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MicroRNA-193a-3p participates in the progression of rheumatoid arthritis by regulating proliferation and apoptosis of MH7A cells through targeting IGFBP5

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Abstract. – OBJECTIVE: This study aims to explore the regulatory effect of microRNA-193a-3p on rheumatoid arthritis (RA) and its underlying mechanism.

PATIENTS AND METHODS: Expression level of microRNA-193a-3p in synovial tissues extracted from 30 RA patients and healthy controls was detected by quantitative Real-time polymerase chain reaction (qRT-PCR). MH7A cells were subjected to TNF-α induction for constructing the in vitro RA model. After transfection of microRNA-193a-3p inhibitor in MH7A cells, proliferation and apoptosis were detected by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively. Enzyme-linked immunosorbent assay (ELISA) was conducted to determine levels of interleukin 6 (IL-6) and IL-8 in MH7A cells. Subsequently, the dual-luciferase reporter gene assay was carried out to verify the binding condition between microRNA-193a-3p and IGFBP5. Rescue experiments were conducted to evaluate the proliferation and apoptosis of MH7A cells with knockdown of microRNA-193a-3p and IGFBP5.

RESULTS: MicroRNA-193a-3p was highly expressed in synovial tissues of RA patients and TNF-α-induced MH7A cells than those of controls. TNF-α induction significantly increased the proliferative rate of MH7A cells, reaching the peak at 96 h. After knockdown of microRNA-193a-3p, the promoted proliferation by TNF-α induction was significantly inhibited. In addition, TNF-α induction significantly inhibited the apoptosis of MH7A cells. After inhibition of microRNA-193a-3p expression, the inhibited apoptosis by TNF-α induction remarkably increased. TNF-α induction upregulated levels of IL-6 and IL-8 in MH7A cells, which were remarkably reduced after microRNA-193a-3p knockdown. Dual-luciferase reporter gene assay confirmed that IGFBP5 could bind to microRNA-193a-3p, and its expression was negatively regulated by microRNA-193a-3p. The regulatory effects of microRNA-193a-3p on proliferation and apoptosis of MH7A cells were reversed by IGFBP5 knockdown.

CONCLUSIONS: MicroRNA-193a-3p is highly expressed in the synovial tissues and cells of rheumatoid arthritis. MicroRNA-193a-3p participates in the process of rheumatoid arthritis by regulating the proliferation, apoptosis and inflammatory response of MH7A cells through targeting IGFBP5.

Key Words:

Fibroblast-like synoviocytes (FLS), Rheumatoid arthritis (RA), MicroRNA-193a-3p, IGFBP5.

Introduction

Rheumatoid arthritis (RA) is a complex systemic autoimmune disease. Globally, the incidence of RA is about 0.5% to 1%, and the amount of female RA patients is 2-3 times than male. The incidence of RA increases with age, which is about 6% in people over 65 years old[1,2]. RA is characterized by chronic, multi-joint, symmetrical, invasive joint inflammation, as well as progressive destruction of bone and cartilage. Uncontrolled RA can cause severe joint damage and even disability, thus severely affecting life quality of RA patients[3]. The pathogenic cause of RA is not yet clear. It is currently considered to be related to genetic, environmental, and endocrine factors.

The main pathological features of RA are the inflammatory infiltration of a large number of plasma cells, macrophages and lymphocytes in the synovium, as well as the inflammatory erosion and damage of the synovial tissue to cartilage, tendons and bone. Inflammatory and immune cells are abundantly accumulated in the synovial membrane of RA patients. The inner layer of the synovial membrane gradually proliferates, thickens and then transforms into vasospasm, eventually destroying articular cartilage.

