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miR-212-3p reduced proliferation, and promoted apoptosis of fibroblast-like synoviocytes via down-regulating SOX5 in rheumatoid arthritis

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Abstract. – OBJECTIVE: Several microRNAs have been reported to contribute the progression of rheumatoid arthritis (RA) due to the ectopic expression of miRNAs in fibroblast-like synoviocytes (FLS). However, the function of miR-212-3p in RA still has not been mentioned before.

PATIENTS AND METHODS: We obtained serum, synovial tissues, and FLS samples from RA patients and normal donors. Quantitative Real-time polymerase chain reaction (qRT-PCR) was used to analysis the expression level of miR-212-3p. By using miR-212-3p mimics and inhibitors, we detected the effects of miR-212-3p on cell proliferation, cell cycle, and apoptosis in RA-FLS. Dual-luciferase and Western-blot were employed to verify the target of miR-212-3p. In addition, we over-expressed the SOX5 in miR-212-3p mimics treatment FLS to emphasize our results.

RESULTS: The level of miR-212-3p in serum, synovial tissues, and FLS from RA patients was lower than these in relative normal group. Up-regulation of miR-212-3p inhibited cell proliferation, promoted cell apoptosis; however, knockdown of miR-212-3p promoted cell growth but reduced cell apoptotic rate. Furthermore, we found SOX5 as a direct target of miR-212-3p in RA-FLS and up-regulation of SOX5 reversed the effects of miR-212-3p over-expression.

CONCLUSIONS: miR-212-3p could reduce cell proliferation and promoted cell apoptosis of RA-FLS via repressing SOX5, which may provide a new biological target for RA treatment.

Key Words:
miR-212-3p, Rheumatoid Arthritis, SOX5, Proliferation, Apoptosis.

Introduction

Rheumatoid arthritis (RA), with high morbidity and morbidity, is a group of autoimmune diseases characterized by chronic, progressive, and aggressive arthritis. Its main pathological features are inflammatory cell infiltration, synovial hyperplasia and bone destruction[1]. According to data of the Centers for Disease Control and Prevention (CDC), the incidence of RA was about 0.5 to 1%, and the prevalence of RA in China was 0.32 to 0.36%[2]. If RA is not treated, it eventually causes damage to the entire joint structure and loss of function, and even death. RA has become one of the major causes of disability and the loss of people in the workforce[3]. Although the exact pathogenesis of RA is not clear, more and more studies confirmed that miRNAs play important roles in the development of RA[4]. MiRNAs, as long as 18-25 nucleotides, are evolutionarily conserved, single-stranded non-coding RNAs[5]. They could silence the expression of specific genes by binding to the unique 3'-untranslated regions (3'-UTR) of target mRNAs on post-transcriptional level[6,7]. At present, several miRNAs have been proved ectopic expressed in RA and to participate in the development and process of RA[8,9]. For instance, Filková et al[10] found serum miR-16 and miR-223 were correlated with RA development. Kawano et al[11] and Nakamachi et al[12] reported miR-124a controlled cell growth and MCP-1 release of synoviocytes from RA patients. Also, Kurows-

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ka-Stolarska et al[13] demonstrated miR-155 was a regulator of proinflammatory in clinical and experimental arthritis. In addition, Stanczyk et al[14,15] confirmed miR-203 altered expressed in RA-FLS and regulated its activation. More and more miRNAs were identified to participate in the development and progress of RA. MiR-212-3p located at 17p13.3, was proved to regulate several progression of different diseases. It could inhibit cell proliferation of glioblastoma via SGK3 and control cell migration and invasion of hepatic carcinoma via YAP1[16]. What's more, miR-212 inhibited renal cell cancer proliferation and invasion by repressing FOXA1 while functioned as a tumor suppressor in non-small cell lung cancer (NSCLC) through SOX4[17-19]. Several miRNA expression analysis studies pointed out that miR-212-3p ectopic expressed in synovial tissues RA patient, but the specific mechanism of miR-212-3p in RA was still unclear[20]. Here, we analyzed for the first time the expression of miR-212-3p in samples of serum, synovial tissues, and FLS from RA patients and normal donors. Next, we measured cell proliferation, cell cycle and cell apoptosis abilities in miR-212-3p up- or down-regulated RA/FLS by some functional experiments. Also, we demonstrated SOX5 as a potential target of miR-212-3p in RA and verified the expression of SOX5 in serum, synovial tissues, and FLS of RA samples. Our study might provide a potential target for RA biotherapy.

Patients and Methods

Samples of Serum, Synovial Tissues, and Fibroblast-Like Synoviocytes

Samples of serum and synovial tissues were collected from 20 RA patients who underwent joint replacement at the Qilu Hospital of Shangdong University. Twelve healthy donors were recruited as normal groups (Normal). Written informed consents from all patients in this study have been collected. This tudy was approved by the Ethical Committee of Qilu Hospital of Shangdong University and complied with the recommendations of the Declaration of Helsinki. By using 2.5 g/L trypsin, RA-FLS were acquired from synovial tissue samples of three RA patients and maintained in Dulbecco's Modified Eagle's medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). The cells were cultured in moist air containing 5% CO_2 at 37°C.

