1 β and IL-10 were analyzed using reverse transcription-quantitative PCR. (C and D) NF- κ Bp65, p-NF- κ Bp65 and TLR4 expression levels were determined using western blotting. β -actin was used as the loading control. * $P < 0.05$ and ** $P < 0.01$ vs. control. # $P < 0.05$ and ## $P < 0.01$ vs. TNF- α + NC mimic. miR, microRNA; p-, phosphorylated; TLR4, toll-like receptor 4; NC, negative control.

miR-23a-5p reverses TNF- α -induced inflammatory injury and TLR4/NF- κ B signaling activation in MH7A cells. The effects of miR-23a-5p on inflammatory injury following TNF- α stimulation was subsequently investigated. Briefly, MH7A cells were stimulated with TNF- α following transfection with miR-23a-5p mimic or NC mimic and the levels of cellular cytokines were determined. Transfection of cells with miR-23a-5p mimic prior to TNF- α stimulation gradually decreased TNF- α -induced IL-6 and IL-1 β concentrations and reversed the TNF- α -induced reduction in IL-10 concentration (Fig. 3A). Similarly, as shown in Fig. 3B, the TNF- α -increased mRNA expression levels of IL-6, IL-1 β and IL-10 were significantly blocked by miR-23a-5p mimic transfection. The TLR4/NF- κ B signaling pathway is considered to be closely related to inflammation (26). Thus, the changes in the activity of the TLR4/NF- κ B signaling pathway in response to miR-23a-5p transfection and TNF- α treatment were investigated. Western blotting data in Fig. 3C and D revealed that TLR4 expression levels and the phosphorylation levels of NF- κ Bp65 were both significantly upregulated following TNF- α stimulation compared with controls. Conversely, miR-23a-5p overexpression significantly inhibited TLR4 and p-NF- κ Bp65 levels, even following TNF- α stimulation (Fig. 3C and D).

TLR4 is a target gene of miR-23a-5p. The underlying mechanisms of the pro-apoptotic and anti-inflammatory effects of miR-23a-5p were subsequently determined. Bioinformatics analysis predicted that TLR4 was a target gene of miR-23a-5p (Fig. 4A). To confirm that miR-23a-5p regulated TLR4 expression levels by directly binding to the TLR4 3'-UTR,

a dual luciferase reporter assay was performed. As shown in Fig. 4B, the relative luciferase activity was significantly decreased in cells co-transfected with miR-23a-5p mimic and TLR4 3'-UTR-wt1 or TLR4 3'-UTR-wt2 (Fig. 4B). Thus, a pcDNA-TLR4 plasmid and TLR4 siRNA were constructed for use in subsequent experiments (Fig. 4C and D). Both at the protein and mRNA level, the expression of TLR4 was increased by pcDNA-TLR4 plasmid transfection and was decreased by TLR4 siRNA transfection in MH7A cells (Fig. 4C and D).

miR-23a-5p overexpression inhibits TNF- α -induced-MH7A cell survival via targeting TLR4. The results demonstrated that miR-23a-5p mimic reversed the TNF- α -induced protective effects over cell damage, as evidenced by the decreased cell viability (Fig. 5A), increased apoptotic rate (Fig. 5B) and the aberrant expression levels of proteins associated with cell survival (Fig. 5C). As expected, transfection with pcDNA-TLR4 plasmid inhibited miR-23a-5p mimic-induced cell damage (Fig. 5A-C). Furthermore, the migration of TNF- α -stimulated MH7A cells was inhibited following miR-23a-5p overexpression. This effect was further strengthened following transfection with pcDNA-TLR4, which was evidenced through the upregulated expression levels of PCNA, MMP2, MMP9 and VEGFB (Fig. 5D and E).

miR-23a-5p overexpression prevents TNF- α -induced inflammatory injury and TLR4/NF- κ B signaling activation by targeting TLR4. Further experiments revealed that transfection of cells with miR-23a-5p mimic significantly