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Therefore, the proliferative synovial membrane is a pathological marker of RA. Fibroblast-like synoviocytes (FLS) are a class of mesenchymal stem cells manifesting with many fibroblast characteristics, which are the main components involved in the inflammatory response of RA. FLS could invade and destroy cartilage, further leading to the degeneration of articular cartilage and bone. Studies have shown that TNF-α can induce apoptosis of FLS, resulting in "tumor-like" abnormal proliferation of FLS. Therefore, inhibition of the abnormally proliferative FLS is an effective method to treat RA. MicroRNAs (miRNAs) are a class of highly conserved, noncoding, single-stranded RNAs discovered in recent years. They are widely present in many eukaryotes, inducing the degradation or inhibiting the translation of targeted mRNAs. MicroRNAs participate in the regulation of cell proliferation, division, differentiation, metabolism and apoptosis[4]. Therefore, microRNAs can serve as diagnostic biomarkers and potential therapeutic targets for a variety of diseases. There are many microRNAs expressing in different tissues or cells of RA patients, including FLS and synovial fluids[5-7]. It is reported that microRNA-146a and microRNA-155 promote RA development by regulating key components in cytokine signaling pathways[8]. MicroRNA-212-3p and microRNA-29a alleviate RA progression by inhibiting FLS proliferation and inducing apoptosis through targeting SOX5 and STAT3, respectively[9,10]. As an oncogene, microRNA-193a-3p is capable of regulating proliferation and apoptosis in prostate cancer, breast cancer, esophageal cancer, lung cancer, and colorectal cancer[11-14]. However, the role of microRNA-193a-3p in RA has not been reported yet.

Patients and Methods

Sample Collection

Synovial tissues were harvested from 30 RA patients undergoing joint replacement and 30 traumatic patients from July 2015 to September 2017. All RA patients were pathologically diagnosed in accordance to the standard of ACR/EULAR published in 2010[15]. Patients were informed consent and this study was approved by the Weifang People's Hospital Ethic Committee.

Cell Culture

MH7A cells were provided by American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) containing 15% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (HyClone, South Logan, UT, USA). Culture medium was replaced every other day. Cell passage was performed until the 80% of cell confluence.

Cell Transfection

MH7A cells were transfected with microRNA-193a-3p inhibitor, inhibitor-NC, si-IGFBP5 or si-NC using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Culture medium was replaced 6 hours later. The plasmid sequences were: MicroRNA-193a-3p inhibitor: 5'GAUAGCAGAGCAGCAGUGCGCC3'; inhibitor NC: 5'UCUACUCUUUCUAGGAGGUUGUG3'; siIGFBP5: 5'GGAUCUGUCUCCUCCUCUAGC3'; siNC: 5'UUCUUCGAAGGUGUCACGUTT3'.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using the TRIzol kit (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into complementary Deoxyribose Nucleic Acid (cDNA). After the cDNA was amplified, qRT-PCR was performed to detect the expressions of related genes. Primers used in this study were as follows: IGFBP5, F: 5'ACGCGTCGACATGGGCTCCTTCGTGCAC-3', 5'CGCGGATCCATCACTC AACGTTGCTGCTG3'; MicroRNA-193a-3p, F: 5'-GCATAACTGGCCTACAAAGT-3', R: 5'-GTGCAGGGTCCGAGGT-3'; GAPDH, F: 5′-CACCCACTCCTCCACCTTTG-3′, R: 5′-CCACCACCCTGTTGCTGTAG-3'; U6, F: 5'-AACGCTTCACGAATTTGCGT-3', R: 5'-CCAAGCTTATGACAGCCATCATC-3'.

Western Blot

Cells were lysed for protein extraction. The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). Protein sample was separated by gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membranes (Millipore, Billerica, MA, USA). After incubation with primary and secondary antibody (Cell Signaling Technology, Danvers, MA, USA), immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

ELISA (Enzyme-Linked Immuno Sorbent Assay)

Cells were seeded in the 96-well plates with 5×10^4 cells per well. Serum-free medium was replaced when the cell confluence was up to 80-90%. After specific treatment, the supernatant of each group was collected for detecting cytokine levels using ELISA kits (Abcam, Cambridge, MA, USA). The absorbance value was recorded at the wavelength of 450 nm with a microplate reader.

CCK-8 (Cell Counting Kit-8) Assay

Cells were seeded into 96-well plates at a density of 1×10^4/μL. 10 μL of CCK-8 solution (cell counting kit-8, Dojindo, Kumamoto, Japan) was added in each well. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA). Each group had 5 replicates.