RNA Isolation and qRT-PCR

Total RNA of serum, synovial tissues FLS and was draw using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reversed using a miRNA Reverse Kit (TaKaRa, Dalian, China). The miR-212-3p expression level was measured by using SYBR Premix Ex Taq kits (TaKaRa, Dalian, China) with ABI Prism 7500 HT (ABI, Waltham, MA, USA) and U6 was used as internal control. The expression of SOX5 was detected using SYBR Kits (TaKaRa, Dalian, China) and GAPDH was used as control. All the expression levels were measured by the $2^{-\Delta\Delta}$CT method relatively. Each experiment was confirmed at least three times.

Mimics and Inhibitors Transfection and pcDNA Treatment

The mimics and inhibitors for miR-212-3p and pcDNA for SOX5 were provided by Genewiz (Suchou, Jiangsu, China). Cells were put into six-well plate and culture to density of 50-70%, then incubated with miR-212-3p mimics (or negative control), miR-212-3p inhibitors (or inhibitors negative control) or pcDNA-SOX5 mixed with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in FBS-free DMEM for 48 h following to the instructions. Next, these cells were maintained in normal condition and the efficiency of transfection was confirmed by qRT-PCR.

Cell Counting Kit-8 (CCK8) Assay

CCK8 (Dojindo Laboratories, Kumamoto, Japan) assay was applied to detect the proliferation activity of cells. The cells seeded in a density of 1×10^3 cells with 100 μL medium were cultured after transfection. 10 μL CCK8 reagent were added into the wells at the time point of 0 h, 24 h, 48 h, 72 h and the absorbance of 450 nm was recorded. Each experiment was confirmed at least three times.

Cell Cycle and Apoptosis Analysis By Flow Cytometry

Flow cytometry was obtained to measure the cell apoptotic rate and cell cycle. For apoptosis, a fluorescein isothiocyanate (FITC) and propidium iodide (PI) kit (Vazyme, Nanjing, China) was employed. Cells were harvested after miR-212-3p mimics or inhibitors treatment and washed with pre-cooling phosphate-buffered saline (PBS). Cells were suspended in 1000 μL of binding buffer mixing 10 μL of FITC and PI, respectively. Next, cell apoptotic rate was measured by flow cytometry (FACS, New York, NY, USA), and the

percentage of apoptotic cells was counted. For cell cycle, cells after harvest were resuspended in 500 μL of binding buffer mixing 5 μL PI (Vazyme, Nanjing, China) and cell cycle distributions were recorded by flow cytometry. Each measurement was repeated three times.

Dual-Luciferase Assay

The luciferase activity of binding was tested by using the reporter system of Dual-Luciferase (Promega, Madison, WI, USA). The SOX5 3'-UTR region containing the wild type or mutant miR-212-3p binding region was duplicated and cloned into pGL3 luciferase vector (Promega, Madison, WI, USA). Then, treated cells were co-transfected with the established vector and miR-212-3p mimics or NC using lipofectamine 2000. The activity of luciferase was determined using luminometer (Promega, Madison, WI, USA) and measured. Each measurement was repeated for three times.

Protein Extraction and Western-Blot

To measure SOX5 protein expression level, total protein of cells were extracted by using RIPA (Beyotime, Shanghai, China) after washed with pre-cooling PBS. The protein was next separated by using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with Bio-Rad electrophoresis system (Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) with electrophoresis transfer system. Non-specific protein interactions on the PVDF membranes were blocked by immersing the membranes in 5% skim milk dissolved in Tris-buffered saline-Tween (TBST) buffer at room temperature for 2 h. The membranes were incubated at 4°C overnight within the primary antibody (1:1000) against SOX5 or GAPDH as internal control (Abcam, Cambridge, MA, USA) dissolved in Tris-buffered saline-tween (TBST) buffer. The membranes were next incubated with horseradish-peroxide (HRP)-conjugated secondary antibody for 1 h after washing with TBST buffer for 10 min × 3 times at room temperature. At last, the bands were detected by using ECL Detection Kit (Millipore, Billerica, MA, USA) following the instructions.

Statistical Analysis

The one-way analysis of variance and *t*-test of independent samples (SPSS, IBM, Armonk, NY, USA) were employed to measure quantitative data. All these data in the study were presented with SPSS 19.0 version (SPSS Inc., Armonk, NY, USA) and GraphPad prism 5.0 version software (Version X; La Jolla, CA, USA). p-value < 0.05 was considered having significant difference. Data was shown as mean ± SD.

Results

Level of miR-212-3p was Downregulated in Samples of RA Patients' Serum, Synovial Tissue and FLS

To evaluate the miR-212-3p expression in RA patients, we collected 20 samples of RA patients' serum and synovial tissue compared with 10 normal samples. After detection by qRT-PCR, we found the expression level of miR-212-3p was markedly lower in RA serum and RA synovial tissue compared to each normal group, respectively (Figure 1A, 1B). Also, the expression level of miR-212-3p in RA-derived FLS was decreased comparing with negative normal control (Figure 1C). These data indicated miR-212-3p could participate in RA progression.

MiR-212-3p Influenced Cell Growth of RA-FLS

To study the influence of miR-212-3p in RA-FLS, we regulated its expression level by using miR-212-3p mimics and inhibitors. The level of miR-212-3p was improved in miR-212-3p mimics (Mimics) treatment group compared to the negative control (NC) group, while decreased in miR-212-3p inhibitors (inhibitors) group compared with inhibitors negative control (INC) group (Figure 2A, 2B). CCK8 assay was employed to detect the cell proliferation ability. Clearly shown in Figure 2C, 2D, cell growth was significantly reduced in miR-212-3p mimics group but enhanced in miR-212-3p inhibitors group compared with relative negative control group. These results indicated miR-212-3p could inhibit cell proliferation in RA-FLS.