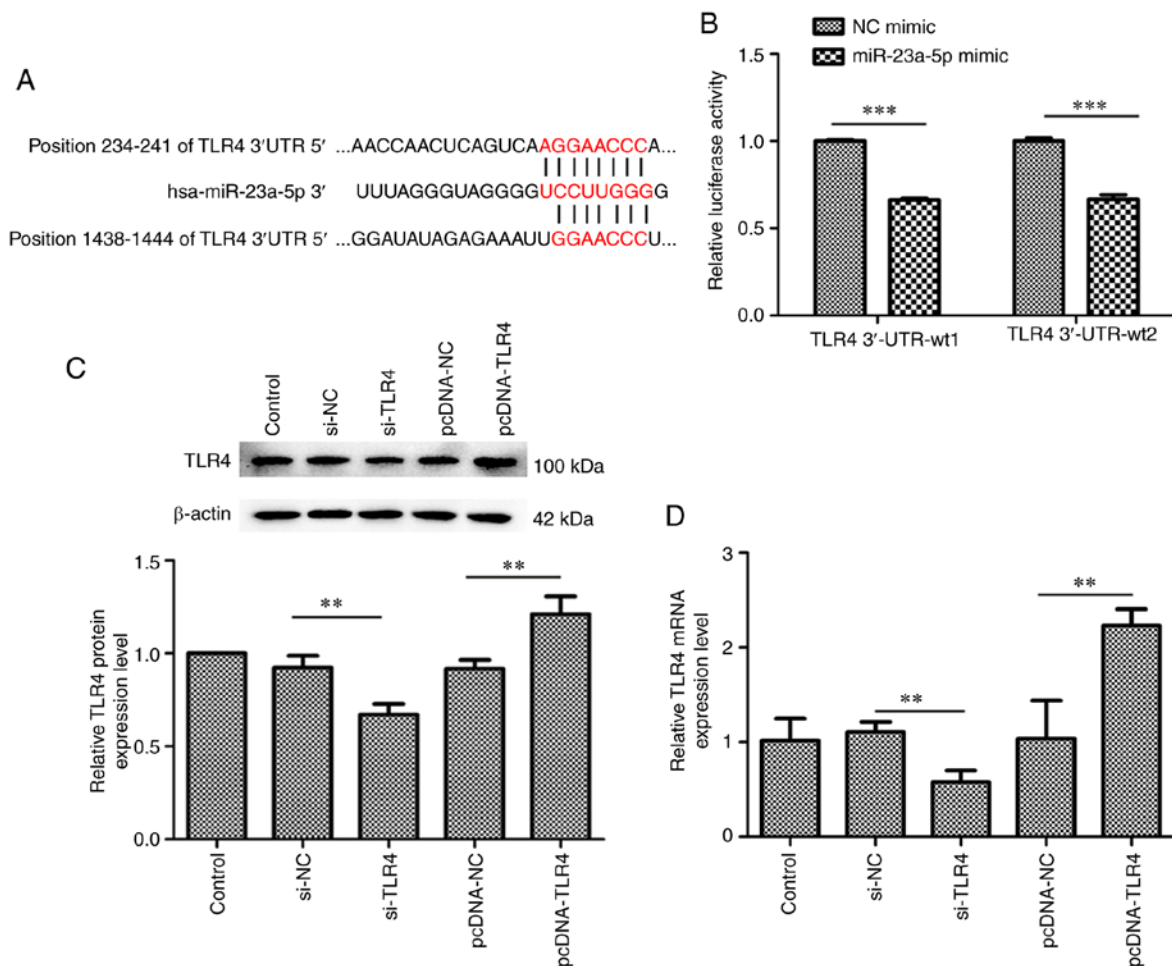


Figure 4. TLR4 is a target gene of miR-23a-5p. (A) Binding regions between miR-23a-5p and the TLR4 3'-UTR sequences are shown in red. (B) Dual luciferase reporter assay was used to determine the interaction between miR-23a-5p and TLR4 3'-UTR sequences. *** $P < 0.001$ vs. NC mimic. (C) Expression levels of TLR4 were analyzed using western blotting. (D) mRNA expression levels of TLR4 were analyzed using reverse transcription-quantitative PCR. β -actin was used as the loading control. ** $P < 0.01$ vs. si-NC/pcDNA-NC. miR, microRNA; TLR4, toll-like receptor 4; UTR, untranslated region; NC negative control; si, small interfering RNA.

