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Original Article

miR-135a-5p mediated down-regulation of STAT6 inhibits proliferation and induces apoptosis of fibroblast-like synoviocytes in rheumatoid arthritis

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Abstract: Objective: Rheumatoid arthritis (RA), as a chronic autoimmune disorder, seriously threatens human health. However, no study has thoroughly illustrated the etiology of RA. The present work focused on investigating the biological functions of STAT6 and the upstream miRNAs that regulate its expression. Methods: Synovial tissues from rheumatoid arthritis (RA) patients and normal participants were acquired. Cell viability, proliferation, apoptosis, concentrations of cytokines, miRNA and protein levels, and relative luciferase activities were detected. Results: WB and qRT-PCR showed that STAT6 was obviously up-regulated in synovial tissues of RA patients as well as RA fibroblast-like synoviocytes (RA FLSs). Functionally, down-regulation of STAT6 significantly inhibited the growth of RA FLSs as indicated by EdU and CCK-8 assays. In addition, inhibition of STAT6 remarkably promoted apoptosis of RA FLSs. Besides, silence of STAT6 notably suppressed inflammatory cytokine levels, such as TNF-α, IL-6 and IL-1β. Mechanistically, STAT6 was predicted to be the direct target of and negatively regulated by miR-135a-5p. Moreover, STAT6 was involved in the regulation of miR-135a-5p on cell growth, apoptosis and inflammatory response of RA FLSs. Conclusion: miR-135a-5p/STAT6 is a potential novel therapeutic target for RA treatment.

Keywords: Fibroblast-like synoviocytes, signal transducers and activators of transcription 6, microrna-135a-5p, rheumatoid arthritis, proliferation, apoptosis

Introduction

Rheumatoid arthritis (RA), a chronic autoimmune disorder, involves abnormal proliferation and thickening of synovial tissues and synovial inflammation, often accompanied by destruction of cartilage and osteogenesis [1]. According to statistics, the incidence of RA is about 0.5%-1% in adults, which is still increasing annually [2]. At present, the treatment of RA is limited to drug therapy, such as methotrexate and some biological agents [3]. However, no study has thoroughly illustrated the etiology of RA [4-6]. Therefore, it is a critical research direction to study RA treatment.

During the development of RA, the synovium is a place where inflammatory and immune cells gather. The inner layer of the synovium gradually proliferates, thickens, and then turns into pannus tissues, finally destroying articular cartilage [7, 8]. In RA, fibroblast-like synoviocytes (FLSs) accumulate in the proliferative synovial tissues, showing tumor cell like invasiveness and have a destructive ability [9-11]. The synovial tissue continues to proliferate and eventually reaches the articular cartilage, leading to bone and articular cartilage degeneration [12-14]. Apart from invasive and destructive abilities, FLSs also participate in the inflammatory response by directly generating some proinflammatory factors or indirectly recruiting other inflammatory cells [15]. Thus, FLSs are considered to be an important factor of RA. Therefore, inhibiting the abnormal proliferation of FLSs can be a potential treatment for RA.

The Signal Transducer and Activator of Transcription (STAT) transcription factor family has been reported to possess important functions

in diverse cell processes, like proliferation, apoptosis, differentiation, inflammation and immune response [16-18]. STAT6, an important member of the STAT family, plays a vital role in the regulation of cytokine generation and cell differentiation [17]. More importantly, STAT6 is found to be over-expressed in synovial tissues from RA patients [18]. Nonetheless, it remains unclear about the role of STAT6 in RA FLSs and its possible mechanism. In recent years, as the important regulatory genes for growth and development in organisms, microRNAs (miRNAs) have been shown to exert critical functions in disease genesis and progression [19, 20]. MiRNAs are noncoding RNAs with a length of about 22 nucleotides (nt) long. MiRNAs induce target mRNA degradation or translation inhibition, inhibit normal protein levels within cells, and regulate post-transcriptional gene levels, thus participating in the occurrence and progression of diseases through binding with the 3'-UTR of target genes [21, 22]. With the further research, more and more miRNAs are found to have abnormal expression in RA, and also participate in the regulation of biological behavior of RA [23].

In this study, we investigated the biological functions of STAT6 and the upstream miRNAs in FLSs, with the hope of promoting research in regard to the pathogenesis of RA.

Materials and methods

Tissue specimens

Synovial tissues were obtained from RA patients and normal participants from the Department of Orthopedics in our hospital. All experimental protocols were approved by the Ethics Committee (2019-K058). Each participant provided written informed consent for participation.

Cell culture and transfection

Human RA FLSs and human normal FLSs were provided by American Type Culture Collection (ATCC; Manassas, VA, USA), cultivated in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Gibico, NY, USA) and incubated in a humid incubator at 37°C and 5% CO_2.

Genechem (Shanghai, China) constructed the specific siRNAs against STAT6 (5'-GGACUCUCGGAUUGUAAGAUU-3') and the corresponding siNC (5'-GGACUCUCGGAUUG UAAGAUU-3'), the STAT6-targeting pcDNA3.1 vector together with the related empty vector (Nucleotide sequences of were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/guide/)). In addition, GenePharma (Shanghai, China) constructed the miR-135a-5p mimic (5'-UAUGGC UUUUUAUUCCUAUGUGA-3') and the NC mimic (5'-UACUGAGAGACAUAAGUUGGU C-3'). Each of the above plasmids was individually transfected into cells via Lipotransfectamine 3000 (Thermo Fisher Scientific) according to the instructions.