Flow Cytometry

Cells were digested with Ethylene Diamine Tetraacetic Acid (EDTA)-free trypsin and cell density was adjusted to 1×10^5/mL. After resuspension with 1×Annexin, cells were labeled with 5 μL of Annexin V and stained with 1 μL of Propidium Iodide (PI) at room temperature in dark. 15 min later, cell apoptosis was detected using flow cytometry (Partec AG, Arlesheim, Switzerland). Each experiment was repeated in triplicate.

Dual-Luciferase Reporter Gene Assay

The binding sequences of microRNA-193a-3p and IGFBP5 were predicted to construct IGFBP5-WT 3'UTR and IGFBP5-MUT 3'UTR. Cells were seeded in 12-well plates and co-transfected with 150 pmol/L microRNA-19a inhibitor or NC and 80 ng IGFBP5-WT 3'UTR or IGFBP5-MUT 3'UTR for 48 h, respectively. Cells were then washed with phosphate-buffered saline (PBS) and incubated with 1×PLB for complete lysis. Luciferase activity was finally detected according to the relative commercial kit instructions.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Quantitative data were represented as mean ± standard deviation ($\bar{x} \pm s$). The *t*-test was used for comparing intergroup differences. $p<0.05$ was considered statistically significant.

Results

MicroRNA-193a-3p was Highly Expressed in Synovial Tissues and FLS of RA

To investigate the role of microRNA-193a-3p in RA, expression level of microRNA-193a-3p in synovial tissues extracted from 30 RA patients and controls was detected by qRT-PCR. MicroRNA-193a-3p was highly abundant in synovial tissue of RA than that of controls (Figure 1A). Meanwhile, we examined the expression of microRNA-193a-3p in TNF-α-induced MH7A cells. Similarly, microRNA-193a-3p expression in MH7A cells increased after TNF-α

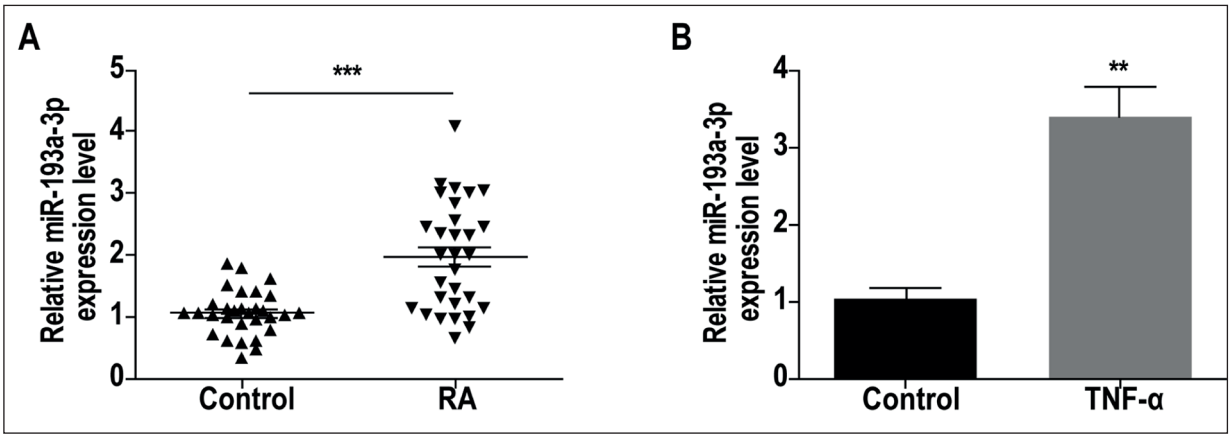

Figure 1. MicroRNA-193a-3p was highly expressed in synovial tissues and FLS of RA. ***A,*** MicroRNA-193a-3p expression in synovial tissue of RA was higher than that of healthy controls. ***B,*** MicroRNA-193a-3p expression increased in MH7A cells after TNF-α induction.

induction (Figure 1B). These results indicated that the high expression of microRNA-193a-3p may be involved in the development of RA.