ameliorated TNF- α -induced cell inflammation, which was evidenced through the downregulated mRNA expression levels of IL-6 and IL-1 β and upregulated mRNA expression levels of IL-10 (Fig. 6A). Compared with the TNF- α + miR-23a-5p mimic group, the mRNA expression levels of IL-6 and IL-1 β were all upregulated, while IL-10 mRNA expression levels were downregulated in the TNF- α + miR-23a-5p mimic + pcDNA-TLR4 group (Fig. 6A). By contrast, TLR4 knockdown further strengthened the effects of the miR-23a-5p mimic by downregulating IL-6 and IL-1 β expression levels and upregulating IL-10 expression levels (Fig. 6A). The effects of TLR4 overexpression on the TLR4/NF- κ B signaling pathway were analyzed in MH7A cells stimulated with TNF- α and co-transfected with miR-23a-5p mimic and pcDNA-TLR4 plasmid. The downregulated expression levels of TLR4 and decreased p-NF- κ Bp65/NF- κ Bp65 ratio induced by TNF- α and miR-23a-5p mimic transfection were reversed in cells treated with TNF- α and co-transfected with miR-23a-5p mimic and pcDNA-TLR4 simultaneously (Fig. 6B and C). Meanwhile, TLR4 knockdown enhanced the inhibitory effects of miR-23a-5p on TLR4 and p-NF- κ Bp65 levels (Fig. 6B and C).

Discussion

In recent years, accumulating studies have reported that miRNAs are closely associated with the pathological process of RA. Previous studies have revealed that the expression levels of mature miR-146a, miR-146a/b precursor, miR-23b and miR-26a-5p were all highly upregulated in fibroblast-like synoviocytes (FLSs) from patients with RA (16,27). In addition, miR-223 and miR-128-3p expression levels were discovered to be upregulated in the T lymphocytes of patients with RA (28,29). Another previous study also reported that miR-126, miR-20a and miR-613 expression levels were markedly downregulated in the peripheral synovial tissues of patients with RA or RASFs (30-32). The results of the present study revealed that the expression levels of miR-23a-5p were significantly downregulated in the peripheral blood plasma of patients with RA and RASFs. miR-23a-5p is located on chromosome 19p13 and was identified to serve a role in regulating the normal growth, differentiation and apoptosis of cells, and has been shown to be widely involved in regulating various physiological and pathological processes (33-35). Previous studies have confirmed that miR-23a-5p may be a potential biomarker for human systemic inflammatory response syndrome and

