

MiR-34a-5p inhibits fibroblast-like synoviocytes proliferation *via* XBP1

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Abstract. – OBJECTIVE: Rheumatoid arthritis (RA) is an autoimmune, inflammatory disease mainly manifested by joint damage. Its mechanism is not completely clear at present. Previous studies have found that microRNA-34a-5p (miR-34a-5p) is involved in the development of many inflammatory diseases. In this study, we intended to study the role and mechanism of miR-34a-5p in the development of RA.

MATERIALS AND METHODS: We predicted that miR-34a-5p could directly inhibit the expression of X-box binding protein 1 (XBP1). We analyzed whether miR-34a-5p could inhibit XBP1 expression by Real-time Quantitative PCR. Cell Counting Kit-8 was used to detect the proliferation of fibroblast-like synoviocytes (FLS). Tumor Necrosis Factor- α (TNF- α) and interleukin-6 (IL-6) secreted by FLS were measured by Enzyme-Linked Immunosorbent assay. Western blot was used to detect the expression of XBP1 and Luciferase assay was used to verify the interaction between miR-34a-5p and XBP1.

RESULTS: We found that miR-34a-5p expression is lower in RA synovial tissue compared to osteoarthritis (OA). Moreover, miR-34a-5p inhibited the proliferation of FLS and inhibited the secretion of TNF- α and IL-6 by FLS. According to the prediction, we found that miR-34a-5p may bind to the 3' untranslated region (3' UTR) of XBP1, thereby inhibiting its expression. Through functional experiments and Luciferase experiments, we showed that miR-34a-5p can directly target XBP1, thereby inhibiting its expression.

CONCLUSIONS: In short, miR-34a-5p can directly inhibit the expression of XBP1, ultimately inhibit the proliferation of FLS, and inhibit the secretion of TNF- α and IL-6 by FLS. This study can provide new ideas for the treatment of RA.

Key Words:

Rheumatoid arthritis, MicroRNA-34a-5p, Fibroblast-like synoviocytes, Proliferation.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that mainly affects the joints. The average prevalence of RA worldwide is estimated at 0.5-1.0%. The incidence is significantly higher in women¹. The joint damage caused by RA seriously affects the quality of patients' life. The onset of RA is slow, and early identification is not easy. As the disease progresses, synovial hyperplasia and cartilage destruction occur, which eventually leads to loss of joint function^{2,3}.

Before the 1990s, RA often resulted in patients with disabilities and even death. Recently, considerable progress has been made in the research of anti-RA drugs, including traditional drugs: methotrexate, hydrochloroquine, sulfadiazine, and the new drugs, like, pan-JAK- and JAK1/2-inhibitors, tumor necrosis factor- α (TNF- α) inhibitors, TNF-receptor inhibitors, interleukin-6 (IL-6) inhibitors, IL-6 receptor inhibitors, B cell depleting antibodies, and inhibitors of co-stimulatory molecule. Although these new drugs have evident advantages in controlling symptoms, they can delay the progress of RA. However, the side effects and high costs are also worth noting⁴. Even so, the etiology of RA is still not completely clear. Furthermore the treatment is far from ideal nowadays. Most importantly, no treatment can prevent the progress of RA⁵.

MicroRNA (miRNA) is a non-coding RNA with a length of 18-25 nucleotides. It can bind to the 3' untranslated region (3'-UTR) of mRNA, then, promote mRNA degradation or inhibit its translation to regulate target gene at the post-transcriptional level⁶. A variety of miRNAs play a key role in RA^{7,8}. MiRNAs can be involved in the regulation of RA's inflammatory response. In

addition, miRNAs can also be involved in regulating the proliferation, apoptosis, and migration of fibroblast-like synoviocytes (FLS)⁹⁻¹¹. MicroRNA-34a-5p (miR-34a-5p) is less expressed in synovial tissue of RA patients¹². We assumed that miR-34a-5p could affect FLS proliferation, but the specific mechanism is not clear. According to StarBase 3.0, we predicted that miR-34a-5p could directly inhibit the expression of X-box binding protein 1 (XBP1). This study mainly focuses on the role of miR-34a-5p in RA and whether miR-34a-5p inhibits the proliferation of FLS by inhibiting XBP1.