CCK-8 assay

To detect the cell viability, we inoculated the transfected RA FLSs (1×10^4/well) into the 96-well plates for 0, 24, 48 and 72 h, respectively. Thereafter, CCK-8 kit (Beyotime Biotechnology, Shanghai, China) was utilized to detect cell viability according to specific instructions. Microplate reader (Tecan Infinite M200 Micro Plate Reader; LabX, Switzerland) was employed to detect absorbance (OD) value at 490 nm.

EdU assay

Proliferation of RA FLSs after transfection was evaluated through EdU assay. In brief, the transfected RA FLSs (4×10^4/well) were inoculated into the 24-well plates for 48 h. Afterwards, 4% paraformaldehyde was used for fixation and nuclear membrane permeabilization was done with Triton X-100, goat serum was used to block RA FLSs for 1 h. Furthermore, RA FLSs were stained in line with specific protocols.

TUNEL assay

Apoptosis of RA FLS after transfection was detected by TUNEL assay (Beyotime Biotechnology, China). Briefly, after dewaxing and 15 min of permeabilization using proteinase K under ambient temperature, 3% H_2O_2 was used to treat RA FLS slices for blocking the endogenous peroxidases; later, terminal deoxynucleotidyl transferase and equilibration buffer were utilized to incubate the slices. At last, the conjugate of antidigoxigenin-peroxidase was used to incubate the slices, and then the slices were developed by DAB and observed under the light microscope (200 ×).

Flow cytometric analysis

Annexin V-FITC kit (Beyotime Biotechnology, Shanghai, China) was utilized to measure the

Table 1. Primer sequences

Gene name	Primer sequences
STAT6	F: 5'-CATTTGGTACAACGTGTCAACCA-3'
	R: 5'-TGTGGCAGGTG GAGGATTATTA-3'
Caspase-3	F: 5'-CAGTGGAGGCCGACTTCTTG-3'
	R: 5'-ATGAACCAGGAGCCATCCTTT-3'
Caspase-9	F: 5'-CGAACTAACAGGCAAGCA-3'
	R: 5'-CAAATCCTCCAGAACCAAT-3'
Bax	F: 5'-GGATGCGTCCACCAAGAA-3'
	R: 5'-ACTCCCGGCCACAAAGATG-3'
Bcl-2	F: 5'-TTCTTTGAGTTCGGTGGGGTC-3'
	R: 5'-TGCATATTTGTTTGGGGCAGG-3'
TNF-α	F: 5'-CGGGACGTGGAGCTGGCCGAGGAG-3'
	R: 5'-CACCAGCTGGTTATCTCTCAGCTC-3'
IL-1β	F: 5'-CAGTGAAATGATGGCTTATTAC-3'
	R: 5'-CTTTCAACACGCAGGACAGGT-3'
IL-6	F: 5'-GGCCCTTGCTTTCTCTTCG-3'
	R: 5'-ATAATAAAGTTTTGATTATGT-3'
miR-135a-3p	F: 5'-TTGGTCTTGTTTCCCGGTCC-3'
	R: 5'-TCACAGCTCCACAGGCTAAC-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3'
	R: 5'-GTACTCAGCGCCAGCATCG-3'
β-actin	F: 5'-AGCCACATCGCTCAGACACC-3'
	R: 5'-CGGCCACATTGTAACTTTG-3'

apoptosis of RA FLSs after transfection. Briefly, at 48 h after culture, the transfected RA FLSs were collected after digestion by trypsin and rinsed with PBS, followed by resuspension within 500 μL binding buffer. Thereafter, Annexin V-FITC (5 μL) together with PI (10 μL) was utilized to treat RA FLSs in the dark for 15 min. At last, flow cytometer (BD Biosciences, USA) was utilized to determine apoptotic cells.

Enzyme-linked immunosorbent assay (ELISA)

Cytokine TNF-α, IL-6 and IL-1β levels in RA FLSs were analyzed through ELISA kits (NanJing JianCheng Bioengineering Institute, Nanjing, China) in line with the specific protocols.

qRT-PCR analysis

TRIzol reagents (Beyotime Biotechnology, Shanghai, China) were utilized to extract total RNA from RA FLSs which were then reverse-transcribed into cDNA by TaqMan one-step reverse transcription (Applied Biosystems, USA). The ABI Prism 7500 system (Applied Biosystems, USA) was used for qRT-PCR following specific the protocols. We measured the relative mRNA levels of STAT6, caspase-3, caspase-9, Bcl-2, Bax, TNF-α, IL-6, IL-1β, and miR-135a-3p by $2^{-\Delta\Delta Ct}$ method. U6 and β-actin served as the endogenous references. Primers used in this study are shown in **Table 1**. The reactions were performed in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min.

Western blot analysis

RIPA lysis buffer was utilized to extract total protein from RA FLSs. Later, the BCA kit (Beyotime Biotechnology, Shanghai, China) was utilized to quantify protein content. After separation with 10% SDS-PAGE, proteins were transferred onto PVDF membranes (Millipore, Bedford, MA, USA). Afterwards, 5% skim milk with TBST was used to block the membranes for 1 h under ambient temperature, followed by overnight incubation using primary antibodies under 4°C. After being rinsed with TBST three times, membranes were further incubated using HRP-labeled secondary antibody (1: 2000, ab6728, abcam, MA, USA) under ambient temperature for 1 h. Finally, the enhanced chemiluminescence kit (ECL, Millipore, Bedford, MA, USA) was used for visualizing the protein blots, whereas Image J software (NIH, version 4.3) was employed for quantification. The primary antibodies (1:1000, abcam, MA, USA) included anti-STAT6 (ab32108), anti-caspase-3 (ab13847), anti-Cleaved caspase-3 (ab32042), anti-caspase-9 (ab32539), anti-Cleaved caspase-9 (ab2324), anti-Bax (ab32503), anti-Bcl-2 (ab32124) and anti-β-actin (ab8226).

