

Influences of miR-320a on proliferation and apoptosis of fibroblast-like synoviocytes in rheumatoid arthritis through targeting MAPK-ERK1/2

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Abstract. – **OBJECTIVE:** To study the expression of micro ribonucleic acid (miR)-320a in synovial tissues of patients with rheumatoid arthritis (RA) and explore the influences of miR-320a on the proliferation and apoptosis of fibroblast-like synoviocytes (FLSs) in RA and its mechanism.

PATIENTS AND METHODS: The expression level of miR-320a in synovial tissues of 40 healthy people and 32 RA patients was detected via reverse transcription-polymerase chain reaction (RT-PCR). The FLSs were isolated from RA patients, cultured *in vitro* and divided into Control group and miR-320a mimic group. The proliferation and apoptosis of FLSs in each group were observed. Finally, the expression level of mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) 1/2 in each group was detected via Western blotting.

RESULTS: The expression level of miR-320a in synovial tissues of RA patients was significantly lower than that in healthy people ($p < 0.05$). After miR-320a mimic was transfected into FLSs cultured *in vitro*, EdU staining and flow cytometry analysis were performed. The results revealed that the proportion of EdU-positive cells significantly declined in miR-320a mimic group, the proportion of cells in G0/G1 phase was increased, while the cells in G2/M and S phases were significantly decreased ($p < 0.05$). Above data indicated that the cell proliferation ability was significantly inhibited. In addition, the results of flow cytometry also showed that the apoptosis rate of FLSs in miR-320a mimic group was significantly higher than that in Control group ($p < 0.05$). The results of Western blotting manifested that the Bcl-2 associated X protein (Bax)/Bcl-2 ratio in miR-320a mimic group was also obviously increased ($p < 0.05$). According to further studies, the phosphorylation level of ERK1/2 in miR-320a mimic group was remarkably inhibited ($p < 0.05$).

CONCLUSIONS: The expression level of miR-320a significantly declined in synovial tissues of RA patients. MiR-320a attenuated proliferation and promoted apoptosis of FLSs through inhibiting the activation of the MAPK-ERK1/2 signaling pathway.

Key Words:

Rheumatoid arthritis, Fibroblast-like synoviocytes, proliferation, apoptosis, miR-320a

Introduction

Rheumatoid arthritis (RA) is a kind of chronic autoimmune disease, which is mainly characterized by synovial hyperplasia, joint destruction and extra-articular manifestation, and ultimately leading to tissue destruction^{1,2}. In addition, RA increased the risk of cardiovascular diseases, threatening the health of 0.5-1% of the global population. About 30% of RA patients will be permanently incapacitated for work within 2-3 years if not treated in time³. Studies have demonstrated that RA-fibroblast-like synoviocytes (FLSs) also possess biological behaviors similar to tumor cells, such as proliferation, apoptosis, differentiation, invasion and migration⁴. RA-FLSs are major inflammatory cells causing joint inflammation and destruction, which play important roles in the occurrence and development of RA⁵. RA-FLSs can spontaneously secrete a large number of pro-inflammatory factors, innate immune cytokines and matrix degradation products, thereby initiating and aggravating the development of RA⁶.

Micro ribonucleic acids (miRNAs) are a group of single-stranded non-coding RNAs existing

with 20-24 nt in length and widely expressed in eukaryotes^{7,8}. MiRNAs were reported to regulate the expression of a variety of genes through targeted binding to specific genes, and thus played important roles in physiological activities of cells, such as proliferation, differentiation and apoptosis⁹. In addition, recent studies have also found that miRNAs could regulate the inflammatory response of immune and non-immune cells¹⁰. For example, the expression level of miR-320 significantly declined in muscle tissues of patients with myasthenia gravis, while up-regulation of miR-320 inhibited the release of inflammatory factors through inhibition on the expression of mitogen-activated protein kinase 1 (MAPK1)¹¹. However, the expression of miR-320a in RA and its influence on RA have not been reported yet.

In this work, the expression of miR-320a in synovial tissues of RA patients and healthy people was detected. The expression of miR-320a in RA-FLSs was regulated to further detect the influences of its expression level on the proliferation and apoptosis of RA-FLSs. Our study provided certain references for the clinical prevention and treatment of RA in the future.