Ectopic Expression of miR-212-3p Effected Cell Cycle and Apoptosis of RA-FLS

Next, to evaluate the functions of miR-212-3p on cell cycle and apoptosis, we detect the cell cycle distribution and apoptotic rate using flow cytometry. The percentage of G0/G1 phase was higher but S phase lower in miR-212-3p over-expressing group compared to NC group (Figure

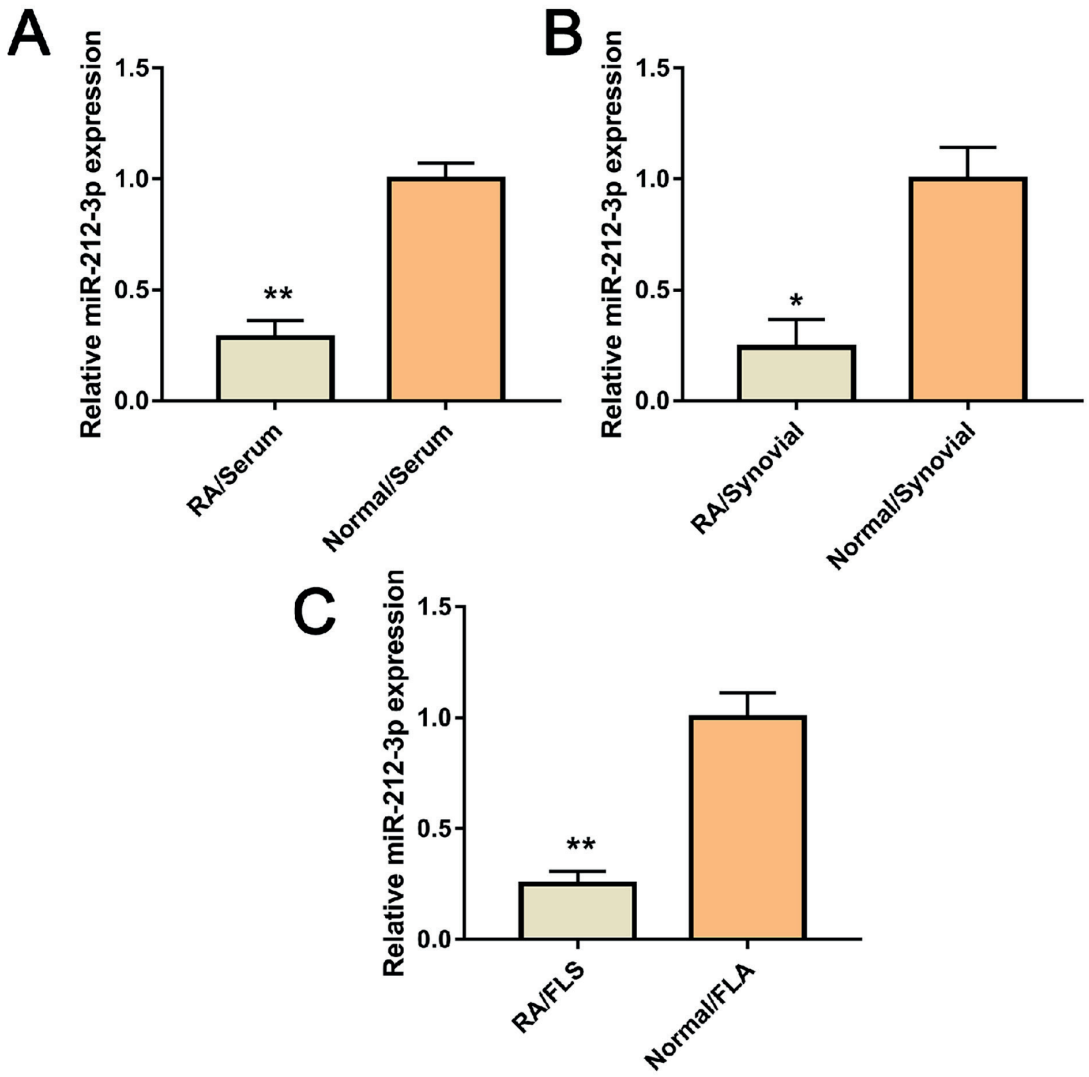

Figure 1. miR-121-3p expression was downregulated in the serum, synovial tissue, and fibroblast-like synoviocytes of rheumatoid arthritis patients. ***A,-C,*** The expression of miR-121-3p was determined in serum, synovial tissue, and FLS of RA patients and normal control (NC) by qRT-PCR. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3A). However, in miR-212-3p knocking-down group, a lower G0/G1 phase and higher S phase distribution was found compared to INC group (Figure 3B). Furthermore, the apoptotic rate was remarkably increased in miR-212-3p mimics transfection group but decreased in miR-212-3p inhibitors treatment when comparing to negative group, relatively (Figure 3C, 3D, 3E). These data suggested miR-212-3p could regulate cell cycle and apoptosis in FA-FLS.