Materials and Methods

Synovial Tissues

Synovial tissues from patients with osteoarthritis (OA) (n = 18) and RA (n = 18) who needed total knee joints were collected in Inner Mongolia People's Hospital. All patients signed informed consent before surgery. All RA and OA patients meet American College of Rheumatology criteria. FLS for the cell experiment are derived from RA patients. The research was approved by the Medical Ethics Committee of Inner Mongolia People's Hospital.

Fibroblast-Like Synoviocytes Culture

After obtaining the synovial tissue under aseptic conditions, the synovial tissue is cut with scissors. Synovial tissue was added to Minimum Essential Medium Eagle- α modification (α -MEM, Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 4 mg/ml type I collagen (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), and digested in a humidified incubator containing 5% CO₂ at 37°C for 2 hours. FLS were filtered using a 70 μ m cell strainer, and FLS were obtained by centrifugation. FLS were cultured in α -MEM containing 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 IU/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified incubator containing 5% CO₂ at 37°C. Cells were passaged when they reached 90% confluency, and FLS at second passage were used in the following experiments.

Transfection

MiR-34a-5p mimic, miR-34a-5p inhibitor, siRNA against XBP1 and XBP1 overexpression plasmid were synthesized by Genepharma (Gene-

pharma, Shanghai, China). According to the manufacturer's instructions, Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to transfect miR-34a-5p mimic, miR-34a-5p inhibitor, XBP1 siRNA and XBP1 overexpression plasmid into fibroblast-like synoviocytes. 48 hours after cell transfection, the total RNA and total protein were extracted for subsequent experiments.

Real-time Quantitative PCR (RT-qPCR)

Total RNA was extracted from synovial tissue and FLS using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The total RNA was reverse transcribed into cDNA using PrimeScript™ RT reagent Kit (Takara Bio, Inc., Dalian, China) according to the manufacturer's instructions. Semi-quantitative analysis was then performed using Quantitative PCR using TB Green® Premix Ex Taq™ II reagent Kit (Takara Bio, Inc., Dalian, China). GAPDH and U6 were used as internal controls. The relative expression was compared using the 2^{- $\Delta\Delta$ Ct} method. Primers used were as follows: GAPDH, Forward: 5'-CTCGCCTA-GAGTGAGCTCC-3', Reverse: 5'-AACTGCT-GCGTTGACGGGTATG-3'; XBP1, Forward: 5'-GAGCAGCAAGTGGTGGATTT3', Reverse: 5'-AAAGGGAGGCTGGTAAGGAA3'; U6, Forward: 5'-CTCGCTTCGGCAGCACATA3', Reverse: 5'-AACGATTCACGAATTTGCGTC3'; miR-34a-5p, Forward: 5'-AGGGGGTGGCAGTGTCTTAG-3', Reverse: 5'-GTGCGTGTCTGGAGTCG-3'; TNF- α , Forward: 5'-CTCGAGTGACAAGC-CCGTAG-3', Reverse: 5'-GGCAGCCTTGTC-CCTTGAAG-3'; IL-6, Forward: 5'-CCTTCCTAC-CCCACTTCCA-3', Reverse: 5'-GAGTTGGATGGTCTTGGTCC-3'.

Cell Counting Kit-8

FLS were seeded into 96-well plates at a density of 2000 cells per well. We added 200 μ L of complete culture medium to each well. At 24, 48, 72, and 96 hours, 90 μ L of complete culture medium and 10 μ L 5. Cell Counting Kit-8 (CKK-8) reagent (Dojindo, Kumamoto, Japan) were added. After an hour incubation at 37°C, absorbance was measured at 450 nm on a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The cell proliferation curve was drawn according to the OD value.

Enzyme-Linked Immunosorbent Assay

FLS were plated in 12-well plates (5 \times 10⁴ cells/well) in 1 ml complete culture medium. The

culture supernatant was collected at 72 hours. TNF- α and IL-6 concentrations were measured using TNF- α and IL-6 ELISA kits (PeproTech, Inc., Suzhou, China) according to the manufacturer's instructions. Briefly, we added 200 μ L of the diluted supernatant sample solution to a coated 96-well plate for 1 hour at room temperature. After washing with PBS-T (TBST, 0.5% Tween), 200 μ L of diluted detection antibody was added to each well, and the plate was incubated at room temperature for 30 minutes. After washing the plate again with PBS-T, the substrate solution was added, and the reaction was stopped after 30 minutes. The absorbance was detected on a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm.