Patients and Methods

Tissue Specimens

This study enrolled 32 cases of synovial tissues of RA patients received joint replacement and 40 cases of synovial tissues of non-RA patients received joint replacement in our hospital from December 2015 to December 2017. After the blood stains were washed away with normal saline, all specimens were cut into pieces, placed into an Eppendorf (EP) tube and stored in a refrigerator at -80°C. All the above procedures were approved by the Medical Ethics Committee of The Second Affiliated Hospital of Wenzhou Medical University.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted from synovial tissues using the TRIzol method (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were detected using an ultraviolet spectrophotometer, and the RNA with absorbance (A_{260}/A_{280}) of 1.8-2.0 was considered qualified. The messenger RNA (mRNA) was synthesized into the complementary deoxyribonucleic acid (cDNA) through RT and stored in the refrigerator at -80°C. RT-PCR system was prepared as

follows: 2.5 μ L 10 \times Buffer, 2 μ L cDNA, 0.25 μ L forward primer (20 μ mol/L), 0.25 μ L reverse primer (20 μ mol/L), 0.5 μ L dNTPs (10 mmol/L), 0.5 μ L Taq enzyme (2×10^6 U/L) and 19 μ L ddH₂O. The amplification system of RT-PCR was the same as above. Primer sequences used in this study were as follows: miR-320a, F: 5'-CCTGGTGTAACCTCCTCGCTG-3', R: 5'-AACTGTGTCGTGTAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Isolation and Culture of Human RA-FLSs

RA-FLSs were isolated according to a previous study¹². Briefly, the synovial tissues were minced and digested with 2 mg/mL type II collagenase (Invitrogen, Carlsbad, CA, USA) in the Dulbecco's modified Eagle medium (DMEM, Gibco, Rockville, MD, USA) at 37°C for 2 h. After centrifugation at 210 \times g for 10 min, the precipitate was resuspended in 1 mL DMEM containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and cultured with 5% CO₂.

Cell Counting kit-8 (CCK-8)

The cells in the logarithmic growth phase in each group were inoculated into a 96-well plate and cultured in an incubator with 5% CO₂ at 37°C for 0, 12, 24, 36 and 48 h. The developing solution was prepared in dark using 1640 medium and CCK-8 (10:1) (Dojindo, Kumamoto, Japan). After the medium was discarded, totally 110 μ L developing solution was added into each well, followed by incubation in the incubator at 37°C for 2 h. The optical density (OD) in each group was detected at 540 nm using an ultraviolet spectrophotometer.

5-Ethynyl-2'-Deoxyuridine (EdU) Staining

RA-FLSs were stained using the EdU staining kit (Invitrogen, Carlsbad, CA, USA) according to the instructions. After that, each glass slide was photographed using a fluorescence microscope in 3 randomly-selected fields of view. Finally, the EdU-positive cells were quantified.

Flow Cytometry

RA-FLSs in the logarithmic growth phase were digested with 0.25% trypsin-EDTA (Ethylene Diamine Tetraacetic Acid) (Thermo Fisher Scientific, Waltham, MA, USA), prepared into the cell suspension and inoculated into a 6-well plate. The apoptosis rate of each group was detected accor-

ding to the operation steps of the Annexin V-FITC (Fluorescein) propidium iodide (PI) apoptosis assay kit (Beyotime, Shanghai, China).

Western Blotting

The cells were washed with phosphate buffered saline (PBS) for 3 times before lysis. After that, a total of 1000 μ L lysis buffer was added into every dish and fully vibrated for 20 min. The cells at the bottom of dish were scraped off using a brush, placed into the EP tube and further lysed using an ultrasonic pyrolyser for about 15 s. After standing for 15 min, the cells were centrifuged at 12000 rpm for 0.5 h. The supernatant was then transferred to new EP tubes. The protein concentration was detected via ultraviolet spectrometry, and all the protein samples were quantified to be the same concentration. The protein was sub-packaged and placed in the refrigerator at -80°C .