Knockdown of microRNA-193a-3p Inhibited Proliferation and Induced Apoptosis of MH7A Cells

To further validate the specific functions of microRNA-193a-3p in regulating biological performances of MH7A cells, we first constructed microRNA-193a-3p inhibitor. Transfection of microRNA-193a-3p inhibitor in MH7A cells remarkably decreased microRNA-193a-3p expression (Figure 2A). TNF-α induction significantly increased the proliferative rate of MH7A cells, reaching the peak at 96 h. After knockdown of microRNA-193a-3p, the promoted proliferation by TNF-α induction was significantly inhibited (Figure 2B). In addition, TNF-α induction significantly inhibited the apoptosis of MH7A cells. After inhibition of microRNA-193a-3p expression, the inhibited apoptosis by TNF-α induction remarkably increased (Figure 2C). Abnormal secretion of cytokines is important in RA progression. Here, we found TNF-α induction upregulated levels of IL-6 and IL-8 in MH7A cells, which were remarkably reduced after microRNA-193a-3p knockdown (Figure 2C and 2D). These results suggested that microRNA-193a-3p could regulate proliferation, apoptosis and inflammation of MH7A cells.

MicroRNA-193a-3p Directly Regulated IGFBP5

Studies[16] have shown that the IGFBP5 pathway is involved in cell proliferation, differentiation and apoptosis. To verify whether IGFBP5 pathway is related to the regulatory effects of microRNA-193a-3p on proliferation and apoptosis of MH7A cells, we constructed the IGFBP5 wild-type sequence IGFBP5-WT 3'UTR and the mutant-type sequence IGFBP5-MUT 3'UTR (Figure 3A). Luciferase activity remarkably decreased in MH7A cells co-transfected with microRNA-193a-3p inhibitor and IGFBP5-WT 3'UTR. However, no significant change in luciferase activity was found after co-transfection of microRNA-193a-3p inhibitor and IGFBP5-MUT 3'UTR (Figure 3B). Both mRNA and protein levels of IGFBP5 were upregulated after transfection of microRNA-193a-3p inhibitor, suggesting a negative correlation between them (Figure 3C and D).

MicroRNA-193a-3p Regulated Proliferation and Apoptosis of MH7A Cells by Targeting IGFBP5

Rescue experiments were conducted to further verify the role of IGFBP5 in RA. We have already pointed out that microRNA-193a-3p knockdown could promote proliferation of MH7A cells. However, knockdown of microRNA-193a-3p and IGFBP5 remarkably reversed the inhibited cell proliferation (Figure 4A). Similarly, knockdown of IGFBP5 reversed the promoted apoptosis induced by knockdown of microRNA-193a-3p (Figure 4B). The above results elucidated that microRNA-193a-3p regulated proliferation and apoptosis of MH7A cells by targeting IGFBP5.

Discussion

RA is a multi-system, inflammatory disease that causes autoimmune disorders, joint damage and multiple systemic complications. The pathological process of RA mainly involves in the infiltration of joint inflammatory cells, as well as the proliferation and invasion of synovial cells, eventually leading to the destruction of bone and cartilage[17,18]. RA severely threatens human health due to its high disability and organ involvement. It is urgent to explore effective treatments for improving clinical outcomes of RA patients. FLS distribute in the inner layer of synovial membrane, which are the most important cells in the pathogenesis of RA. FLS extracted from RA patients exert tumor-like characteristics, including high proliferative, anti-apoptotic, migratory and invasive abilities. Therefore, regulations on proliferation and apoptosis of FLS contribute to control the occurrence and progression of RA. IGFBP5, a member of the insulin growth factor binding protein family, competes with the insulin growth factor receptor by binding to IGF[19]. Studies[16,20] have shown that IGFBP5 exerts an important role in regulating cell proliferation, differentiation and apoptosis, as well as tumor growth. Yang et al[21] have shown that IGFBP5 regulates the proliferation and apoptosis of FLS and participates in the pathological process of RA. Evidence showed the biological functions of microRNAs in RA development. MiRNA-188-5p is differentially expressed in activated FLS, directly or indirectly regulating the expression of extracellular matrix in RA[22]. In addition, over-expression of microRNA-203 directly upregulates MMP-1 and IL-6 through activating NF-κB signaling pathway[23].

