

MiR-30e-5p deficiency exerts an inhibitory effect on inflammation in rheumatoid arthritis via regulating AtI2 expression

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ABSTRACT

Objectives: This study aims to investigate the inflammatory effect of the microRNA (miRNA) miR-30e-5p on rheumatoid arthritis (RA) development in RA mice and fibroblast-like synoviocytes (FLS).

Materials and methods: MiR-30e-5p and atlastin GTPase 2 (AtI2) expression in RA tissues and RA-FLS was evaluated using real-time quantitative polymerase chain reaction. The function of miR-30e-5p in inflammation of RA mice and RA-FLS was analyzed by enzyme-linked immunosorbent assay (ELISA) and Western blotting. 5-ethynyl-2'-deoxyuridine (EdU) assay was used to detect RA-FLS proliferation. Luciferase reporter assay was used to confirm the interaction between miR-30e-5p and AtI2.

Results: MiR-30e-5p expression was upregulated in the tissues from RA mice. Silencing miR-30e-5p alleviated inflammation in RA mice and RA-FLS. MiR-30e-5p negatively modulated AtI2 expression. AtI2 knockdown exerted a proinflammatory effect on RA-FLS. AtI2 knockdown rescued the inhibitory effect of miR-30e-5p knockdown on proliferation and inflammatory response of RA-FLS.

Conclusion: MiR-30e-5p knockdown inhibited the inflammatory response in RA mice and RA-FLS through AtI2.

Keywords: AtI2, fibroblast-like synoviocytes, inflammatory response, MiR-30e-5p, rheumatoid arthritis.

As a commonly diagnosed systemic autoimmune disease, rheumatoid arthritis (RA) causes chronic inflammation of the synovial joint, cartilage damage, as well as bone erosion,¹ with an incidence of about 0.3 to 1.1% of the population worldwide.² Patients with RA often experience stiffness, fatigue, work disorders, or even disability.³ Notably, the inflammatory response has been confirmed to be a main cause of RA, and the expression of inflammatory factors such as tumor necrosis factor (TNF), interleukin (IL)-1, IL-17, and IL-6 are upregulated in RA, and effective small molecule kinase inhibitors targeting inflammatory cytokines have

greatly improved the clinical efficacy of RA.⁴ Fibroblast-like synoviocytes (FLS) are in the intima of the synovium membrane and participate in the proinflammatory cytokine network and play a key role in RA development.⁵⁻⁸ However, RA is still currently incurable, thus, to explore the role of possible molecule in inflammatory response of RA-FLS or RA mice is important for further RA study.

Micro ribonucleic acids (miRNAs) can regulate gene expression by binding in the 3'-UTR of target messenger RNAs (mRNAs).⁹ Many researchers have investigated the role of several miRNAs in

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RA-FLS or in animal models. To illustrate, miR-20a regulates FLS proliferation and apoptosis in RA.¹⁰ The miR-21 relieves RA in rats by downregulating the Wnt signaling pathway.¹¹ The miR-124a exerts an inhibitory effect on RA-FLS proliferation and inflammation via PIK3/NF- κ B signaling pathway.¹² According to the results of GSE115885, miR-30e-5p has been confirmed as one of the upregulated miRNAs in the blood samples from early RA patients, compared to the blood samples from healthy controls. Moreover, miR-30e-5p is also involved in the prediction of coronary events in RA.¹³ However, the potential role of miR-30e-5p in inflammatory response of RA-FLS and RA mice is still undocumented.

As a member of the Atlantin GTPase (AtI2) group, AtI2 has been reported as one of the direct targets of miR-30b-5p in the mammary epithelial cells.¹⁴ The AtI2 expression was also regulated by miR-214-5p in gastric cancer cells.¹⁵ However, the mechanism of miR-30e-5p/AtI2 in RA have not been discussed, yet.

In the present study, we aimed to detect the impact of miR-30b-5p targeting AtI2 on inflammatory response of in RA mice and RA-FLS, which identified a promising new research strategy for RA development.