After the total protein was extracted from RA-FLSs, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Then, the protein in the gel was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and incubated with the primary antibody at 4°C overnight, and then incubated again with the goat anti-rabbit secondary antibody in a dark place for 1 h. The protein band was scanned and quantified using the Odyssey scanner, and the level of proteins to be detected was corrected using actin.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for the analysis of all data. Measurement data were expressed as mean \pm standard deviation, and t-test was used for the comparison of data between two groups. $p < 0.05$ suggested that the difference was statistically significant.

Results

Expression Level of miR-320a in Synovial Tissues of RA Patients and Healthy People

The expression level of miR-320a in synovial tissues of healthy people and RA patients was detected via RT-PCR. The results revealed that the expression level of miR-320a in synovial tissues of RA patients was significantly lower than (about 0.143 times) that in Control group ($p < 0.05$) (Figure 1).

Influence of miR-320a Mimic on Proliferation of RA-FLSs

After 12, 24, 36 and 48 h of miR-320a mimic transfection, the OD_{540} value of RA-FLSs significantly declined ($p < 0.05$) (Figure 2), indicating that the miR-320a overexpression could inhibit the proliferation ability of RA-FLSs.

EdU staining Results in Both Groups

As shown in Figure 3, miR-320a mimic significantly reduced the rate of EdU-positive RA-FLSs. The EdU-positive rate of Control group and miR-320a mimic group was $(25.12 \pm 1.93)\%$ and $(7.22 \pm 2.16)\%$, respectively ($p < 0.05$).

Influence of miR-320a Mimic on Cell Cycle of RA-FLSs

As shown in Figure 4, the cell cycle of RA-FLSs was obviously altered after miR-320a mimic was added. In miR-320a mimic group, the proportion of cells in G0/G1 phase was markedly increased ($p < 0.05$), while decreased in G2/M and S phases ($p < 0.05$), suggesting that miR-320a mimic had an inhibitory effect on the cell cycle of RA-FLSs.

Flow Cytometry Results in Both Groups

The results of flow cytometry showed that the apoptosis rate of Control group and miR-320a mimic group was $(3.12 \pm 1.72)\%$ and $(8.83 \pm 1.05)\%$,

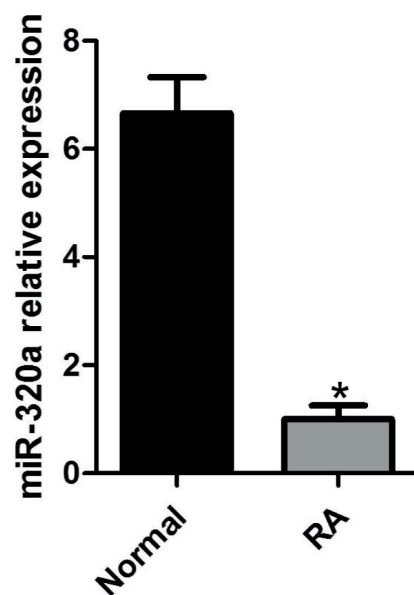


Figure 1. Expression level of miR-320a in synovial tissues of RA patients and healthy people. Normal: healthy people. RA: RA patients. * $p < 0.05$, the RA group vs. the Control group.

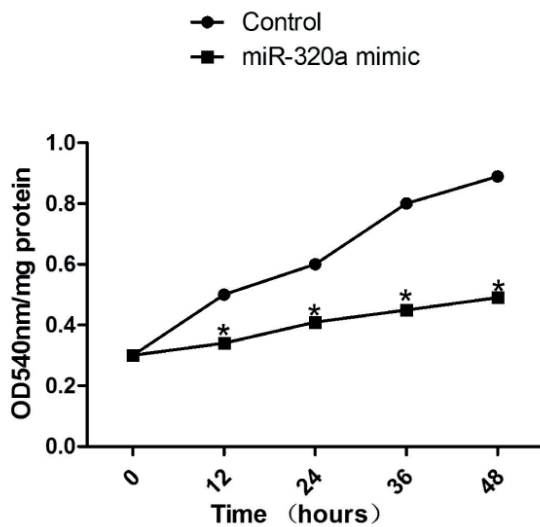
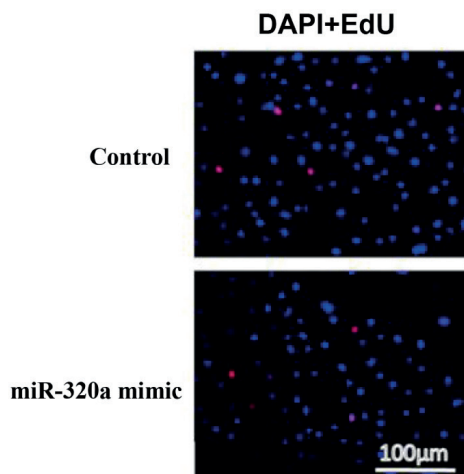