Western Blot

Total protein in synovial tissue and FLS was extracted using RIPA (Beyotime Institute of Biotechnology, Shanghai, China). The protein concentration was measured using BCA kit (Beyotime Institute of Biotechnology, Shanghai, China). A total of 60 μ g proteins were separated by 10% SDS-PAGE. Then, the protein was transferred from the gel to a PVDF membrane (Millipore, Burlington, MA, USA). After washing the PVDF membrane, XBP1 primary antibody (1:1,000; cat. no. ab37152; Abcam, Shanghai, China) containing non-fat milk was added and incubated at 4°C overnight. After washing with PBS, horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. no. ab205718; Abcam, Shanghai, China) was added and incubated for 2 hours at room temperature. Finally, the blots were visualized using beyoECL Plus kit (Beyotime Institute of Biotechnology, Shanghai, China).

Dual-Luciferase Reporter Gene Assay

The XBP1 reporter plasmid with miR-34a-5p wild-type and mutant-type binding sites (pmirGLO-XBP1-wt, pmirGLO-XBP1-mut) were synthesized by Genepharma (Genepharma, Shanghai, China). FLS were seeded on 96-well plates. After 24 hours, Luciferase reporter plasmid and miR-34a-5p mimic were transfected into FLS using Lipofectamine 2000. Changes in fluorescence intensity were detected using the Dual-Luciferase Reporter Gene Assay Kit (Genepharma, Shanghai, China).

Statistical Analysis

The data were analyzed by SPSS 19 (version 26; IBM Corp., Armonk, NY, USA). Data were expressed as mean \pm standard deviation. The

comparison between two groups used Student's *t*-test. The comparison between multiple groups used Two-way ANOVA with Tukey's post-hoc test. $p < 0.05$ was considered statistically significant.

Results

The Expression of MiR-34a-5p is Low in RA Synovial Tissue

We examined miR-34a-5p expression in synovial tissue of RA and OA patients. It was found that miR-34a-5p expression was significantly lower in RA patients (Figure 1).

MiR-34a-5p Inhibits FLS Proliferation and Inhibits the Secretion of TNF- α and IL-6

To study the function of miR-34a-5p, we used miR-34a-5p mimic and miR-34a-5p inhibitor to up-regulate and down-regulate miR-34a-5p expression, respectively (Figure 2A). MiR-34a-5p mimic significantly inhibited the proliferation of FLS at 72 and 96 hours, and miR-34a-5p inhibitor significantly increased the proliferation of FLS at 72 and 96 hours (Figure 2B). MiR-34a-5p

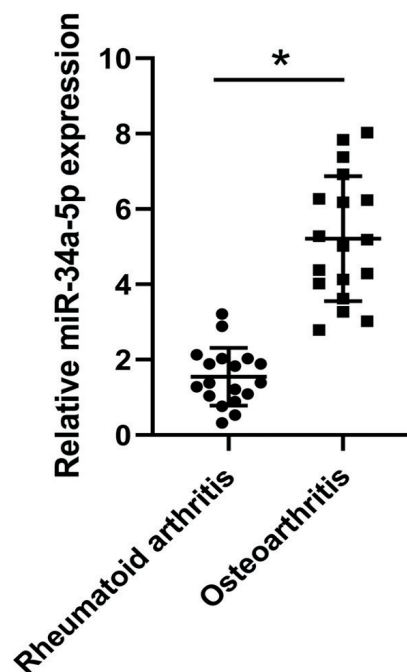


Figure 1. MiR-34a-5p expression in RA and OA synovial tissue. * < 0.05 .