Dual-luciferase reporter assay

Targetscan was used to predict the up-stream regulatory gene of STAT6, and miR-135a-3p was proved to regulate STAT6. STAT6 WT/MUT was sub-cloned into the pmirGLO dual-luciferase vector (Promega) to generate pmirGLO-STAT6 WT/MUT, which was later transfect into cells respectively, and then co-incubated with NC mimics or miR-135a-3p mimics. After co-transfection for 48 h, the relative luciferase activity was measured utilizing the dual-luciferase reporter assay system (Promega).

Statistical methods

Data were analyzed using GraphPad Prism 5.0 and presented in a form of mean ± SD. Differences between groups were compared by

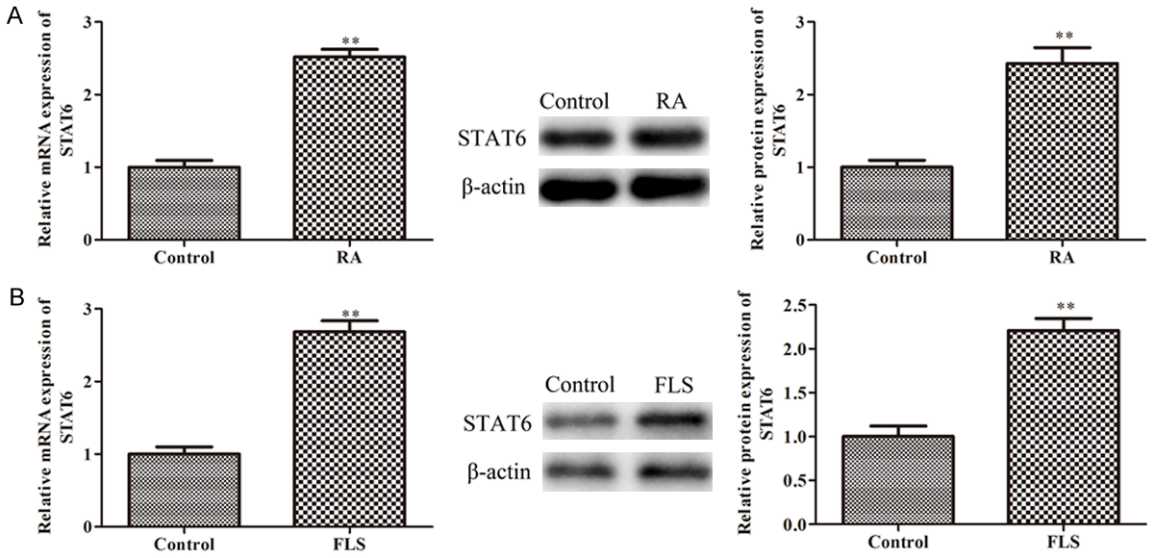

Figure 1. STAT6 is up-regulated in RA tissues and RA FLSs. A: The mRNA and protein expressions of STAT6 in clinical synovial tissues were assessed by qRT-PCR and western blot assays; B: The mRNA and protein expressions of STAT6 in RA FLSs were assessed by qRT-PCR and western blot assays. Compared with control group, $^{**}P < 0.01$. All data were presented as mean ± SD, n = 3. RA FLSs: RA fibroblast-like synoviocytes.

one-way ANOVA and Turkey's poc host test. $P < 0.05$ indicated statistical significance.

Results

STAT6 was over-expressed in RA tissues and RA FLSs

To explore the possible role of STAT6 in RA, a total of 30 clinical synovial tissues were collected. WB and qRT-PCR were conducted for evaluating STAT6 expression in RA tissues. **Figure 1A** illustrates that STAT6 was obviously over-expressed in RA tissues ($P < 0.01$). In addition, STAT6 expression in RA FLSs was further investigated by WB and qRT-PCR analyses. Expectedly, STAT6 was highly expressed in RA FLSs (**Figure 1B**; $P < 0.01$).

Down-regulation of STAT6 inhibited proliferation and induced apoptosis of RA FLSs

Proliferation of RA FLSs plays a vital role in RA [24]. Therefore, we first conducted CCK-8 assay to evaluate the effect of STAT6 on proliferation of RA FLSs. As shown in **Figure 2A**, down-regulation of STAT6 inhibited the viability of RA FLSs in a time-dependent manner. In addition, we conducted EdU assay to evaluate the effect of si-STAT6 on the growth of RA FLSs. As shown in **Figure 2B**, si-STAT6 remarkably down-regulated EdU-positive cell proportion in RA FLSs. Besides, TUNEL and flow cytometry were utilized to evaluate the effect of si-STAT6 on apoptosis of RA FLSs, and the results showed that the number of apoptotic cells was markedly elevated following si-STAT6 transfection (**Figure 2C** and **2D**). In addition, qRT-PCR and WB assays were conducted to detect the mRNAs and proteins levels of apoptosis related molecules. As shown in **Figure 2E** and **2F**, STAT6 down-regulation remarkably suppressed Bcl-2 expression but promoted Cleaved caspase-3/9 and Bax expression.