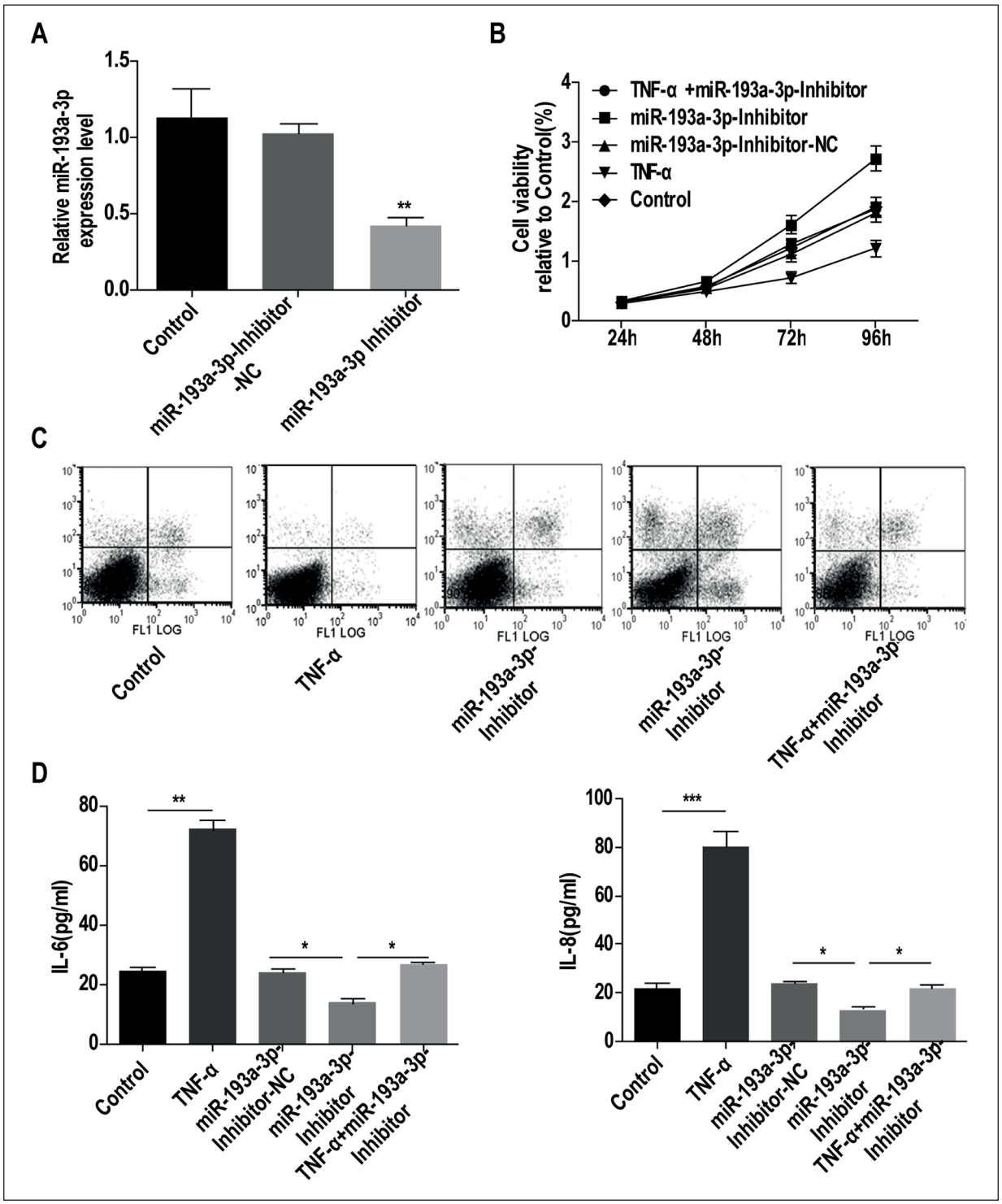

Figure 2. Knockdown of microRNA-193a-3p inhibited proliferation and induced apoptosis of MH7A cells. ***A,*** Transfection of microRNA-193a-3p inhibitor in MH7A cells remarkably decreased microRNA-193a-3p expression. ***B,*** TNF-α treatment significantly increased the proliferation of MH7A cells, reaching the peak at 96 h. After knockdown of microRNA-193a-3p, the promoted proliferation by TNF-α was significantly inhibited. ***C,*** TNF-α treatment significantly inhibited the apoptosis of MH7A cells. After inhibition of microRNA-193a-3p expression, the inhibited apoptosis by TNF-α remarkably increased. ***C, D,*** TNF-α treatment increased levels of IL-6 and IL-8 in MH7A cells, which were remarkably reduced after microRNA-193a-3p knockdown.