MATERIALS AND METHODS

Establishment of a mouse model with RA

Forty-eight male DBA/1 mice (23-25 g, 9 to 10-week-old) were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. and housed in quiet and clean cages with normal circadian rhythm of water and food intake for one week with the temperature of $25\pm 2^{\circ}\text{C}$. Then, all mice were divided into four groups as follows: sham (n=12); RA (n=12); RA+ Adeno-associated virus (AAV)-NC (n=12); and RA+AAV-miR-30e-5p inhibitor (n=12). Thirty-six mice were used to induce RA according to the previous study.¹⁶ Specifically, mice were treated with bovine-II collagen (200 mg) via intradermal injection. Twenty-one days later, mice were treated with bovine-II collagen agonist via intradermal injection again. The sham operation group was given the same amount of phosphate-buffered saline (PBS) as control. Adeno-associated

virus vectors carrying miR-30e-5p inhibitor, or a negative control were generated by the GeneChem Technology Company (Shanghai, China). The AAV-miR-30e-5p inhibitor or AAV-NC were injected intradermally in PBS (50 μL) per RA mouse. After three weeks, when the RA mice showed adequate transfection, mouse foot swelling was assessed with a vernier caliper. At the end, all mice were euthanized with pentobarbital sodium (100 mg/kg), synovial tissues were collected, and the hind paws were prepared for the following experiments.

RA-FLS culture and transfection

The isolation and culture of FLS were performed as previously described.¹⁷ Synovial tissues from the RA mice model were digested and then cultured in prescribed Dulbecco's modified eagle medium (DMEM; Sigma-Aldrich, St. Louis, Missouri, USA) for seven days at 5% carbon dioxide (CO_2) and 37°C . Cells were, then, emerged and subcultured in complete DMEM containing 10% FCS. The FLSs at passages 3 to 4 were used for the present study. For subsequent experiments, cells (1×10^4) were treated with lentiviruses encoding miR-30e-5p inhibitor, sh-AtI2 and their controls (Sangon Biotechnology Co., Ltd., Shanghai, China) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) for 48 h.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The total RNA was extracted from synovial tissues and FLSs using a TRIzol kit (Invitrogen, Carlsbad, CA, USA) and was reverse transcribed to complementary deoxyribonucleic acid DNA (cDNA). Then, the relative expression levels were evaluated using an ABI real-time qPCR System (Thermo Fisher Scientific, Massachusetts, USA). The GAPDH and U6 were used as internal parameters. The data were analyzed by $2^{-\Delta\Delta\text{Ct}}$ method. Sequences of primers are provided in Table 1.

Enzyme-linked immunosorbent assay (ELISA)

The synovial tissues of the mice and supernatant of RA-FLS were collected, and the corresponding ELISA kits (Abcam Co., Ltd., NY, USA) were used to detect the protein levels of TNF-alpha (TNF- α), IL-1 β , and IL-6.

Table 1. Primers used for quantitative RT-PCR

Name	Sequence (5'-3') forward	Sequence (5'-3') reverse
miR-30e-5p	TGTAACATCCTTGACTGGAAGG	CCAGTGCGAATACCTCGGAC
AtI2	ATGGAACAGGTATGTGGAGG	CACTTCCTTGAGATCCAAGTG
GAPDH	TCAAGATCATCAGCAATGCC	CGATACCAAAGTTGTCATGGA
U6	GGTCGGGCAGGAAAGAGGGC	GCTAATCTTCTCTGTATCGTTCC

RT-PCR: Reverse transcription-polymerase chain reaction.

Western blotting analysis

Total protein from synovial tissues and RA-FLS was extracted and separated by SDS-PAGE (10%). Subsequently, the equal amount of protein was transferred onto polyvinylidene fluoride (PVDF) membrane, which was then blocked for 2 h using 5% skim milk. The membranes were, then, incubated with primary antibody against IL-1 β (ab254360; 1:1000; Abcam Co., Ltd., NY, USA), IL-6 (ab259341; 1:1000; Abcam Co., Ltd., NY, USA), TNF- α (ab183218; 1:1000; Abcam Co., Ltd., NY, USA), AtI2 (ab224825; 1:1000; Abcam Co., Ltd., NY, USA) and GAPDH (ab8245; 1:500; Abcam Co., Ltd., NY, USA) at 4°C for 24 h. After washing, the membranes were incubated with horseradish peroxidase (HPR)-conjugated secondary at room temperature for 2 h. The ImageJ software (NIH, Bethesda, MD, USA) was used to scan blots and evaluate the optical density (OD).