Figure 2. Influence of miR-320a mimic on proliferation of RA-FLSs. Control: Control group, miR-320a mimic: miR-320a overexpression group. * $p < 0.05$, the miR-320a mimic group vs. the Control group.

respectively ($p < 0.05$) (Figure 5), demonstrating that the miR-320a overexpression remarkably promoted the apoptosis of RA-FLSs.

Influence of miR-320a Mimic on the Expression of Apoptosis-Related Genes in RA-FLSs

The influence of miR-320a mimic on the expression of apoptosis-related genes, including B-cell lymphoma-2 (Bcl-2) and Bcl-2 associated X protein (Bax), in RA-FLSs was further detected. We found that the Bax/Bcl-2 ratio was obviously increased after miR-320a overexpression ($p < 0.05$) (Figure 6).



Influence of miR-320a Mimic on MAPK-ERK1/2 Signaling Pathway in RA-FLSs

Considering the role of the MAPK-ERK1/2 signaling pathway in the proliferation and apoptosis of RA-FLSs, the MAPK-ERK1/2 protein expression in both groups was further detected via Western blotting. The results exhibited that the phosphorylation level of ERK1/2 in miR-320a mimic group was remarkably inhibited ($p < 0.05$) (Figure 7).

Discussion

RA is a systemic immune disease affecting multiple tissues and organs and mainly occurs in the synovial joint^{13, 14}. The basic pathological changes of RA mainly refer to the inflammatory factors abnormally released in the synovium, among which FLSs are the most important effector cells¹⁵. In addition, RA-FLSs attack cartilage tissues in the synovium via proliferation and invasion, ultimately leading to the joint injury¹⁶. At the same time, RA-FLSs promote the synovial hyperplasia in RA through inhibiting apoptosis¹⁷. Therefore, searching the specific diagnostic markers and therapeutic targets are the main goal of RA prevention and treatment in the future.

With the rapid development of transcriptomics in recent years, more and more disease-related differentially-expressed genes have been uncovered increasingly. A lot of studies have also confirmed the role of miRNAs in the incidence and development of RA¹⁸. For example, miR-146a affected the IL-17 expression in peripheral blood mononuclear cells of RA patients¹⁹. Moreover, the expression

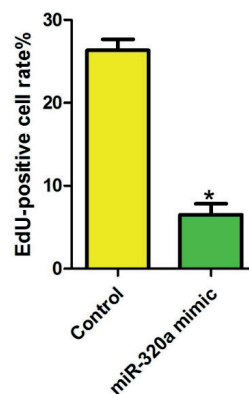


Figure 3. EdU staining results in both groups. Control: Control group, miR-320a mimic: miR-320a overexpression group. * $p < 0.05$, the miR-320a mimic group vs. the Control group.