SOX5 Acted as a Direct Target of miR-212-3p

To investigate underlying mechanism of miR-212-3p in RA, we searched TargetScan Database (http://www.targetscan.org/) and found SOX5 as a potential target of miR-212-3p. The binding region of miR-212-3p of SOX5 3'-UTR was shown in Figure 4A. Next, we conducted dual-luciferase assay to verify the assumption by using wild type or mutant binding site of SOX5. miR-212-3p decreased the luciferase activity of wild type SOX5 3'-UTR reporter plasmid group but had no difference in the mutant group (Figure 4B). In addition, the protein level of SOX5 was detected using Western-blot. Results showed remarkably decreased SOX5 expression in miR-212-3p mimics group. However, SOX5 was significantly higher in miR-212-3p inhibitors group than

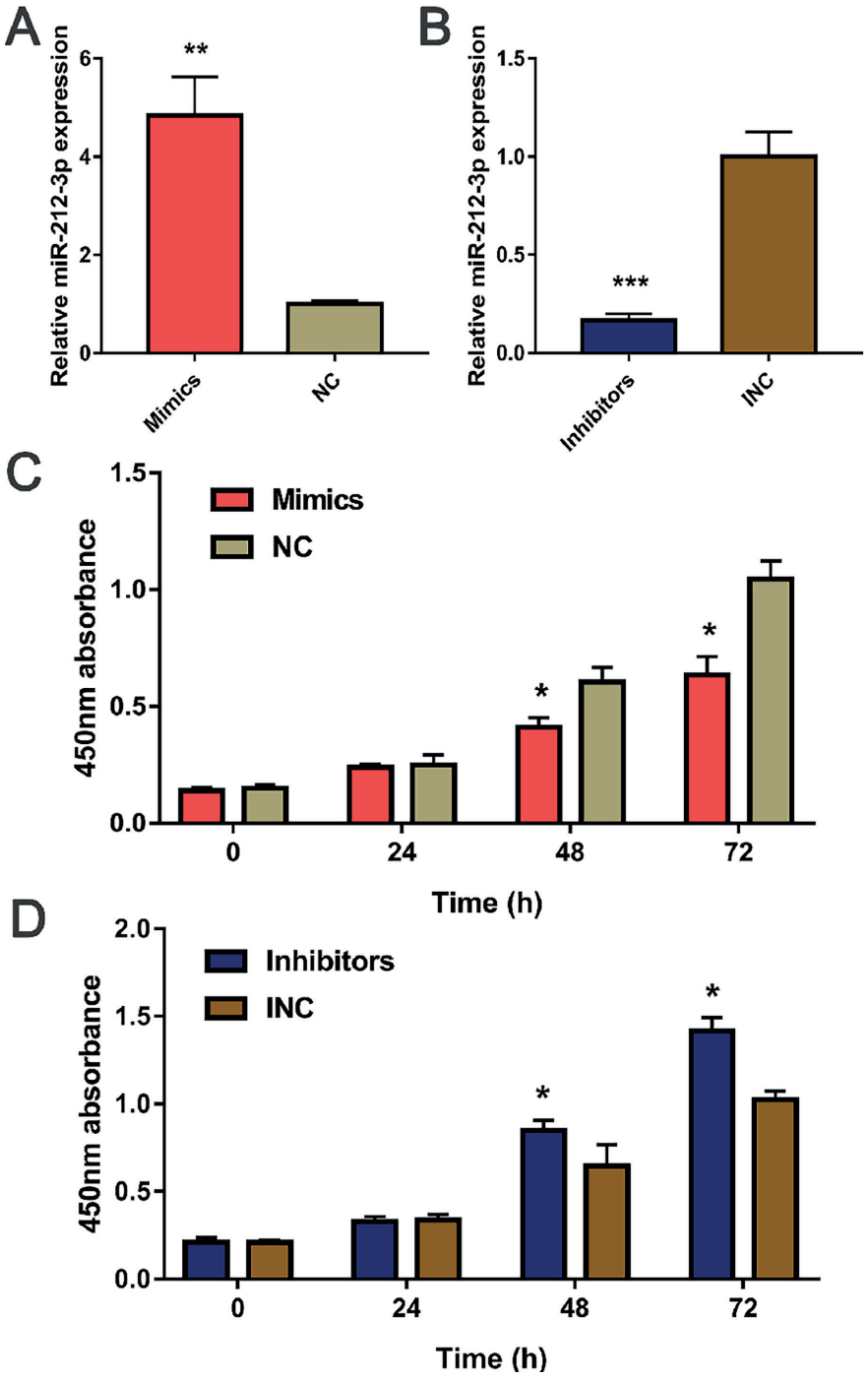

Figure 2. Ectopic miR-121-3p expression effected cell proliferation of RA-FLS. ***A,*** The expression of miR-121-3p was detected by qRT-PCR in RA-FLS after transfection of miR-121-3p mimics or NC. ***B,*** The expression of miR-121-3p was detected by qRT-PCR in RA-FLS after transfection of miR-121-3p inhibitors or inhibitors NC (INC). ***C,-D,*** RA-FLS proliferation was evaluated using CCK-8 assays after transfection of miR-121-3p mimics ***(C)*** or inhibitors ***(D)*** compared to NC or INC group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

that in control group (Figure 4C, 4D, 4E). All these data indicated SOX5 was a direct target of miR-212-3p.