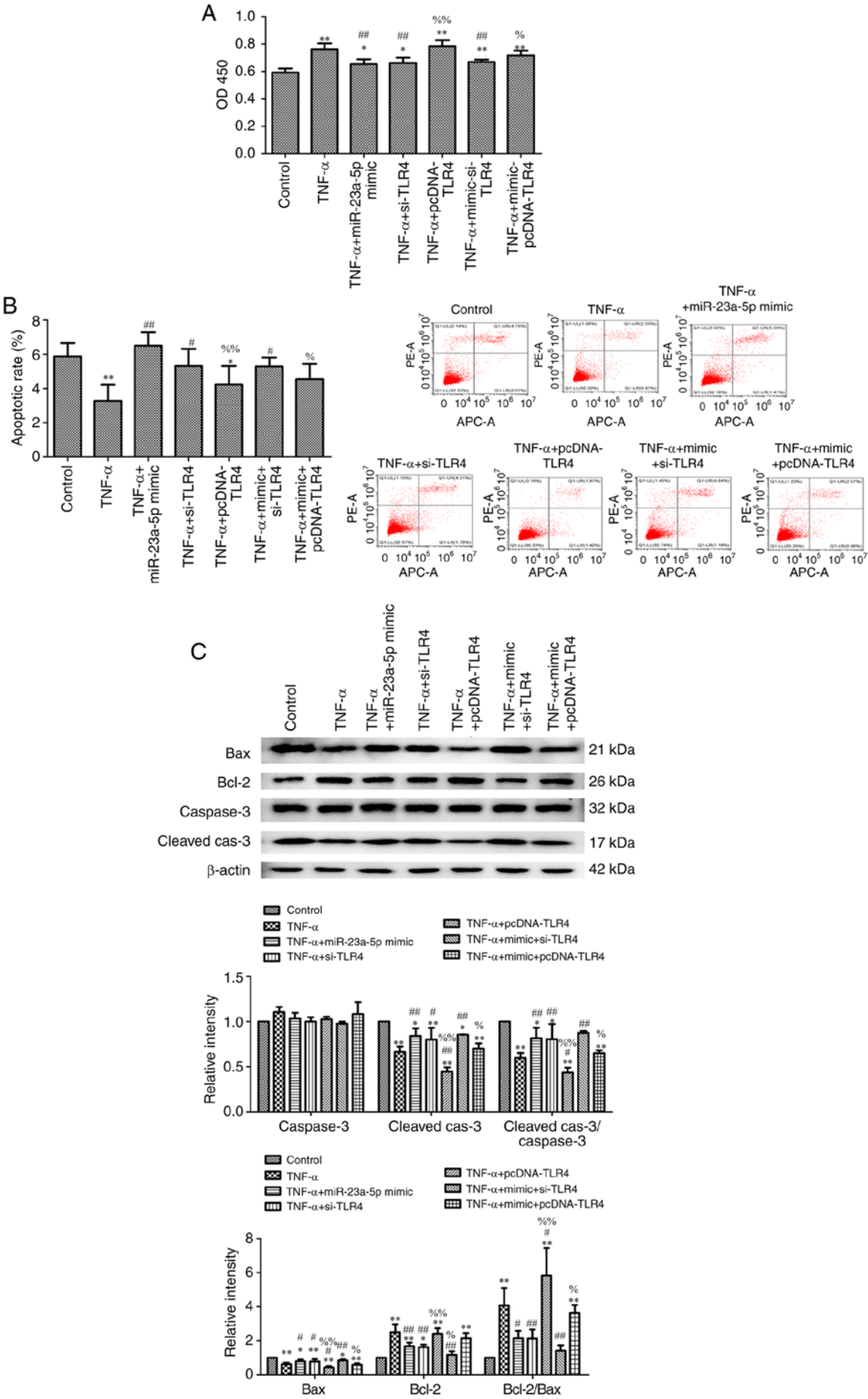


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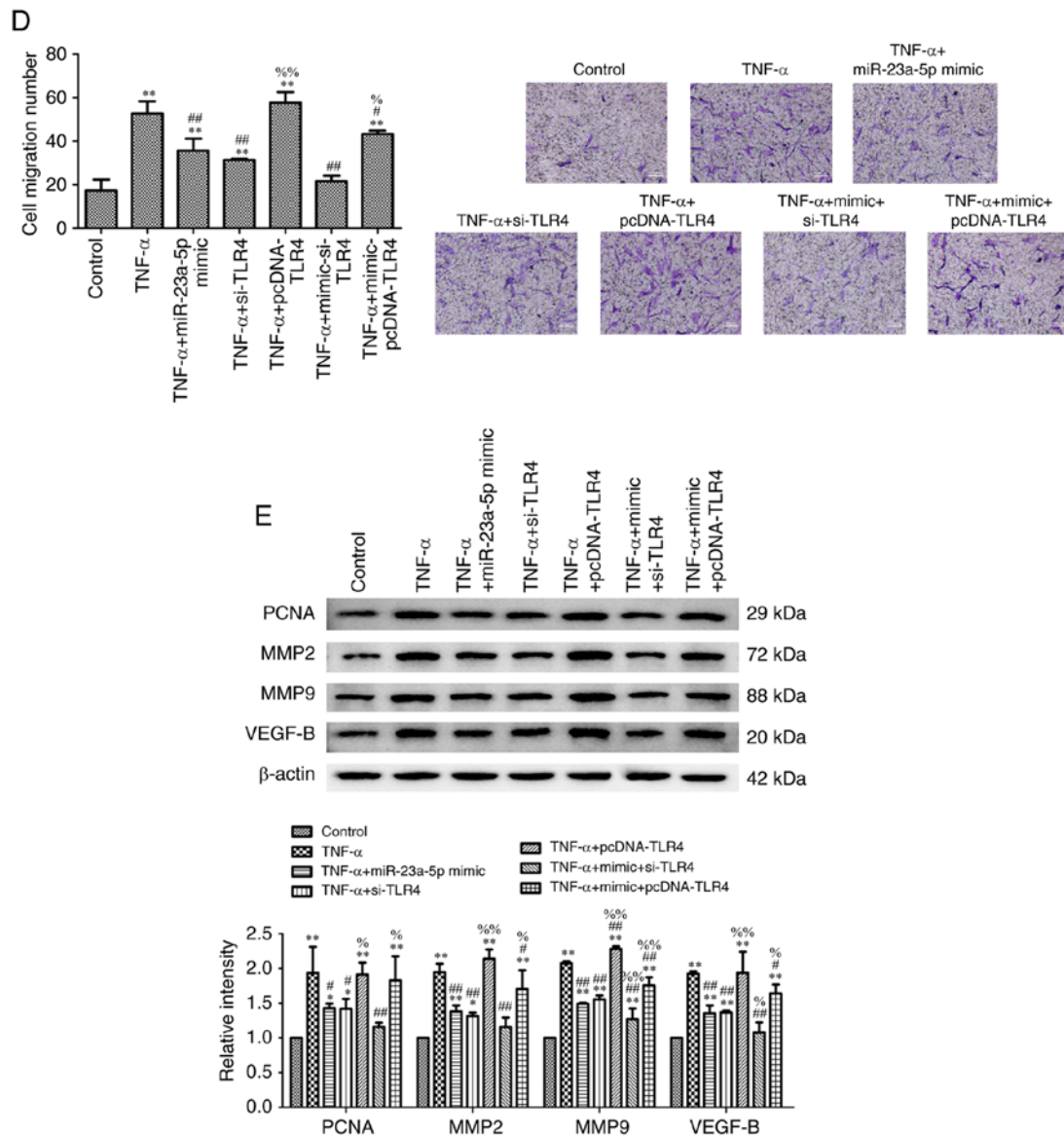


Figure 5. miR-23a-5p overexpression inhibits TNF- α -induced-MH7A cell survival by targeting TLR4. (A) Proliferation of MH7A cells was evaluated using a Cell Counting Kit-8 assay. (B) Apoptosis of MH7A cells was measured using an Annexin V-FITC/PI staining kit. (C) Bax, Bcl-2, caspase-3 and cleaved caspase-3 expression levels were determined using western blotting. β -actin was used as the loading control. (D) Migratory ability of MH7A cells was evaluated using a Transwell assay (magnification, x100). (E) PCNA, MMP2, MMP9 and VEGF-B expression levels were determined using western blotting. β -actin was used as the loading control. * P <0.05 and ** P <0.01 vs. control. # P <0.05 and ## P <0.01 vs. TNF- α . % P <0.05 and %% P <0.01 vs. TNF- α + miR-23a-5p mimic. miR, microRNA; TLR, toll-like receptor 4; PI, propidium iodide; PCNA, proliferating cell nuclear antigen; MMP, matrix metalloproteinase; si, small interfering RNA; Cleaved-cas3, cleaved caspase-3.

seizures (36,37). miR-23a-5p overexpression was found to regulate the proliferation, migration, invasion and apoptosis of PDAC, RCC and bladder cancer (17,19,38). The results of the present study revealed that miR-23a-5p overexpression could inhibit cell proliferation, invasion and inflammation, as well as promote cell apoptosis in TNF- α -treated RASFs. These results suggested that miR-23a-5p may serve a crucial role in RA pathogenesis.