5-ethynyl-2'-deoxyuridine (EdU) labeling assay

The RA-FLSs (5×10^3 cells/well) were seeded into each well of 96-well plates and exposed to EdU solution (100 μ L) for 4 h at 37°C. Subsequently, cells were fixed in formaldehyde (4%) for half an hour at room temperature and permeabilized in Triton X-100 (0.5%) for 10 min. Next, the cells were washed with PBS and added with Apollo staining reaction solution in the dark. Nuclei were labeled using 4',6-diamidino-2-phenylindole 2hci (DAPI) for 30 min, and the images were captured using a fluorescence microscope (Olympus, Corporation, Tokyo, Japan).

Luciferase activity assay

The TargetScan (https://www.targetscan.org/vert_71/) forecasted the binding site between

miR-30e-5p and AtI2 3'UTR. Wild sequences and mutant sequences of the binding site were designed and then cloned into luciferase reporter vector pmirGLO (Promega, Madison, WI, USA) to obtain AtI2 3'UTR wild type plasmid (AtI2-WT) and AtI2 3'UTR mutant type plasmid (AtI2-MUT). Cells were, then, seeded in 96-well plates and co-transfected with the luciferase reporter plasmid and LV-miR-30e-5p inhibitor or LV-NC using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) for 48 h, and then the luciferase activity was tested with luciferase detection kit (BioVision, San Francisco, CA, USA).

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 21.0 software (IBM Corp., Armonk, NY, USA). Descriptive data were expressed in mean \pm standard deviation (SD). Comparisons between two groups and among multiple groups were assessed by t-test and one-way analysis of variance (ANOVA), followed by Tukey's post-hoc analysis. A *p* value of <0.05 was considered statistically significant.

RESULTS

Silencing of miR-30e-5p alleviates inflammation in RA mice

MiR-30e-5p expression was higher in the synovial tissues of RA mice than that in control group, as shown by RT-qPCR. While miR-30e-5p knockdown significantly downregulated miR-30e-5p expression, compared to that in RA+AAV-NC group (Figure 1a). In addition, the RA scores and swelling in mice was also reduced by miR-30e-5p knockdown (Figure 1b, c). The ELISA and Western blotting indicated that the