Level of SOX5 was Upregulated and Negatively Correlated With miR-212-3p Expression in RA Synovial Tissues

In addition, we measured the expression of SOX5 mRNA level in the RA serum, synovial tissue, and FLS of RA patients compared with normal groups using qRT-PCR. Expression level of SOX5 was significantly improved in RA patients' group compared with normal controls (Figure 5A, 5B, 5C). Also, we next analyzed the relationship between miR-212-3p and SOX5 in 20 synovial tissue samples of RA patients and found the level of miR-212-3p was negatively correlated with the mRNA level of SOX5 (Figure

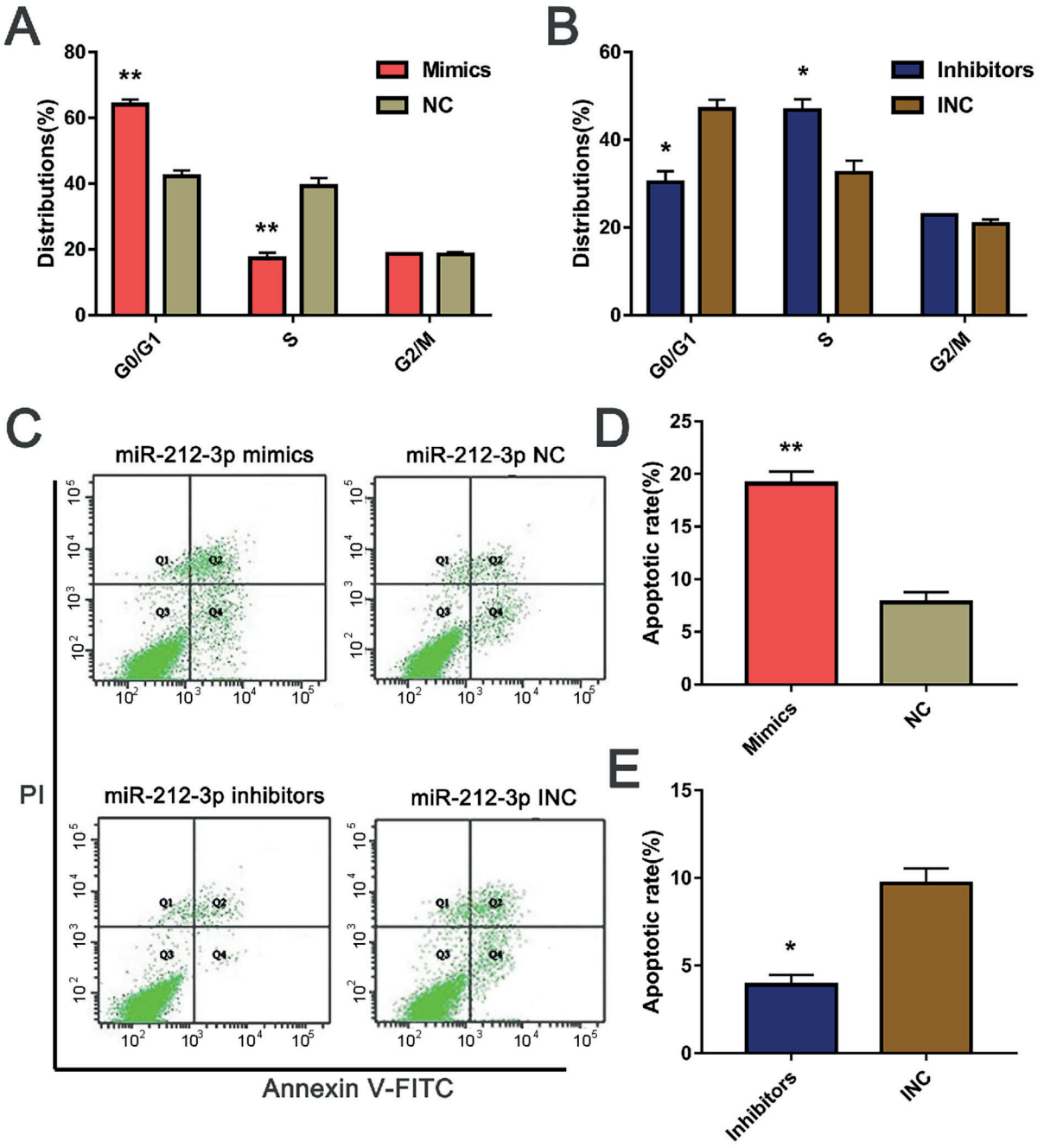

Figure 3. miR-121-3p induced cell cycle and apoptosis of RA-FLS. (A, B) RA-FLS cycle was measured using flow cytometry after transfection of miR-121-3p mimics ***(A)*** or inhibitors ***(B)*** compared to NC or INC group. ***(C, D, E)*** Apoptosis of RA-FLS was evaluated using flow cytometry after transfection of miR-121-3p mimics ***(C, D)*** or inhibitors ***(C, E)*** compared to NC or INC group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

5D). These results proved SOX5 as a target of miR-212-3p in RA.