TNF- α , a physiological inflammatory mediator produced by activated monocytes and macrophages, has been identified to serve an important role in the pathogenesis of RA (39). TNF- α was found to stimulate RASF proliferation and the secretion of IL-6, granulocyte-macrophage colony stimulating factor, MMPs, prostaglandins and other effector molecules (40-42). RA pathogenesis is related to the immune

inflammatory response and is accompanied by secretory changes to various pro- and anti-inflammatory factors, such as TNF- α , IL-1 β , IL-6, IL-10 and IL-17 (5,43). A recent study reported that miR-206 promoted bone metabolism and the secretion of pro-inflammatory cytokines, including IL-16 and IL-17, during the pathological process of RA (44). In addition, miR-451 inhibited the secretion of pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 in synovial fibroblasts by downregulating the expression levels of p38 MAPK protein (45). The data in the present study revealed that TNF- α promoted the inflammation of MH7A cells, while the overexpression of miR-23a-5p significantly inhibited the secretion of pro-inflammatory factors, IL-6 and IL-1 β , and stimulated the secretion of the anti-inflammatory factor, IL-10.

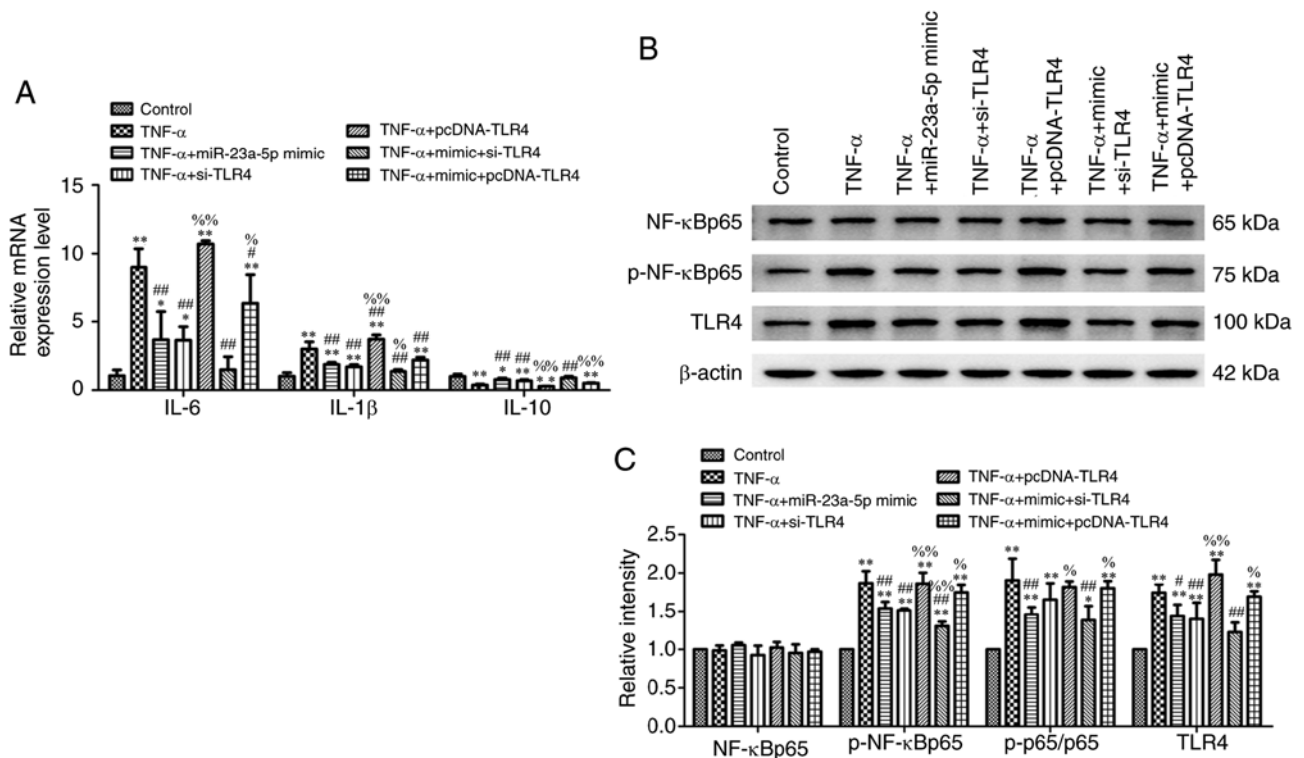


Figure 6. miR-23a-5p overexpression prevents TNF- α -induced inflammatory injury and TLR4/NF- κ B signaling activation by targeting TLR4. (A) mRNA expression levels of IL-6, IL-1 β and IL-10 were analyzed using reverse transcription-quantitative PCR. (B and C) NF- κ Bp65, p-NF- κ Bp65 and TLR4 expression levels were determined using western blotting. β -actin was used as the loading control. * P <0.05 and ** P <0.01 vs. control. # P <0.05 and ## P <0.01 vs. TNF- α . % P <0.05 and %% P <0.01 vs. TNF- α + miR-23a-5p mimic. miR, microRNA; TLR4, toll-like receptor 4; p-, phosphorylated; si, small interfering RNA.