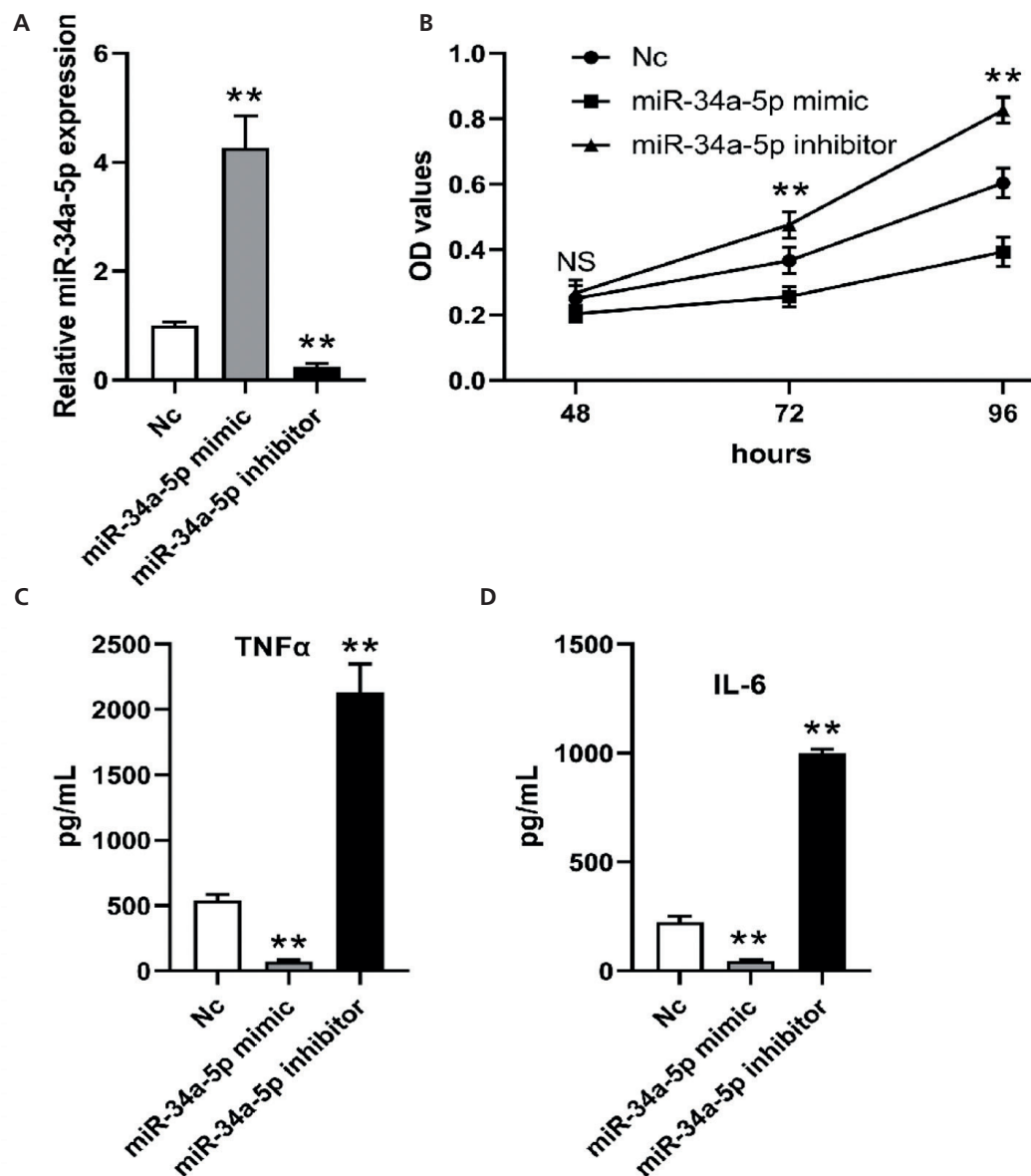


Figure 2. (A) Expression of miR-34a-5p in FLS after miR-34a-5p mimic and miR-34a-5p inhibitor transfection (B). Cell proliferation in FLS after miR-34a-5p mimic and miR-34a-5p inhibitor transfection, The concentration of TNF- α (C) and IL-6 (D) secreted by FLS after miR-34a-5p mimic and miR-34a-5p inhibitor transfection. Nc=Negative control, NS=No significant, **<0.01.

mimic can inhibit the secretion of TNF- α (Figure 2C) and IL-6 (Figure 2D) in FLS. miR-34a-5p inhibitor can significantly promote the secretion of TNF- α (Figure 2C) and IL-6 (Figure 2D).

There Is a Negative Correlation Between MiR-34a-5p and XBP1 Expression in Synovial Tissue

Through StarBase 3.0 prediction, we found that miR-34a-5p may bind to the 3' UTR of XBP1 (Figure 3A). We tested the expression of XBP1 in

the synovial tissue of RA and OA patients, and found that the XBP1 expression (Figure 3B) in the synovial tissue of RA patients was significantly higher than in the synovial tissue of OA patients.