Over-expression of SOX5 Reversed the Influences of miR-212-3p in RA-FLS

Furthermore, to verify SOX5 was functional molecular target of miR-212-3p, we next over-expressed SOX5 in miR-212-3p mimics treatment RA-FLS using plasmids. The expression level of SOX5 protein was confirmed using Western-blot and SOX5 expression was restored (Figure 6A-B). Also, CCK8 showed the cell proliferation activity was rescued by SOX5 plasmids (Figure 6C). The effects on cell

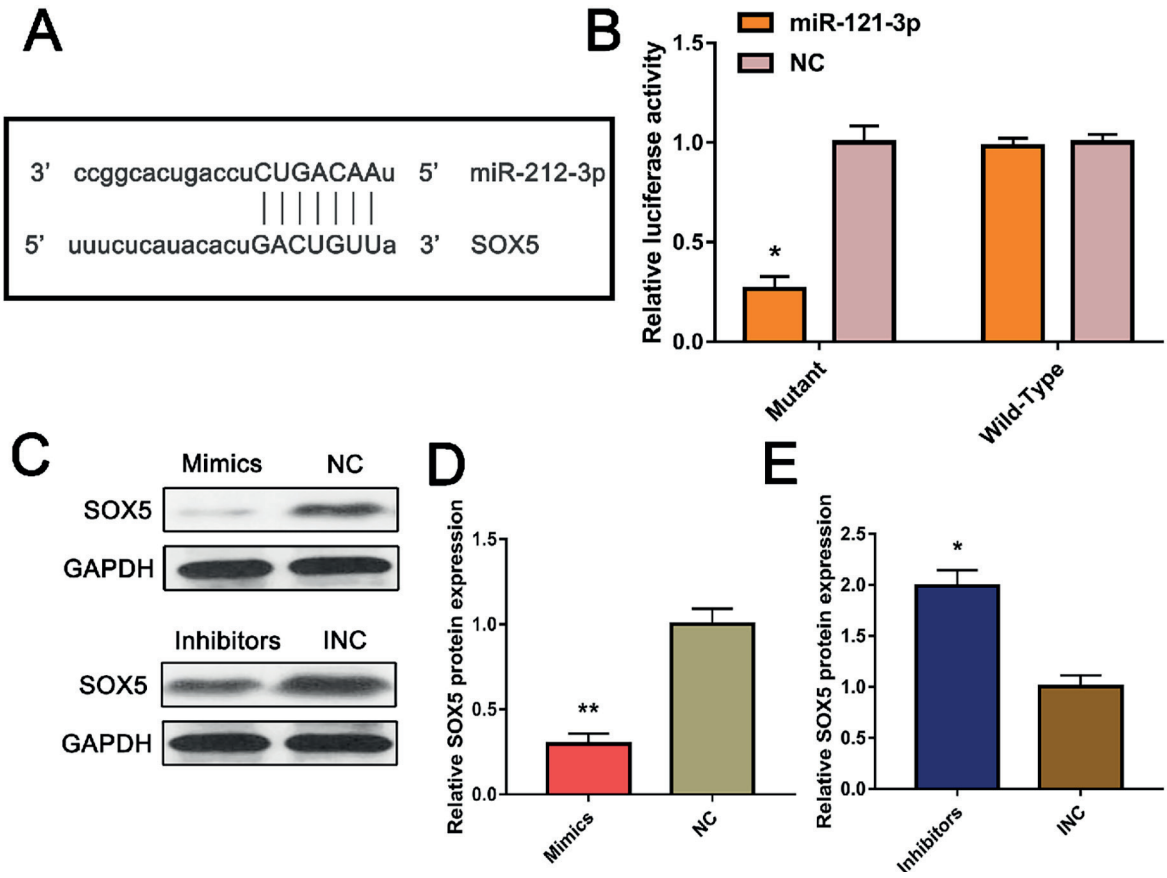

Figure 4. miR-212-3p directly targeted SOX5 in RA-FLS. ***A,*** TargetScan predicted that SOX5 was a target of miR-29a. ***B,*** Luciferase activity was determined in RA-FLS co-transfected with miR-121-3p mimics/NC and SOX5 3′-UTR Wild type/Mutant reporter plasmid. ***C-E,*** SOX5 protein expression was determined by Western-blot in RA-FLS cells transfected with miR-29a mimics ***(C-D)*** or miR-inhibitors ***(C-E)*** compared to NC or INC group. GAPDH was used as an internal control. *$p < 0.05$, **$p < 0.01$.

cycle and apoptosis of miR-212-3p mimics were also partially reversed by SOX5 over-expression (Figure 6D, 6E, 6F). These results verified miR-212-3p could regulate RA-FLS functions via SOX5.

Discussion

Rheumatoid arthritis (RA) has become one of the most common connective tissue diseases. Its main pathological changes are thickening of synovium, infiltration of a large number of inflammatory cells, severe edema of the interstitium, severe destruction of articular cartilage and bone, and angiogenesis. RA has become one of the major causes of disability and the loss of people in the workforce[3,21]. In recent years, the role of miRNAs in the process of rheumatoid arthritis has drawn great attention[9]. Several studies have identified expressions of miRNAs in synovial fibroblasts and synovial tissues in RA were ectopic regulated[8]. Hong et al[22] reported miR-143 and miR-145 could modulate the phenotype of synovial fibroblasts in RA; Philippe et al[23] found miR-19 could regulate TKR2 and inhibit the RA-FLS proliferation. Also, Alsaleh et al[24] demonstrated miR-30a-3p was a negative regulator of BAFF synthesis in RA. In addition, Shi et al[25] found miR-27a functioned as factor to inhibit RA-FLS migration and invasion via repressing follistatin-like protein 1 in RA. However, more studies are still needed to elucidate the specific mechanism of miRNAs in RA. In our study, we first demonstrated miR-212-3p was down-regulated in the serum, synovial tissues and RA-FLS compared to normal groups, which indicated