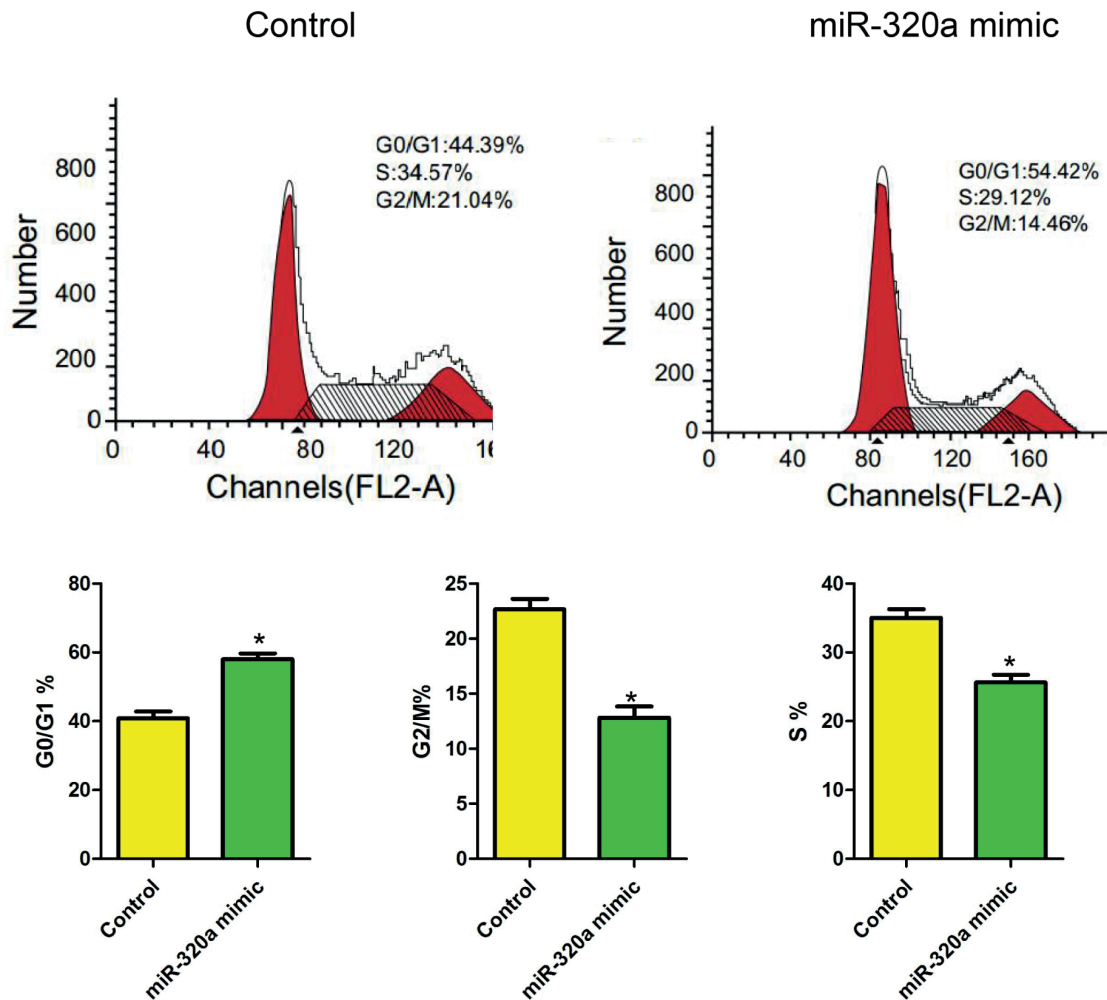


Figure 4. Influence of miR-320a mimic on cell cycle of RA-FLSs. Control: Control group, miR-320a mimic: miR-320a over-expression group. * $p < 0.05$, the miR-320a mimic group vs. the Control group.

of miR-34a was inhibited via methylation in RA-FLSs, thus leading to the up-regulated expression of its direct target (X-linked inhibitor of apoptosis protein) and promoting the apoptosis of RA-FLSs²⁰. The expression of miR-223 in RA patients was significantly increased compared with that in osteoarthritis patients. In addition, miR-223 inhibited the number of tartrate-resistant acid phosphatase-positive multinucleated cells in a dose-dependent manner, indicating that miR-223 might regulate the osteoclast differentiation in RA patients²¹. Besides, the level of miR-21 in the synovium of RA patients significantly declined, accompanied by the increase in the expression of activated signal transducer and activator of transcription 3 (STAT3) and the decrease in levels of STAT5/pSTAT5 protein and Foxp3 mRNA. Ac-

cording to further studies, miR-21 affecting the T cell differentiation through regulating the balance of Th17/Treg cell population²². Besides, miR-126 was reported to regulate the PI3K/AKT signaling pathway through targeted inhibition on PIK3R2, thereby suppressing the proliferation and promoting the apoptosis of RA-FLSs²³.