MiR-34a-5p Directly Inhibits XBP1 Expression

MiR-34a-5p mimic can inhibit the XBP1 expression at mRNA (Figure 4A) and protein (Figure 4B) level, and miR-34a-5p inhibitor can increase the XBP1 expression at mRNA (Figure

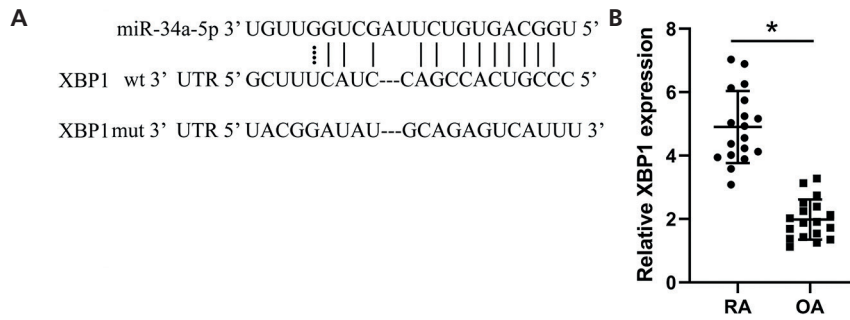


Figure 3. (A) MiR-34a-5p and XBP1 predicted binding sites (B). XBP1 gene expression in RA and OA synovial tissue. WT=wild type, MUT=mutant type, Nc=Negative control, NS=No significant, **<0.01.

4A) and protein (Figure 4B) level. The results of the Dual-Luciferase Reporter Gene Assay experiment showed that the fluorescence intensity of the pmirGLO-XBP1-wt and miR-34a-5p mimic co-transfection group was significantly lower (Figure 4C). This indicates that miR-34a-5p can directly act on the 3' UTR of XBP1 gene.

XBP1 Promotes FLS Proliferation and Promotes Secretion of TNF- α and IL-6

To further study the function of XBP1. We used XBP1 overexpression plasmid and XBP1 siRNA to up-regulate and down-regulate XBP1 expression, respectively (Figure 5A). The results showed that up-regulation of XBP1 gene expres-

sion significantly increased the proliferation of FLS at 72 and 96 hours, while down-regulation of XBP1 gene expression significantly inhibited the proliferation of FLS at 72 and 96 hours (Figure 5B). Moreover, up-regulation of XBP1 gene expression can significantly stimulate FLS to secrete TNF- α (Figure 5C and 5E) and IL-6 (Figure 5D, 5F).

Discussion

RA is an autoimmune inflammatory disease that mainly affects the synovial joints. The pathogenesis of RA is still unknown. Recent studies¹³⁻¹⁵