Down-regulation of STAT6 inhibited the inflammatory response of RA FLSs

ELISA and qRT-PCR was conducted to assess the effect of si-STAT6 on inflammatory response in RA FLSs, and the results showed that TNF-α, IL-6 and IL-1β levels were obviously decreased in RA FLSs after transfection with si-STAT6 (**Figure 3A** and **3B**).

miR-135a-5p was predicted to be an upstream regulator of STAT6

TargetScan was adopted to explore the candidate miRNAs that regulated STAT6, and miR-135a-5p was predicted to have a putative target sites to bind with STAT6 3'-UTR (**Figure 4A**).

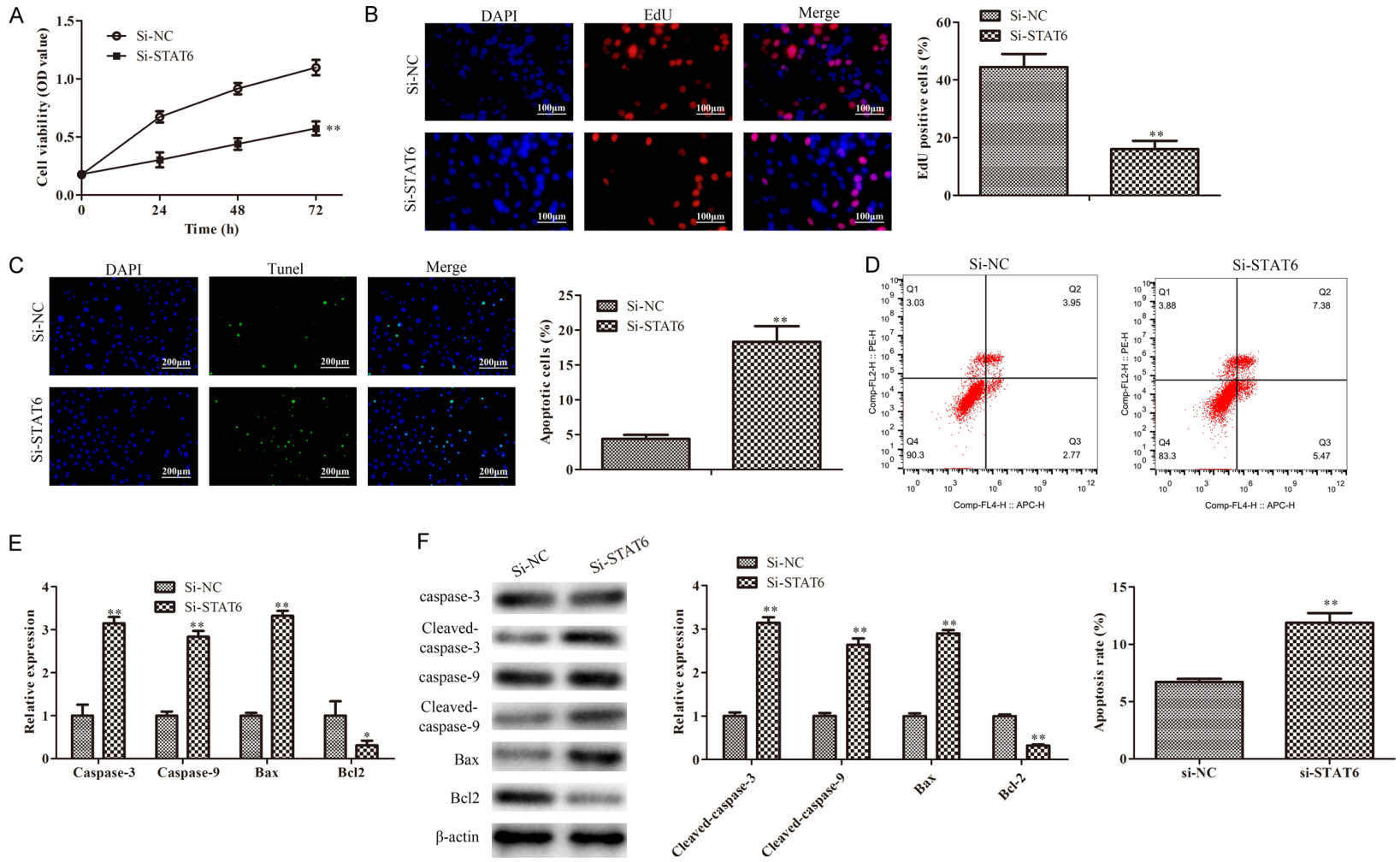

Figure 2. Down-regulation of STAT6 inhibits proliferation and induced apoptosis of RA FLSs. (A) The viability of RA FLSs transfected with si-STAT6 was assessed by CCK-8 assay at indicated times; (B) The proliferation of RA FLSs transfected with si-STAT6 was evaluated by EdU assay (400 ×); The apoptotic rate of RA FLSs transfected with si-STAT6 was determined by (C) TUNEL (400 ×) and (D) flow cytometry assays. The expression levels of mRNAs and proteins related to apoptosis in RA FLSs transfected with si-STAT6 were evaluated by (E) qRT-PCR and (F) western blot assays. Compared with si-NC group, $^{*}P < 0.05$, $^{**}P < 0.01$. All data were presented as mean ± SD, n = 3. STAT6: signal transducers and activators of transcription 6; FLSs: fibroblast-like synoviocytes.