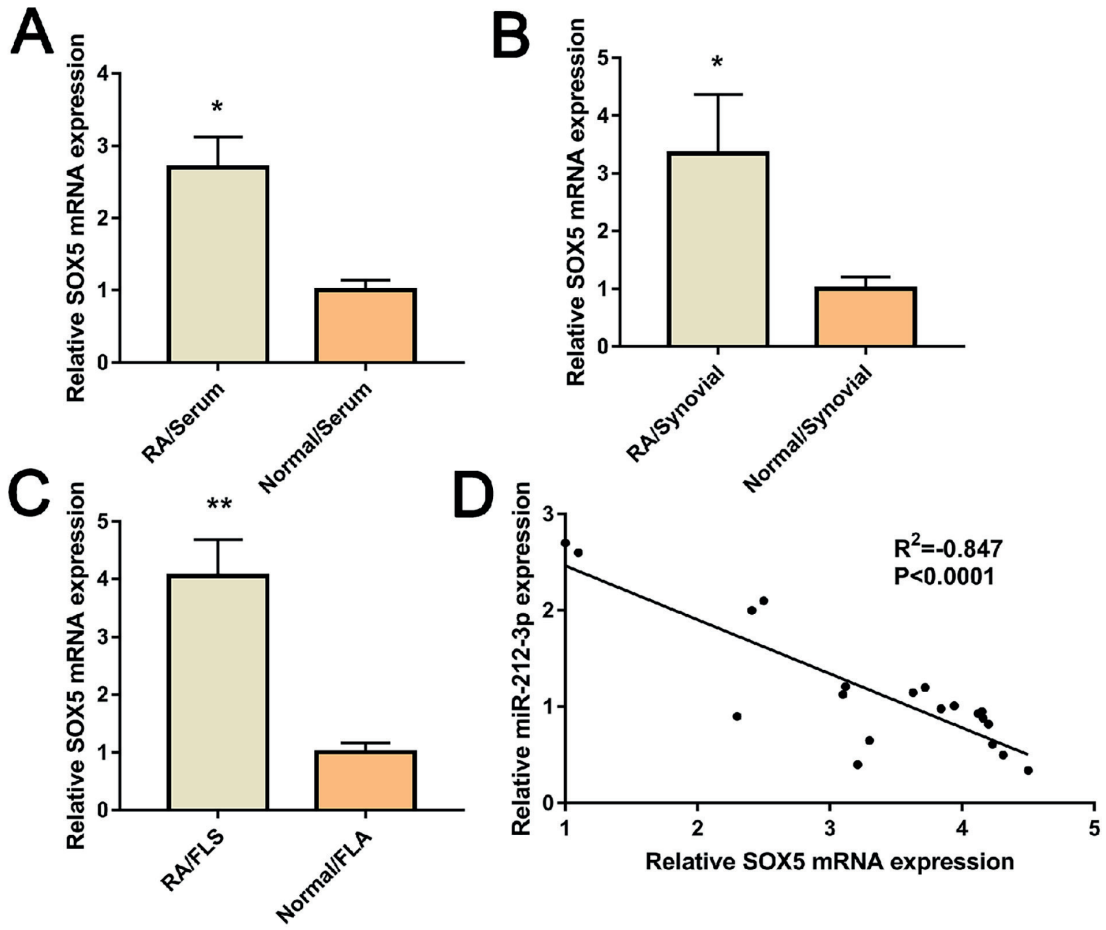

Figure 5. SOX5 expression was upregulated and inversely correlated with miR-121-3p expression. ***A, B, C,*** SOX5 mRNA level was determined in the serum, synovial tissue, and FLS of RA patients and normal control by qRT-PCR. ***D,*** The inverse relationship between SOX5and miR-121-3p expression was determined by Spearman's correlation in synovial tissues (n = 20). *$p < 0.05$, **$p < 0.01$.

miR-212-3p might act a regulatory factor in RA progression. Next, we conducted loss- and gain- of functional experiments to evaluate the effects of miR-212-3p in RA-FLS. The ability of cell proliferation was significantly reduced by miR-212-3p over-expression but increased by miR-212-3p knockdown, and cell cycle was arrested in G0/G1 phase by miR-212-3p up-regulation. Furthermore, cell apoptotic rate was decreased while miR-212-3p inhibitors treatment. These indicated that miR-212-3p acted as a target for RA management. Furthermore, we found SOX5 as a potential target of miR-212-3p based on the database TargetScan. SOX5 (sex determining region Y-box protein 5), a member of huge sox family, was a regulator of determination of cell fate and embryonic development[26]. It can cooperate with the other SOX family member including SOX9, SOX6 to diver chondrogenesis though super enhancers. Also, in some cancers, SOX5 could promote the tumorigenesis and progression[27]. Such as SOX5 promoted cell invasion, migration and epithelial-mesenchymal transition via Twist1 in hepatocellular cancer[28]. What's more, SOX5 could act as a target gene of miR-132, miR-15a and miR-16 to regulate cell proliferation, invasion and migration of pituitary tumor[29]. SOX5 also found to be linked to RA. Wang et al[30] reported SOX5 together with CCR3 and LC3 were correlated with elderly onset rheumatoid arthritis and showed a clinical significance. Several studies[31,32] found SOX5 was a regulatory factor in the modulation of RANKL expression induced by IL-6 in RA synovial tissue and that RANKL polymorphism was relative to