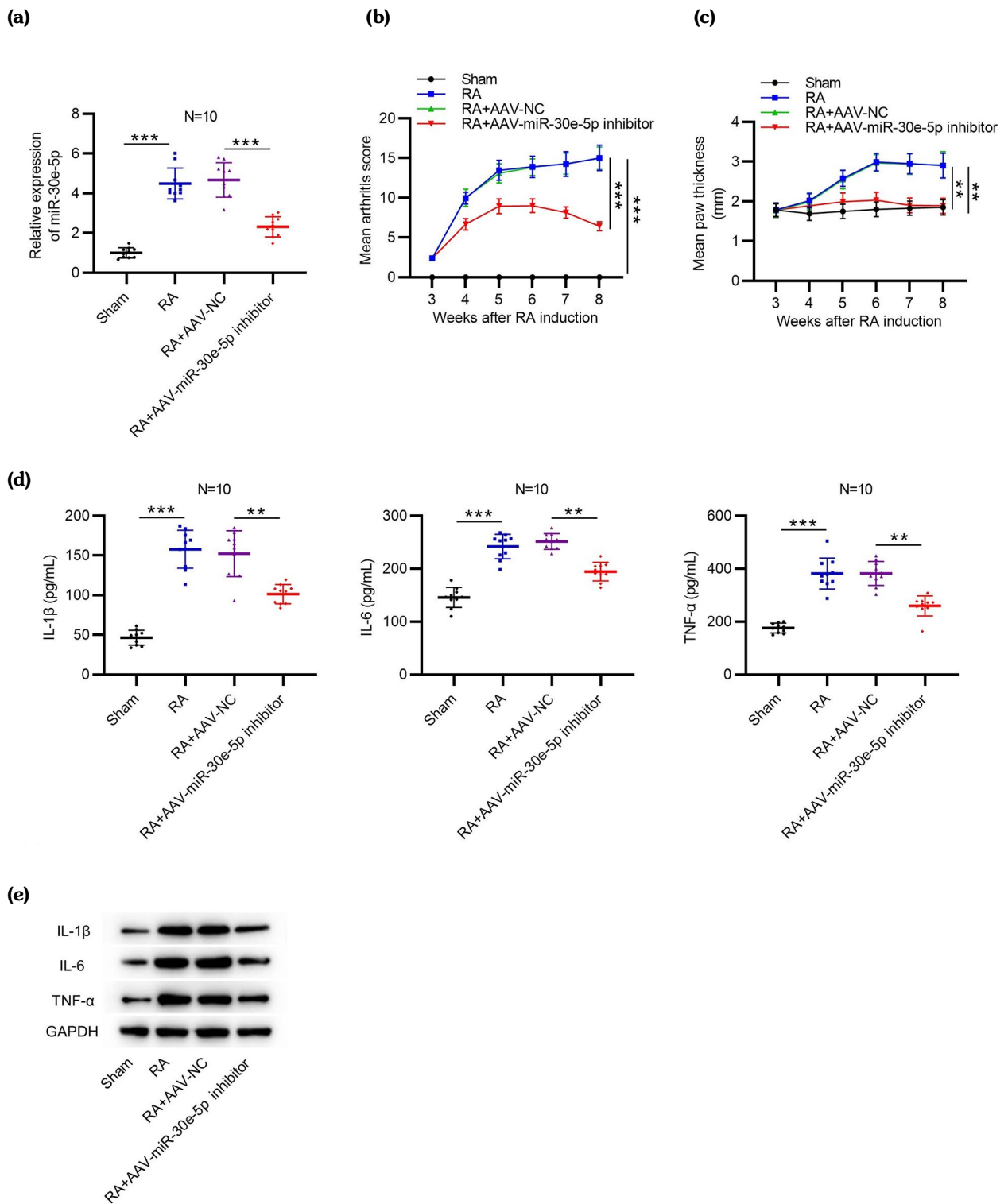


Figure 1. Silencing of miR-30e-5p alleviates inflammation in RA mice. **(a)** MiR-30e-5p expression in the synovial tissues was evaluated by RT-qPCR. **(b, c)** Arthritis score and paw thickness in Sham, RA, RA+AAV-NC, and RA+AAV-miR-30e-5p inhibitor. **(d, e)** ELISA analysis and western blotting revealed the levels of IL-1 β , IL-6, and TNF- α in Sham, RA, RA+AAV-NC, and RA+AAV-miR-30e-5p inhibitor.

RA: Rheumatoid arthritis; RT-qPCR: Reverse transcription quantitative polymerase chain reaction; AAV: Adeno-associated virus; NC: Normal control; miR: microRNA; IL: Interleukin; TNF: Tumor necrosis factor; ** $p < 0.01$; *** $p < 0.001$.

levels of IL-1 β , IL-6, and TNF- α were significantly elevated in the synovial tissues of the RA mice compared to that in control group. However, miR-30e-5p knockdown markedly decreased the levels of IL-1 β , IL-6, and TNF- α in RA mice (Figure 1d, e). Thus, miR-30e-5p knockdown mitigated the inflammatory response in RA mice.

MiR-30e-5p knockdown exerts an anti-inflammatory effect on RA-FLS

We, then, extracted RA-FLS from the synovial tissues to conduct the subsequent experiments. The miR-30e-5p expression was downregulated in RA-FLS silencing miR-30e-5p (Figure 2a). The number of

EdU-positive cells was obviously reduced by miR-30e-5p knockdown relative to the control group (Figure 2b, c). According to the results of ELISA, the levels of IL-1 β , IL-6, and TNF- α in the cells were decreased after miR-30e-5p knockdown, and the similar alterations were observed in Western blotting (Figure 2d, e). Collectively, miR-30e-5p knockdown inhibited the inflammatory response in RA-FLS.