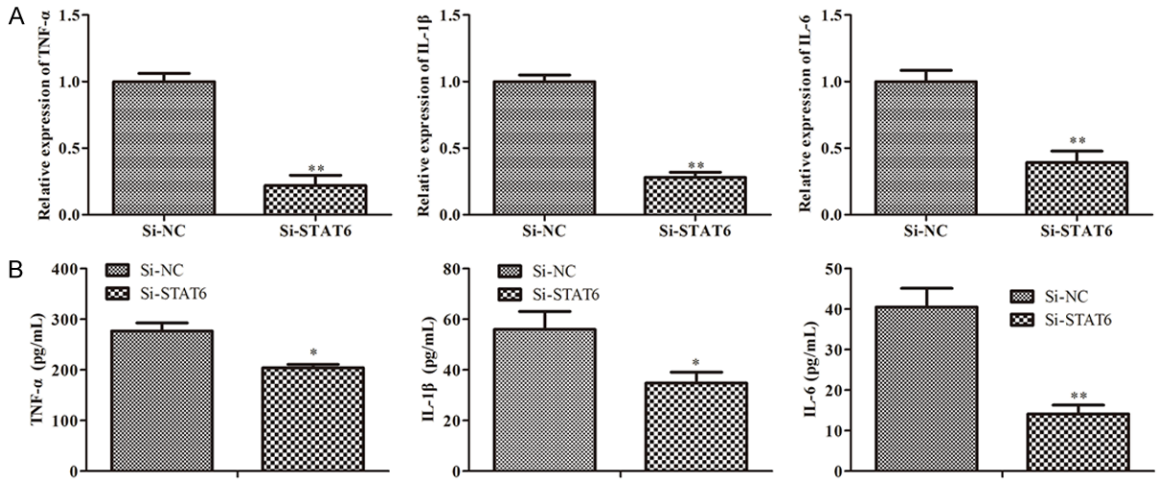

Figure 3. Down-regulation of STAT6 inhibits inflammatory response of RA FLSs. The expressions of TNF-α, IL-1β and IL-6 in RA FLSs transfected with si-STAT6 was determined by (A) qRT-PCR and (B) ELISA assays. Compared with si-NC group, $^{*}P < 0.05$, $^{**}P < 0.01$. All data were presented as mean ± SD, n = 3. STAT6: signal transducers and activators of transcription 6; FLSs: fibroblast-like synoviocytes.

Then, qRT-PCR was conducted to evaluate the miR-135a-5p expression in RA tissues and RA FLSs. As shown in **Figure 4B** and **4C**, miR-135a-5p was remarkably down-regulated in RA tissues and FLSs (all $P < 0.01$). For better confirming the targeting association of miR-135a-5p with STAT6, miR-135a-5p mimics were transfected into RA FLSs. As shown in **Figure 4D**, miR-135a-5p was highly expressed in miR-135a-5p-transfected RA FLSs. Then, luciferase assay further confirmed that STAT6 was the direct target of miR-135a-5p (**Figure 4E**). Moreover, WB and qRT-PCR assays were conducted for evaluating STAT6 expression in RA FLSs under NC mimics or miR-135a-5p mimic transfection. As revealed by **Figure 4F**, the protein and mRNA expression of STAT6 was notably suppressed in RA FLSs under miR-135a-5p mimic transfection.

STAT6 could reverse the effect of miR-135a-5p on proliferation, apoptosis and inflammatory response of RA FLSs

Given that miR-135a-5p negatively regulated STAT6, various experiments were carried out for exploring the role of miR-135a-5p in RA FLSs through targeting STAT6. Firstly, NC mimics or miR-135a-5p mimics and pcDNA NC or pcDNA STAT6 were transfected into RA FLSs. As expected, miR-135a-5p over-expression reduced the STAT6 protein level, while pcDNA STAT6 co-transfection restored STAT6 protein expression (**Figure 5A**). According to CCK-8 and EdU assays, miR-135a-5p mimics notably suppressed viability and proliferation of RA FLSs (**Figure 5B** and **5C**). In addition, up-regulation of miR-135a-5p obviously promoted apoptosis of RA FLSs as indicated by TUNEL and flow cytometric analyses (**Figure 5D** and **5E**). Additionally, qRT-PCR assay illustrated that miR-135a-5p mimics promoted mRNA levels of Bax, caspase-3 and caspase-9, but reduced mRNA level of Bcl-2 (**Figure 5F**). In addition, WB showed that miR-135a-5p over-expression suppressed Bcl-2 protein expression but promoted protein expression of Cleaved caspase-3/9 and Bax (**Figure 5G**).

Furthermore, over-expression of STAT6 partly restored the effects of miR-135a-5p mimics on the proliferation, apoptosis and inflammatory response of RA FLSs.

Discussion

In our study, STAT6 was obviously over-expressed in clinical synovial tissues and RA FLSs. Down-regulation of STAT6 notably suppressed the viability and proliferation, promoted the apoptosis, and reduced the expression levels of inflammatory factors in RA FLSs. These data were consistent with previous studies about STAT families' effect on RA. For instance, STAT1 is obviously up-regulated in RA relative to osteoarthritis (OA) and reactive arthritis.