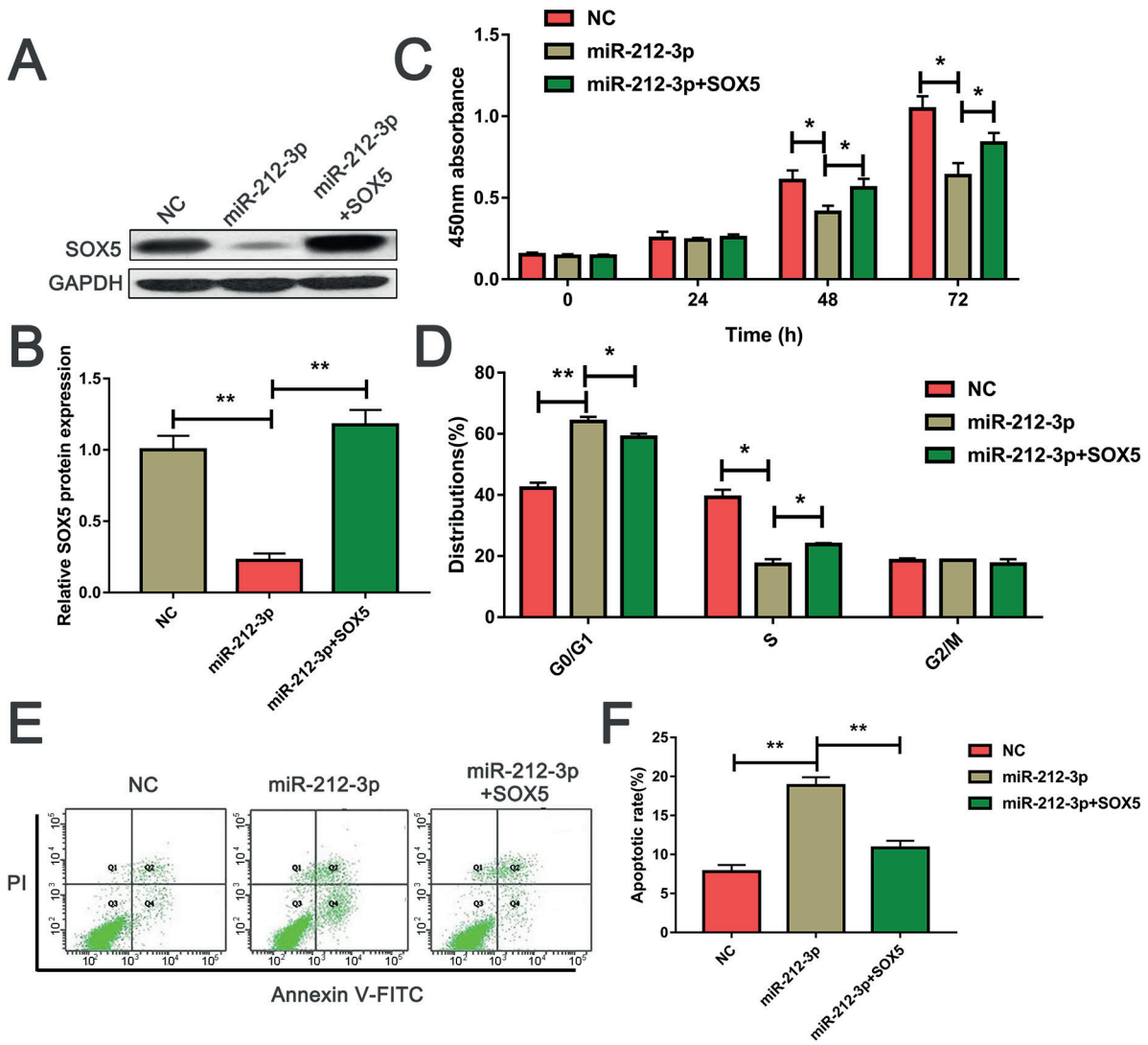

Figure 6. SOX5 over-expression rescued the miR-29a-mediated effect on RA-FLS. ***A-B,*** SOX5 protein expression was measured by Western blot in RA-FLS transfected with miR-121-3p mimics/NC and with/without SOX5 overexpression plasmid. GAPDH was used as an internal control. ***C, D, E, F,*** Cell proliferation (***C***), cell cycle ***(D)***, and apoptosis ***(E-F),*** were determined in RA-FLS transfected with miR-121-3p with/without SOX5 overexpression plasmid. $^{*}p < 0.05$, $^{**}p < 0.01$.

younger age at the beginning of RA. Here, we demonstrated SOX5 as a direct target of miR-212-3p in RA-FLS. SOX5 protein level was decreased when miR-212-3p level up-regulated. In addition, the mRNA level of SOX5 in RA synovial tissues was negative correlated with miR-212-3p expression. Furthermore, up-regulation of SOX5 could reverse the influences of miR-212-3p over-expression in RA-FLS, which emphasized miR-212-3p effected RA-FLS via a functional target SOX5.

Conclusions

We for the first time demonstrated that the expression level of miR-212-3p was reduced in RA patients' serum, synovial tissue and RA-FLS. Also, miR-212-3p could inhibit cell proliferation and promote cell apoptosis via repressing SOX5 in RA-FLS. Although more investigations, including *in vivo* assays, are essential to provide more evidence, this study indicates that miR-212-3p could act as a novel target for RA biotherapy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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