MiR-30e-5p negatively modulates AtI2 expression

AtI2 was predicted as a potential target of miR-30e-5p by the starBase with the screening condition of CLIP data ≥ 5 and Degradome

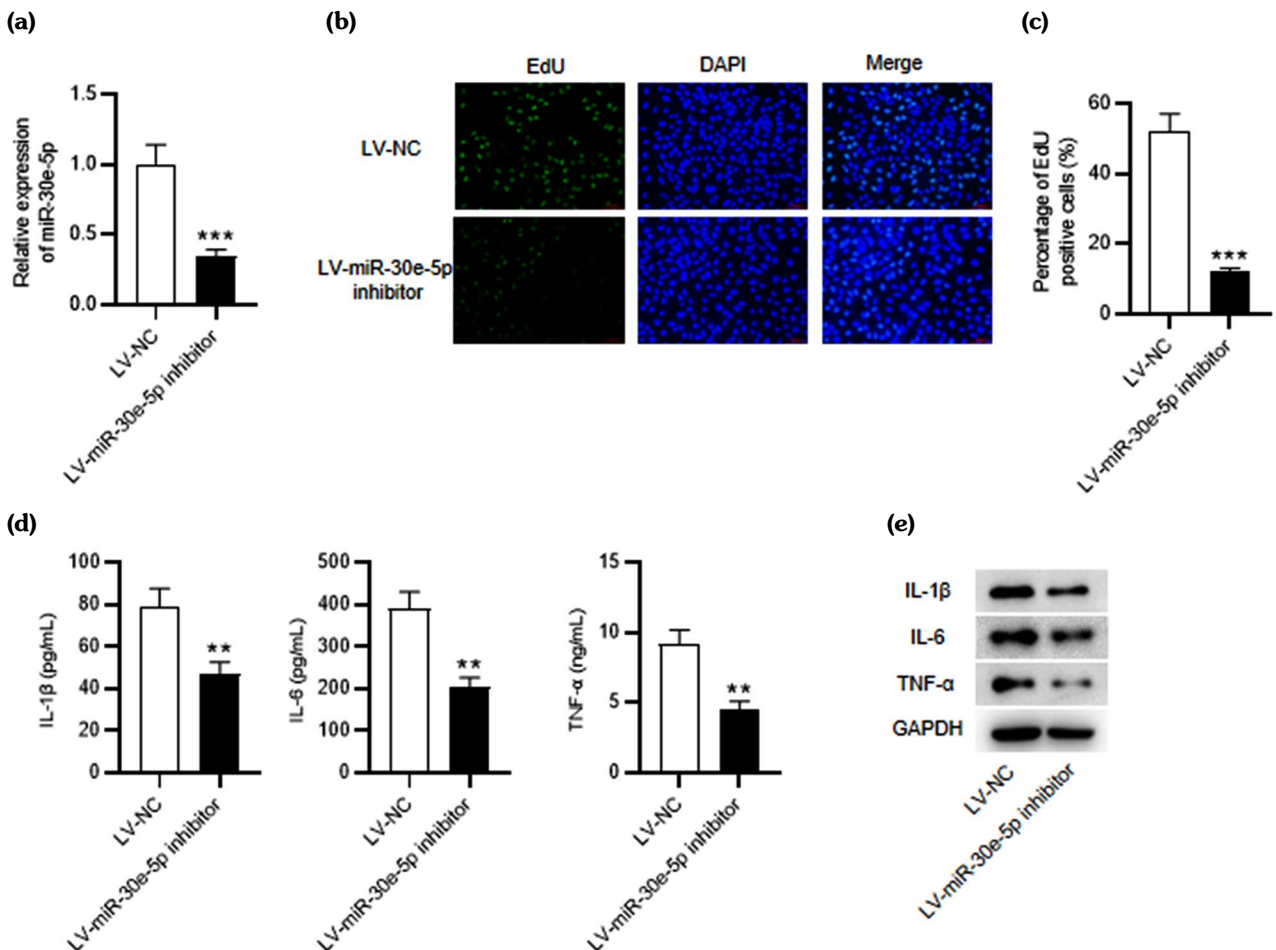


Figure 2. MiR-30e-5p knockdown exerts an anti-inflammatory effect on RA-FLS. **(a)** MiR-30e-5p expression in RA-FLS silencing miR-30e-5p evaluated by RT-qPCR. **(b, c)** EdU assay detected the cell proliferation in RA-FLS silencing miR-30e-5p. **(d, e)** The levels of IL-1 β , IL-6, and TNF- α in RA-FLS silencing miR-30e-5p were evaluated by ELISA and western blotting.