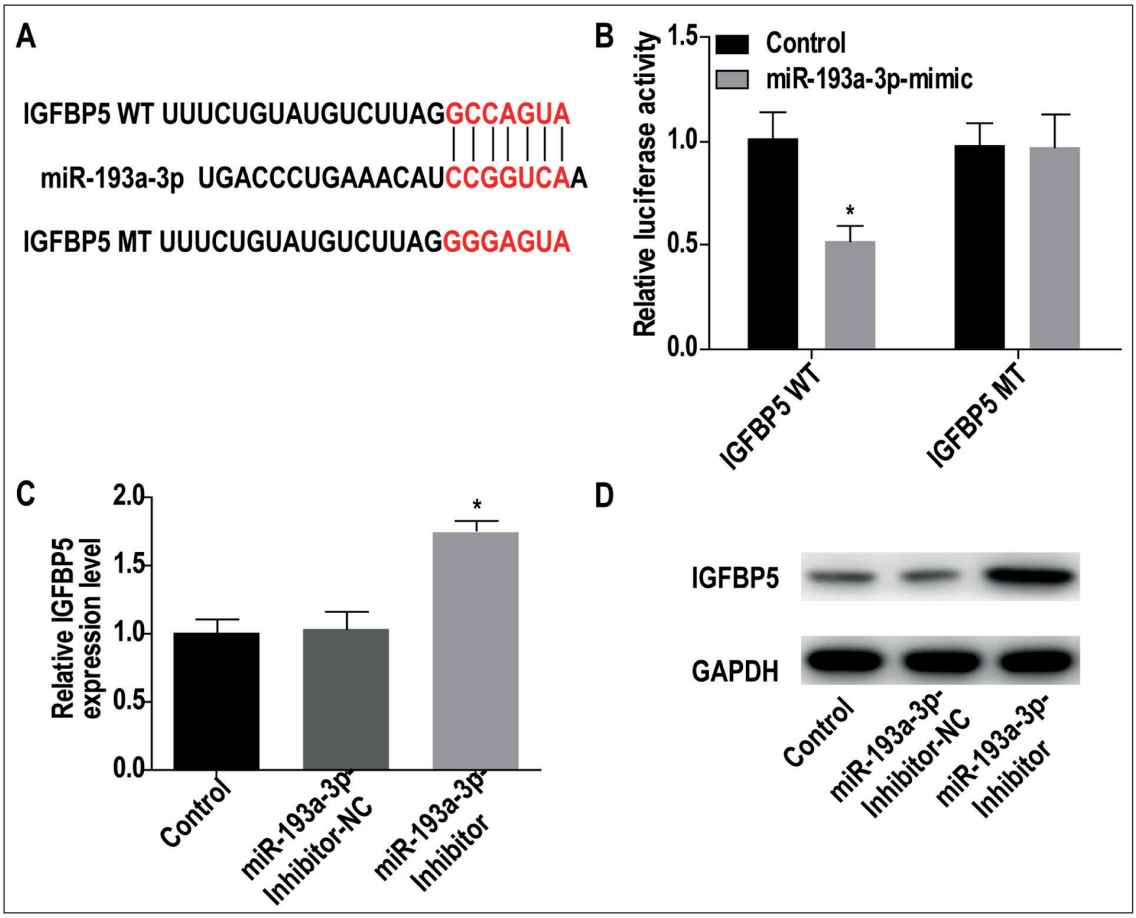

Figure 3. MicroRNA-193a-3p directly regulated IGFBP5. ***A,*** Construction of IGFBP5 wild-type sequence IGFBP5-WT 3'UTR and the mutant-type sequence IGFBP5-MUT 3'UTR. ***B,*** Luciferase activity remarkably decreased in MH7A cells co-transfected with microRNA-193a-3p inhibitor and IGFBP5-WT 3'UTR. No significant change in luciferase activity was found after co-transfection of microRNA-193a-3p inhibitor and IGFBP5-MUT 3'UTR. ***C, D,*** Both mRNA and protein levels of IGFBP5 were upregulated after transfection of microRNA-193a-3p inhibitor.