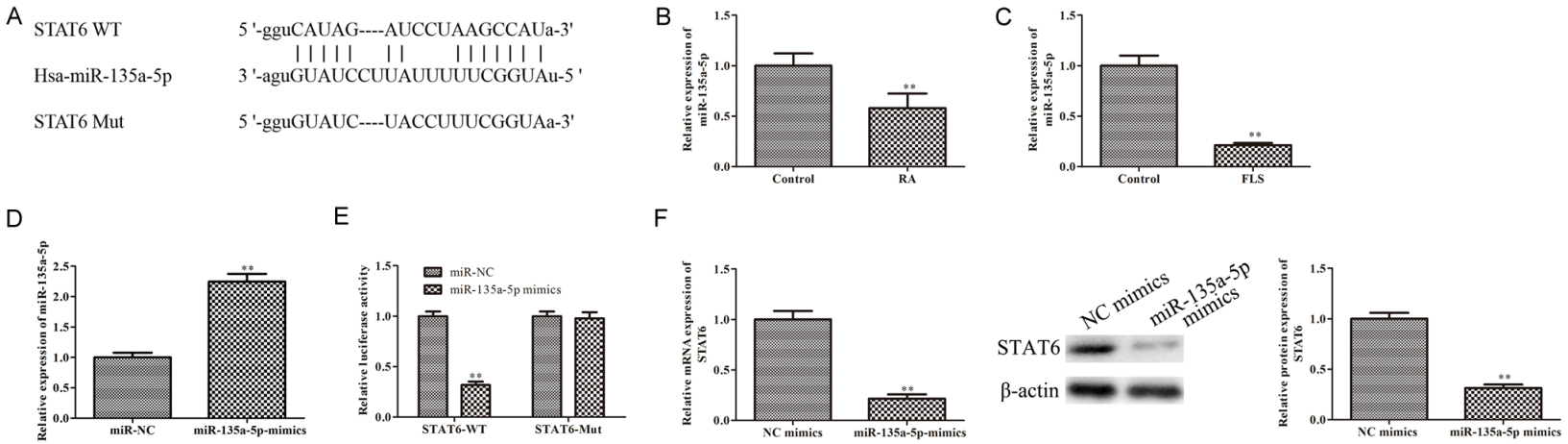

Figure 4. STAT6 is direct target of miR-135a-5p. A: Binding sites between miR-135a-5p and STAT6; B: qRT-PCR assay was performed to evaluate the miR-135a-5p expression in RA tissues; C: qRT-PCR assay was performed to evaluate the miR-135a-5p expression in RA FLSs; $^{**}P < 0.01$. D: qRT-PCR assay was performed to evaluate the miR-135a-5p expression in RA FLSs after transfection with miR-135a-5p mimics; E: Dual-luciferase reporter analysis was employed to validate the coactions between miR-135a-5p and STAT6; F: qRT-PCR and western blot assays were performed to evaluate the STAT6 levels in RA FLSs after transfection with miR-135a-5p mimics. Compared with NC mimics group, $^{*}P < 0.05$, $^{**}P < 0.01$. All data were presented as mean ± SD, n = 3. STAT6: signal transducers and activators of transcription 6.