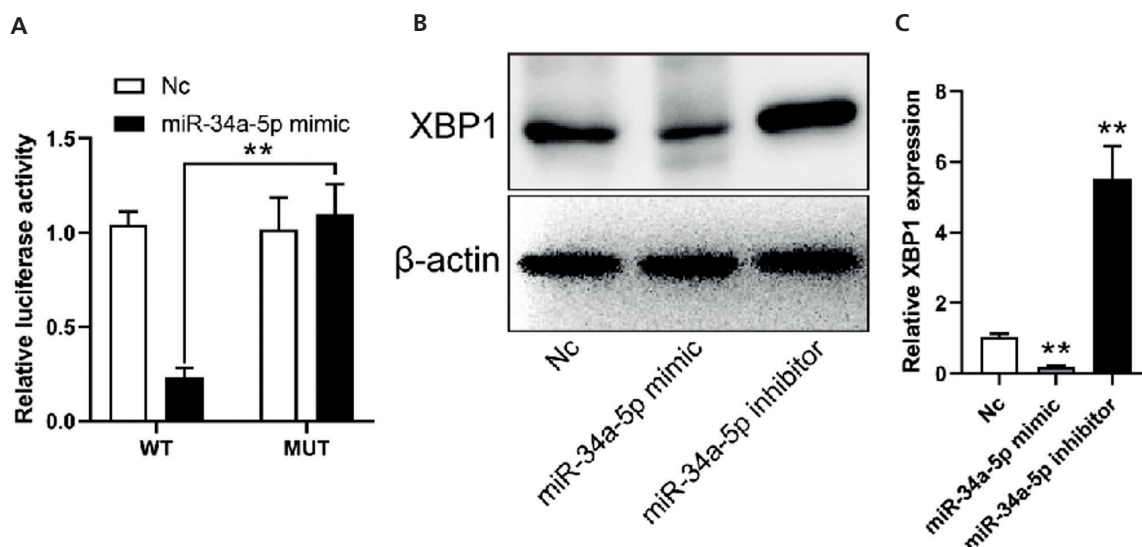


Figure 4. (A) XBP1 gene expression in FLS after miR-34a-5p mimic and miR-34a-5p inhibitor transfection, (B) XBP1 protein level in FLS after miR-34a-5p mimic and miR-34a-5p inhibitor transfection (C) Fluorescence intensity changes after miR-34a-5p mimic and pmirGLO-XBP1-wt reporter plasmid co-transfection or miR-34a-5p mimic and pmirGLO-XBP1-mut reporter plasmid co-transfection. Nc=Negative control, wt=wild type, mut= mutant type, NS=No significant, **<0.01.