RA: Rheumatoid arthritis; FLS: Fibroblast-like synoviocytes; NC: Normal control; miR: microRNA; RT-qPCR: Reverse transcription quantitative polymerase chain reaction; EdU: 5-ethynyl-2'-deoxyuridine; IL: Interleukin; TNF: Tumor necrosis factor; ** $p < 0.01$; *** $p < 0.001$.

data ≥ 2 . A lower AtI2 expression was observed in RA mice, which was, then, upregulated by miR-30e-5p knockdown (Figure 3a). The RT-qPCR and Western blotting revealed that silencing of miR-30e-5p increased AtI2 expression in RA-FLS (Figure 3b, c). The TargetScan predicted the binding site between miR-30e-5p and AtI2 (Figure 3d). The luciferase reporter activity of pmirGLO-AtI2 3'UTR Wt was significantly increased by LV-miR-30e-5p inhibitor, compared to that in the LV-NC group. While The luciferase reporter activity of pmirGLO-AtI2 3'UTR Mut had

no significant change in the cells transfected with LV-miR-30e-5p inhibitor and LV-NC (Figure 3e). Furthermore, AtI2 expression was negatively correlated with miR-30e-5p expression in the synovial tissues (n=20), according to the results of Spearman rank correlation analysis (Figure 3f). Thus, AtI2 was a target of miR-30e-5p.

AtI2 knockdown exerts a proinflammatory effect on RA-FLS

Subsequently, we transfected RA-FLS with LV-sh-AtI2 and LV-NC to explore the effect of