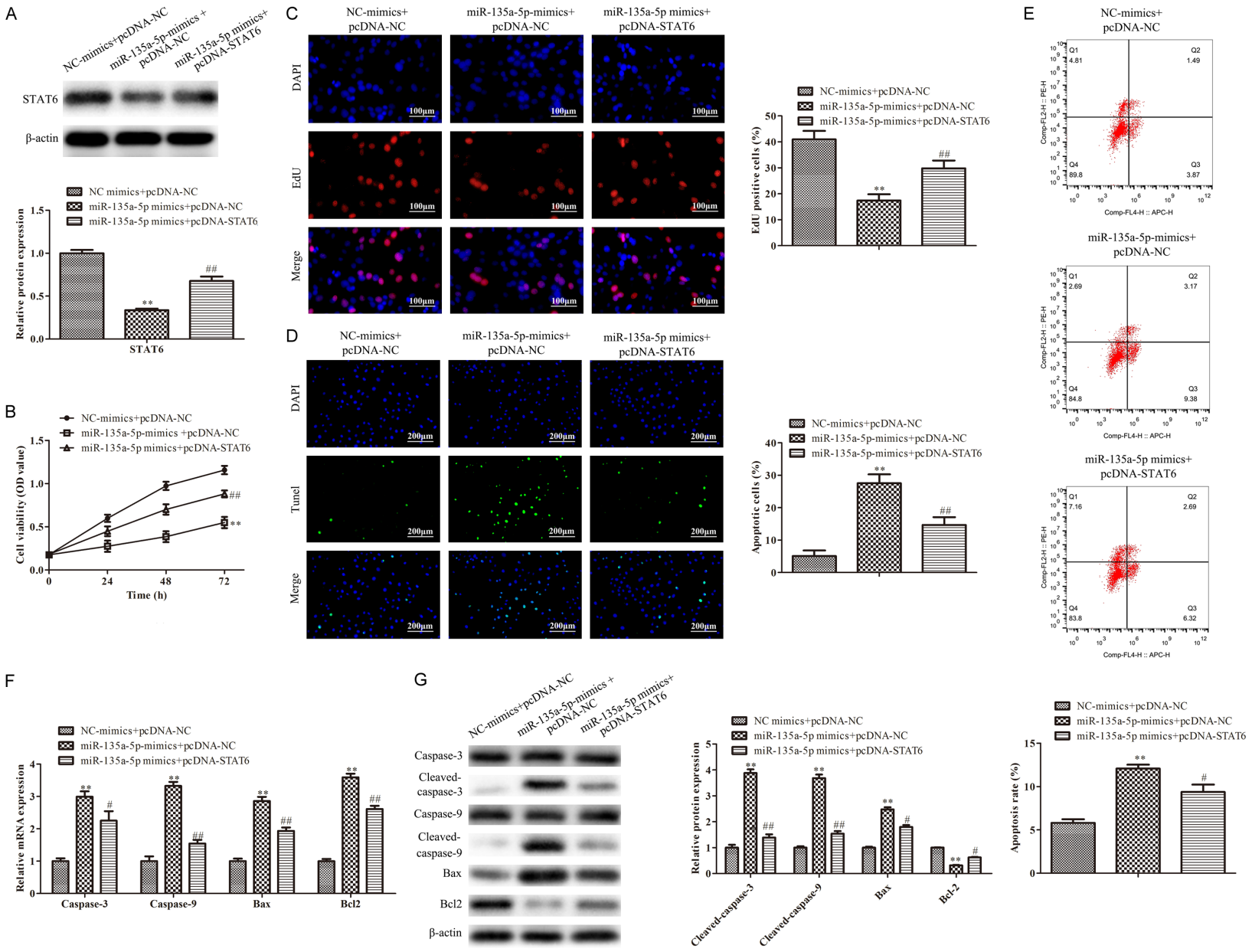
A
NC-mimics+pcDNA-NC
miR-135a-5p-mimics + pcDNA-NC
miR-135a-5p mimics+ pcDNA-STAT6
STAT6
β-actin
NC mimics+pcDNA-NC
miR-135a-5p mimics+pcDNA-NC
miR-135a-5p mimics+pcDNA-STAT6
Relative protein expression
STAT6
B
NC-mimics+pcDNA-NC
miR-135a-5p-mimics +pcDNA-NC
miR-135a-5p mimics+pcDNA-STAT6
Cell viability (OD value)
Time (h)
C
NC-mimics+ pcDNA-NC
miR-135a-5p-mimics+ pcDNA-NC
miR-135a-5p mimics+ pcDNA-STAT6
DAPI
EdU
Merge
100μm
NC-mimics+pcDNA-NC
miR-135a-5p-mimics+pcDNA-NC
miR-135a-5p-mimics+pcDNA-STAT6
EdU positive cells (%)
D
NC-mimics+ pcDNA-NC
miR-135a-5p-mimics+ pcDNA-NC
miR-135a-5p mimics+ pcDNA-STAT6
DAPI
Tunel
Merge
200μm
NC-mimics+pcDNA-NC
miR-135a-5p-mimics+pcDNA-NC
miR-135a-5p-mimics+pcDNA-STAT6
Apoptotic cells (%)
E
NC-mimics+ pcDNA-NC
Q1 4.81
Q2 1.49
Q4 89.8
Q3 3.87
miR-135a-5p-mimics+ pcDNA-NC
Q1 2.69
Q2 3.17
Q4 84.8
Q3 9.38
miR-135a-5p mimics+ pcDNA-STAT6
Q1 7.16
Q2 2.69
Q4 83.8
Q3 6.32
Comp-FL2-H :: PE-H
Comp-FL4-H :: APC-H
F
NC-mimics+pcDNA-NC
miR-135a-5p-mimics+pcDNA-NC
miR-135a-5p mimics+pcDNA-STAT6
Relative mRNA expression
Caspase-3
Caspase-9
Bax
Bcl2
G
NC-mimics+pcDNA-NC
miR-135a-5p-mimics + pcDNA-NC
miR-135a-5p mimics+ pcDNA-STAT6
Caspase-3
Cleaved-caspase-3
Caspase-9
Cleaved-caspase-9
Bax
Bcl2
β-actin
NC mimics+pcDNA-NC
miR-135a-5p mimics+pcDNA-NC
miR-135a-5p mimics+pcDNA-STAT6
Relative protein expression
Cleaved-caspase-3
Cleaved-caspase-9
Bax
Bcl-2
NC-mimics+pcDNA-NC
miR-135a-5p-mimics+pcDNA-NC
miR-135a-5p mimics+pcDNA-STAT6
Apoptosis rate (%)

Figure 5. STAT6 mediates the functional effects of miR-135a-5p on proliferation and apoptosis in RA FLSs. (A) The expression of STAT6 in RA FLSs transfected with miR-135a-5p mimics or negative control and pcDNA NC or pcDNA STAT6 were detected by western blot assay; (B) The viability of RA FLSs after transfection was assessed by CCK-8 assay at indicated times; (C) The proliferation of RA FLSs after transfection was evaluated by EdU assay (400 ×). The apoptotic rate of RA FLSs after transfection was determined by (D) TUNEL (400 ×) and (E) Flow cytometry assays. The expression levels of mRNAs and proteins related to apoptosis in RA FLSs after transfection were evaluated by (F) qRT-PCR and (G) Western blot assays. Compared with NC mimics + pcDNA-NC group, $^{**}P < 0.01$; Compared with miR-135a-5p mimics + pcDNA-NC group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. All data were presented as mean ± SD, n = 3. STAT6: signal transducers and activators of transcription 6; FLSs: fibroblast-like synoviocytes.