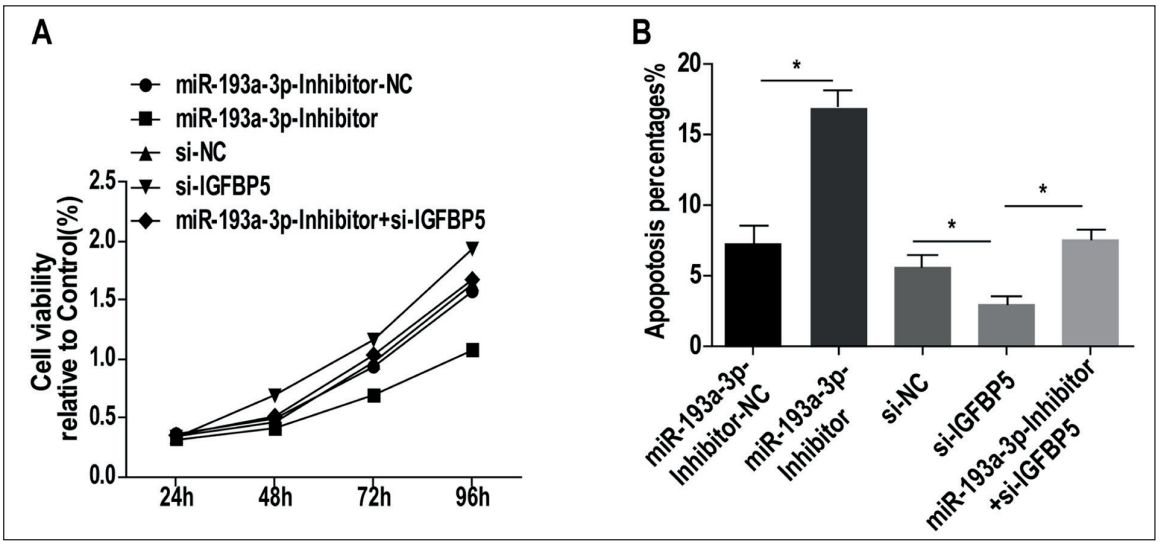

Figure 4. MicroRNA-193a-3p regulated proliferation and apoptosis of MH7A cells by targeting IGFBP5. ***A,*** Knockdown of microRNA-193a-3p and IGFBP5 remarkably reversed the inhibited cell proliferation. ***B,*** Knockdown of microRNA-193a-3p and IGFBP5 remarkably reversed the promoted cell apoptosis.

MicroRNA-193a-3p function has been extensively studied in tumors. It can inhibit tumorigenesis in aldosterone-producing adrenocortical adenomas by downregulating CYP11B2[24]. At the same time, microRNA-193a-3p promotes proliferation and migration of renal cell carcinoma by inhibiting ST3GalIV through the PI3K/AKT pathway[25]. This study found that microRNA-193a-3p was highly expressed in synovial tissues and FLS of RA, suggesting that microRNA-193a-3p was closely related to the occurrence of RA. Knockdown of microRNA-193a-3p inhibited proliferation and inflammation of MH7A cells. Dual-luciferase reporter gene assay verified that IGFBP5 was the target gene of microRNA-193a-3p. Furthermore, rescue experiments confirmed that the regulatory effects of microRNA-193a-3p on RA mainly relied on targeting IGFBP5.

Conclusions

We showed that microRNA-193a-3p is highly expressed in the synovial tissues and cells of rheumatoid arthritis. MicroRNA-193a-3p regulates the proliferation, apoptosis and inflammatory response of MH7A cells by targeting IGFBP5, thereby participating in the process of rheumatoid arthritis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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