The invasion of RASFs into the cartilage and the destruction of bone tissue are important characteristics of RA (46). The erosive damage caused in RA is attributed to MMPs, PCNA and VEGF, amongst other factors (47). miR-221 silencing was discovered to play a role in RA pathogenesis via downregulating the expression levels of MMP3 and MMP9 and promoting the release of inflammatory cytokines and chemokines in FLSs (48). As a sponge of miR-138, the long non-coding RNA HOX transcript antisense RNA significantly decreased the lipopolysaccharide-induced upregulation of PCNA, IL-1 β and TNF- α expression levels, thereby alleviating chondrocyte proliferation, migration and inflammation (48). In addition, miR-143 and miR-145 were found to modulate RASF susceptibility to TNF- α and VEGF₁₆₅ stimuli through downregulating insulin-like growth factor binding protein 5 and semaphorin 3A expression levels, respectively (49). In the present study, TNF- α stimulation upregulated MMP2, MMP9, VEGFB and PCNA expression levels, which were subsequently partially reversed by miR-23a-5p overexpression.

Numerous miR-23a-5p target genes have been identified, including activating transcription factor 3, extracellular matrix protein 1, Runt-related transcription factor 2, insulin-like growth factor 2 and TLR2 (17,21,50,51). Mechanistically, the results of the present study identified TLR4 as a direct target of miR-23a-5p in RASFs. TLR4, as a member of the TLR family, is widely distributed in various cells, such as T cells, B cells, lung macrophages, adipocytes and intestinal epithelial cells (52-55). The extracellular domain of the TLR4 structure can combine with the myeloid differentiation-2/CD14 complex, identify pathogen-associated molecular patterns and

eventually activate NF- κ B signaling (56). The TLR4/NF- κ B pathway has been reported to promote the expression of inflammatory factors and be involved in the inflammatory response of RA (57,58). Previous studies have demonstrated that miRNAs participated in the inflammatory damage of RA by regulating the TLR4/NF- κ B signaling pathway. For example, miR-146a and miR-548a-3p expression levels were reported to mediate the proliferation and inflammation of rheumatoid arthritis fibroblast-like synoviocytes by downregulating the TLR4/NF- κ B signaling pathway (57,59). The findings of the present study suggested that TLR4 expression may be epigenetically regulated as a miR-23a-5p target and may be involved in the proliferation and inflammation of MH7A cells. To address this hypothesis, the cell viability, migratory ability and pro-inflammatory responses of MH7A cells following TLR4 knockdown and overexpression were investigated. The data demonstrated that TLR4 knockdown strengthened miR-23a-5p mimic-induced reduction in cell viability and migration in TNF- α -treated MH7A cells, which simultaneously further inhibited NF- κ B signaling activation and the production of pro-inflammatory factors. Conversely, TLR4 overexpression reversed the effects of miR-23a-5p overexpression on MH7A cells. Collectively, these data suggested that the knockdown of TLR4, which was linked to upregulated miR-23a-5p expression levels, may reduce the sensitivity of MH7A cells to TNF- α .

In conclusion, the findings of the present study provided evidence to suggest that the miR-23a-5p/TLR4/NF- κ B signaling pathway may exert roles in the pathogenesis of RA and serve as promising targets for RA diagnosis and treatment.

However, the role of miR-23a-5p *in vivo* in RA requires further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XB and CH conceived and designed the study; XB, CH and LM performed the experiments; XB and LM analyzed the data; XB, CH and LM confirmed the authenticity of the raw data; XB, CH and LM wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Southwest Medical University Affiliated Hospital (Luzhou, China) and written informed consent was obtained from all participants prior to the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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