Current studies have revealed that the apoptosis signal of RA-FLSs involves multiple signal transduction pathways, such as Fas/FasL, TNF/TNF-R and TRAIL/TRAIL-R. RA-FLSs are also regulated by a variety of genes and proteins, which are all regulated by Fas-associated protein with death domain²⁴. In this study, the synovial tissues were collected from RA patients and healthy people, and the expression of miR-320a in synovial tissues in both groups

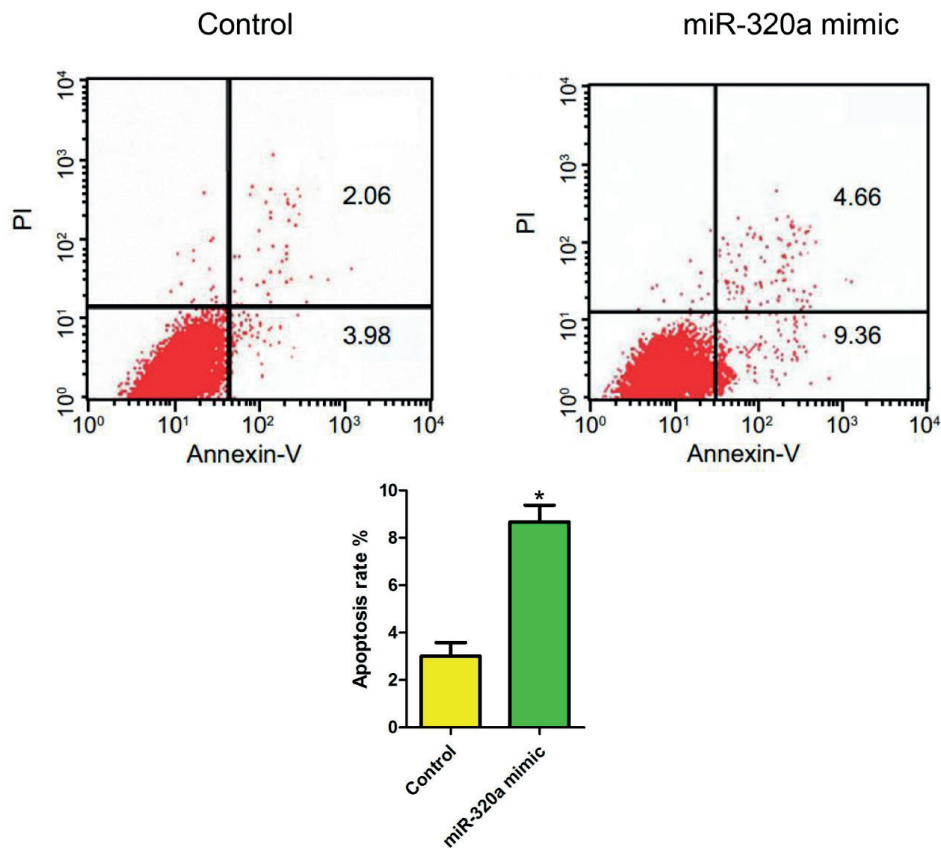


Figure 5. Flow cytometry results in both groups. Control: Control group, miR-320a mimic: miR-320a overexpression group. * $p < 0.05$, the miR-320a mimic group vs. the Control group.

was detected via RT-PCR. The results showed that the expression of miR-320a in synovial tissues of RA patients was significantly down-regulated. Meanwhile, FLSs were isolated from

synovial tissues of RA patients, and miR-320a cells were constructed with transfection of miR-320a mimic. Then, the cell proliferation in both groups was detected via CCK-8 cell prolifera-