STAT1 promotes synovitis occurrence by inducing the expression of inflammatory genes [25]. Besides, STAT3 is continuously activated in the arthritis model induced by yeast polysaccharide, which leads to the persistence of chronic inflammatory response [26, 27]. Additionally, IL-12 exerts a pathogenic effect on upregulating adhesion molecules and inflammatory cytokine receptors, stimulating IFN-γ secretion, and accelerating the immune imbalance between Th1 and Th2 in RA patients, which is transmitted by STAT4 [28]. Moreover, Finnegan *et al*. reported that the severity of arthritis induced by proteoglycan was significantly regulated by IL-4 through STAT-dependent mechanisms [29]. These data suggest that STAT might exhibit an essential role in the occurrence of RA.

Although the role of STAT6 has been well explored, the STAT6 upstream regulators remained greatly unclear. MiRNAs have important functions in regulating translational or post-transcriptional gene expression [30]. Until now, just a few studies have examined miRNAs that regulate STAT6 [31-33]. The present work searched and validated miRNAs regulating STAT6 according to bioinformatic analysis. As a result, miR-135a-5p had negative regulation on STAT6 expression in RA FLSs. Human miR-135 family (miR-135s) is a group of miRNAs with complex functions [34, 35]. miR-135a-5p, the specific miRNA with high expression in bud stage during tooth development in humans, is also expressed in mesenchymal cells and the epithelium [36]. At the same time, miR-135a-5p can regulate allergic inflammatory responses [37, 38]. Interestingly, low miR-135a level is detected in RA synovial tissues. miR-135a upregulation suppresses the growth, invasion and migration of synovial fibroblasts (SFs), but promotes the apoptosis of RA through increasing PIK3R2 expression and inactivating the PI3K/AKT signal transduction pathway [39]. However, the detailed role and relationship of miR-135a-5p and STAT6 as well as the possible mechanism in RA remains unknown. In this study, miR-135a-5p over-expression notably inhibited the viability, expression of inflammatory factors and enhanced the apoptosis of RA FLSs. Moreover, those mechanisms by which miR-135a-5p regulated STAT6 were further explored through rescue experiment. As expected, over-expression of STAT6 partly restored the impact of miR-135a-5p mimics on growth, apoptosis and inflammatory responses of RA FLSs.

Inevitably, there are still some limitations in our research. First of all, more detailed clinical experimental data and related *in vivo* animal experiments are still needed, which would be our next stage of work. Second, the mechanisms of miR-135a-5p/STAT6 regulating the occurrence and development of RA is still largely unclear, which need further exploration.

To sum up, STAT6 is markedly increased in synovial tissues of RA patients, and RA FLS. Down-regulation of STAT6 can remarkably suppress the growth and inflammatory responses but enhance the apoptosis of RA FLSs. Moreover, STAT6 mediated the effect of miR-135a-5p on the growth, apoptosis and inflammatory response of RA FLSs. Taken together, miR-135a-5p/STAT6 is a candidate therapeutic target for RA treatment (**Figure 6**).

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Disclosure of conflict of interest

None.

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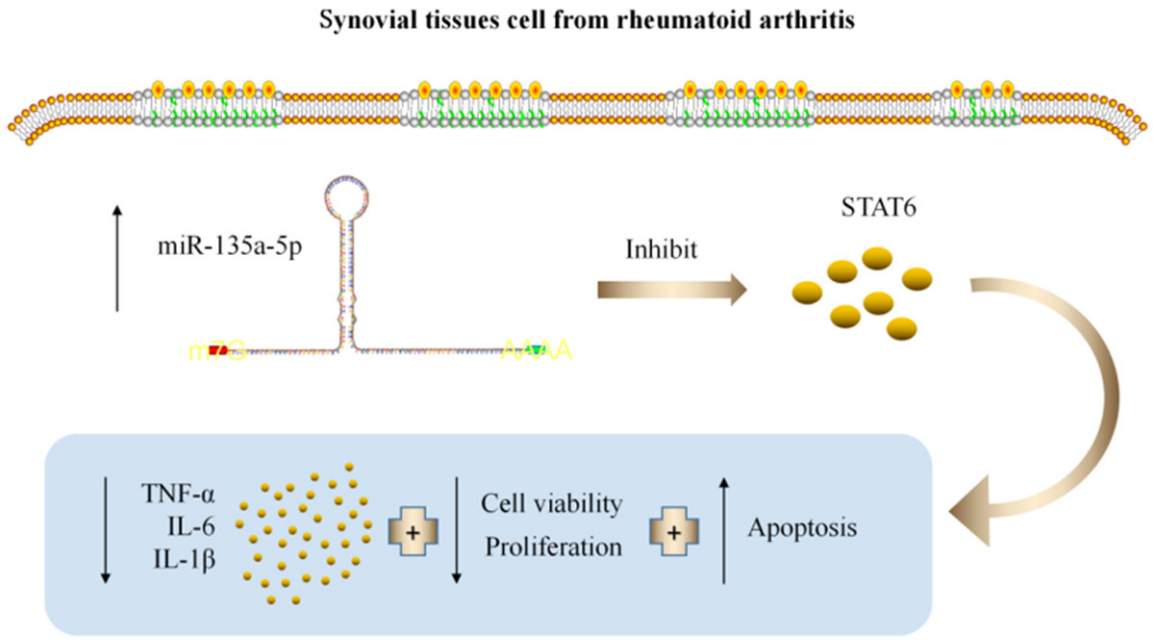

Figure 6. The mechanism of miR-133a-5p in rheumatoid arthritis.

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