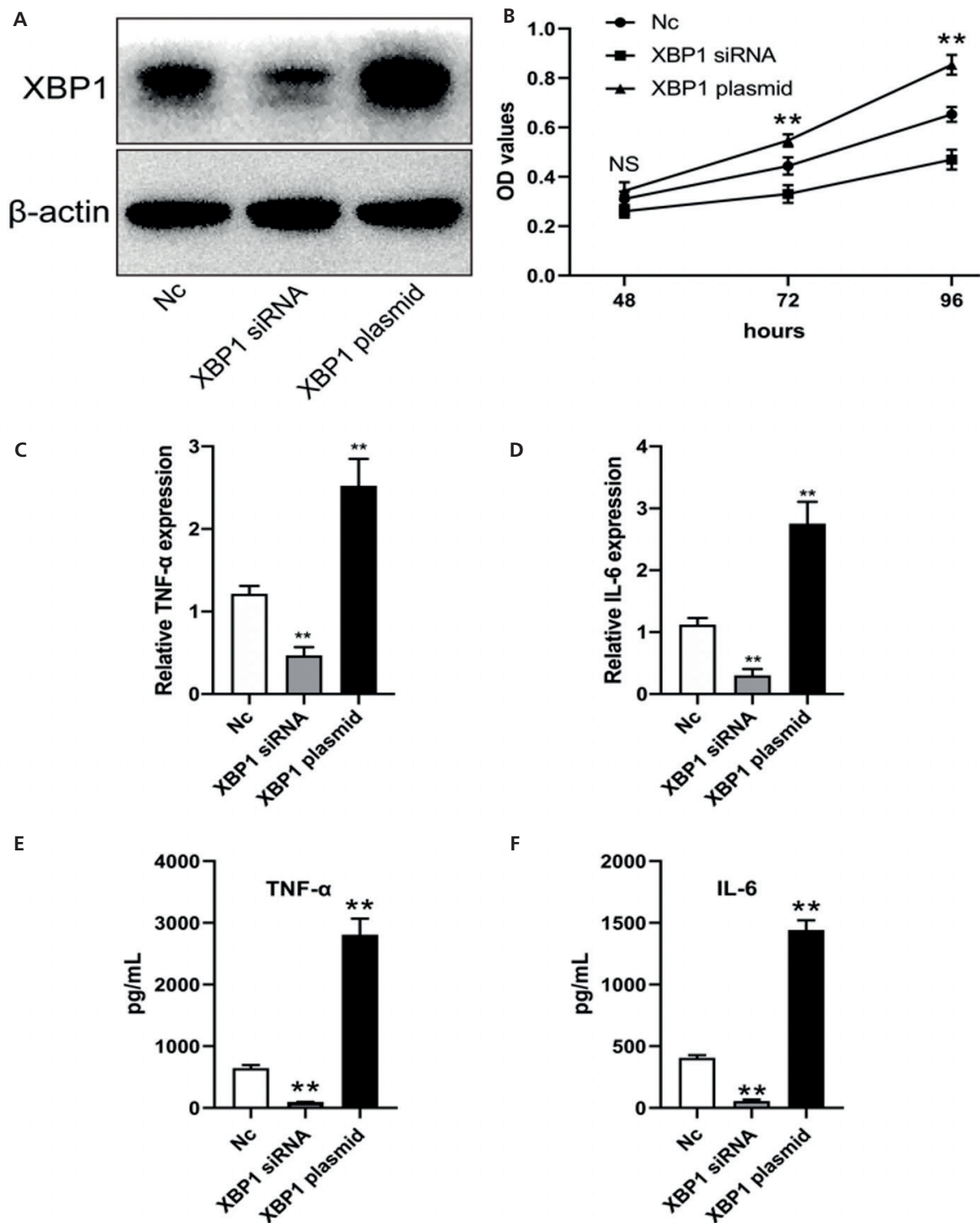


Figure 5. (A) XBP1 protein level in FLS after XBP1 siRNA and XBP1 overexpression plasmid transfection (B). FLS proliferation after XBP1 siRNA and overexpression plasmids transfection, the mRNA level (C) of TNF- α and IL-6 (D) in FLS after XBP1 siRNA and XBP1 overexpression plasmid transfection, the concentration of TNF- α (E) and IL-6 (F) secreted by FLS after XBP1 siRNA and XBP1 overexpression plasmid transfection. Nc=Negative control, NS=No significant, **<0.01.

have shown that multiple miRNAs play an important role in the pathogenesis of RA. MiR-34a has been shown to be involved in inflammatory responses in a variety of diseases¹⁶⁻¹⁸. Similarly, miR-34a has also been shown to be involved in the pathogenesis of RA. Dang et al¹⁹ found that inhibition of miR-34a could induce arthritis in mice, downregulate T cell percentage and cytokine expression. Niederer et al¹² found that down-regulating the expression of miR-34a can increase XIAP gene expression, thereby inhibiting the apoptosis of RA FLS.

One of the characteristics of RA is the hyperplasia of the synovial tissues and increased inflammatory factors secretion². In our study, we found that the expression of miR-34a-5p in synovial tissue of patients with RA was significantly lower than in synovial tissue of patients with OA. This expression difference suggests that miR-34a-5p may play a role in the hyperplasia of the synovial tissues. And further functional experiments, we proved that miR-34a-5p mimic can inhibit the proliferation of FLS.

Synovial tissues of patients with RA have increased secretion of various inflammatory factors, including TNF- α , IL-1, IL-6 and IL-8. These inflammatory factors cause the progress of RA and eventually cause joints destruction²⁰. Our research also found that miR-34a-5p mimic can inhibit the secretion of inflammatory factors (TNF- α and IL-6) by FLS. Through *in vitro* experiments, we proved that miR-34a-5p can inhibit the proliferation of FLS and the secretion of inflammatory factors, and then inhibit the development of RA.

MiRNA can act on the 3'UTR of mRNA, thereby degrading mRNA or inhibiting its expression²¹. We predicted through StarBase 3.0 that we found that miR-34a-5p can bind to 3' UTR of XBP1, suggesting that miR-34a-5p may directly act on XBP1 and inhibit its expression. XBP1 is a regulator of endoplasmic reticulum stress and XBP1 is involved in the inflammatory response of many diseases²²⁻²⁴, including RA²⁵. Compared with healthy controls and remission patients, the active form of XBP1 in active rheumatoid arthritis group was significantly overexpressed. Active form of XBP1 was induced in FLS by TLR4 and TLR2, resulting in XBP1-dependent IL-6 and TNF- α production. Moreover, TNF- α itself induces the activation of XBP1 in FLS, thus generating a potential feedback loop. Examination of synovial tissue in patients with RA and OA showed that the expression of XBP1 in synovial tissue of

patients with RA was higher than that of XBP1 in synovial tissue of patients with OA. There was a negative correlation between miR-34a-5p and XBP1 expression in synovial tissue of patients with RA. MiR-34a-5p can inhibit the expression of XBP1. Further Dual-Luciferase Reporter Gene Assay experiments proved that miR-34a-5p can target directly on XBP1 and inhibit its expression. In addition, XBP1 can promote the proliferation of FLS and the secretion of inflammatory factors, such as TNF- α and IL-6.

Conclusions

Our study revealed that miR-34a-5p inhibits fibroblast-like synoviocytes proliferation by directly targeting XBP1, which may provide a new perspective for the treatment of rheumatoid arthritis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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