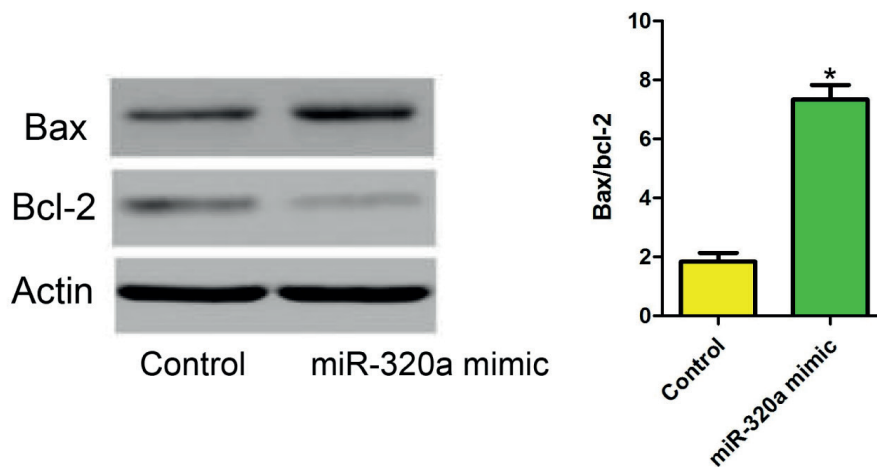


Figure 6. Influence of miR-320a mimic on expression of apoptosis-related genes in RA-FLSs. Control: Control group, miR-320a mimic: miR-320a overexpression group. * $p < 0.05$, the miR-320a overexpression group vs. the Control group.

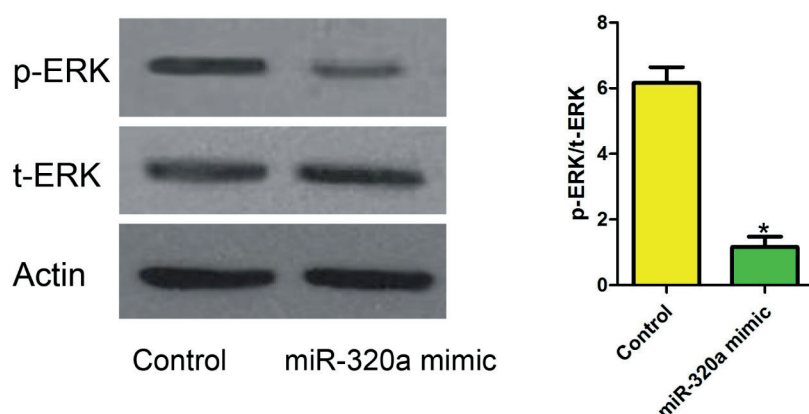


Figure 7. Influence of miR-320a mimic on MAPK-ERK1/2 signaling pathway in RA-FLSs. Control: Control group, miR-320a mimic: miR-320a overexpression group. * $p < 0.05$, the miR-320a overexpression group vs. the Control group.

tion assay and EdU staining, and the proportion of cells in each proliferation cycle was detected via flow cytometry. It was found that the miR-320a overexpression could inhibit the proliferation of RA-FLSs. Moreover, the apoptosis and the expression levels of apoptosis-related proteins in both groups were detected via flow cytometry and Western blotting. The results manifested that miR-320a could significantly promote the apoptosis of RA-FLSs. Furthermore, the classical signaling pathway affecting the proliferation and apoptosis of RA-FLSs was detected. It was found that the phosphorylation of ERK1/2 was significantly inhibited in miR-320a mimic cells. Therefore, it was speculated that the influences of miR-320a on the proliferation and apoptosis of RA-FLSs were mediated by the ERK1/2 signaling pathway. However, there were still certain limitations in this experiment. First, the direct target of miR-320a was not found. Second, no animal experiments were performed for verification. Last, there are many pathways initiating apoptosis, however, the signaling pathway promoting RA-FLS apoptosis was not found in this study.

Conclusions

We indicated for the first time that miR-320a was lowly expressed in synovial tissues of RA patients. The miR-320a overexpression significantly inhibited the proliferation and promoted the apoptosis of RA-FLSs, which might be related to the inhibition of the MAPK-ERK1/2 signaling

pathway caused by. Therefore, miR-320a was expected to be a new targeted therapeutic drug for RA.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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