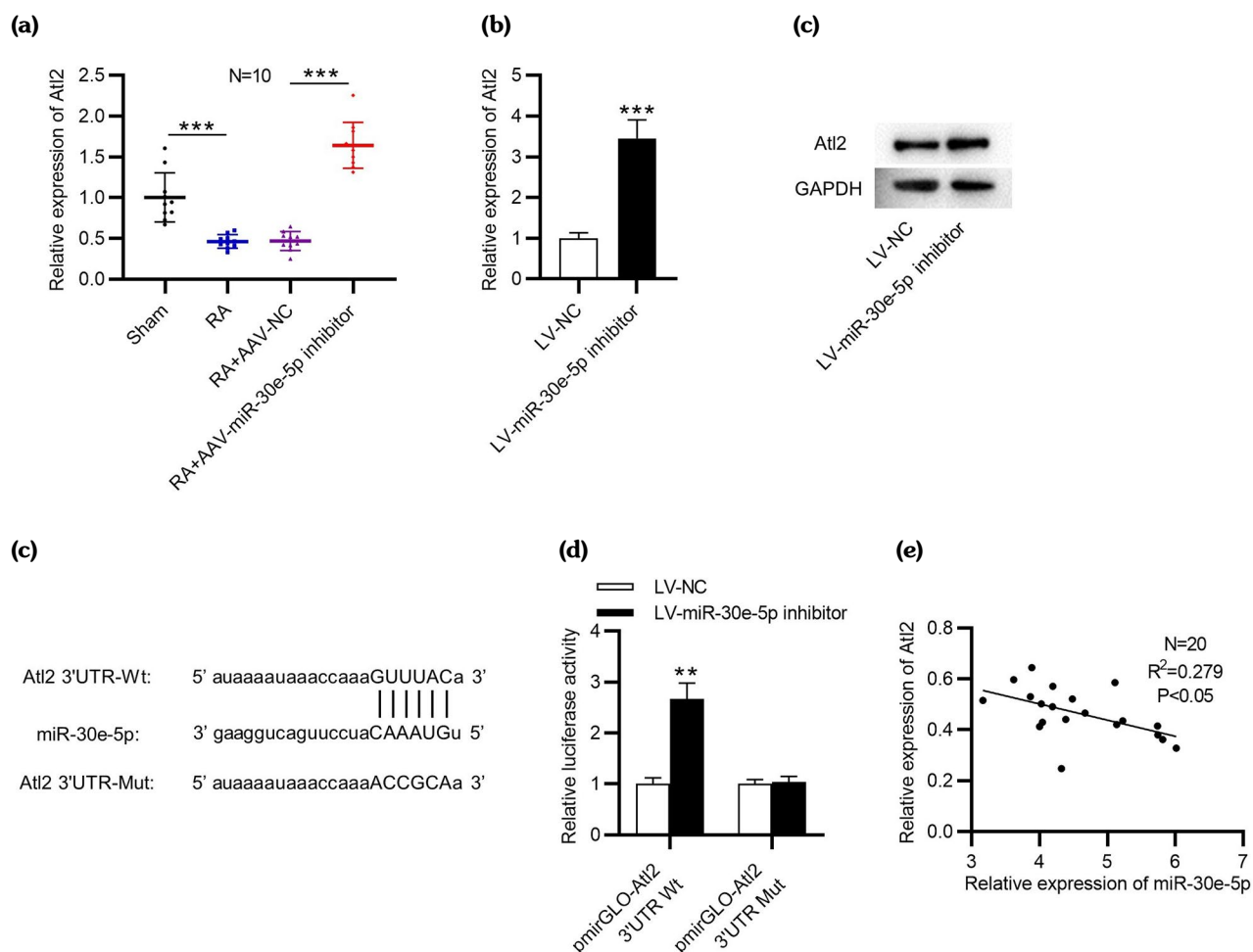


Figure 3. MiR-30e-5p negatively modulates AtI2 expression. **(a)** AtI2 expression level in the synovial tissues of RA mice was evaluated by RT-qPCR. **(b, c)** RT-qPCR and western blotting revealed AtI2 expression in RA-FLS silencing miR-30e-5p. **(d)** TargetScan predicted the binding site between miR-30e-5p and AtI2. **(e)** The luciferase activities of pmirGLO-AtI2 3'UTR Wt and pmirGLO-AtI2 3'UTR Mut were evaluated in the cells transfected with LV-miR-30e-5p inhibitor and LV-NC. **(f)** The correlation between miR-30e-5p and AtI2 expression in the synovial tissues (n=20) was revealed by spearman's rank correlation analysis revealed.

miR: microRNA; RA: Rheumatoid arthritis; RT-qPCR: Reverse transcription quantitative polymerase chain reaction; FLS: Fibroblast-like synoviocytes; pmirGLO: pmirGLO vector; LV-NC: Lentivirus-mediated normal control; ** $p<0.01$; *** $p<0.001$.

AtI2 knockdown on inflammation. The AtI2 expression was significantly decreased in RA-FLS silencing AtI2 (Figure 4a). The EdU assay showed the number of proliferating cells was obviously increased by AtI2 knockdown (Figure 4b, c). The AtI2 knockdown aggravated the inflammation with increased levels of IL-1 β , IL-6, and TNF- α expression, as shown in ELISA and Western blotting (Figure 4d, e). It was inferred that AtI2 knockdown exerted a proinflammatory effect on RA-FLS.

AtI2 knockdown rescues the effect of miR-30e-5p knockdown on inflammatory response of RA-FLS

We, then, transfected sh-AtI2 into miR-30e-5p silencing RA-FLS. As shown in EdU assay, the proliferation of RA-FLS was inhibited

by miR-30e-5p knockdown. However, AtI2 knockdown partially recovered the results (Figure 5a, b). The levels of IL-1 β , IL-6, and TNF- α was downregulated in RA-FLS silencing miR-30e-5p, compared to those in the LV-NC group. While AtI2 knockdown significantly upregulated the levels of IL-1 β , IL-6, and TNF- α relative to those in LV-miR-30e-5p group (Figure 5c, d). Collectively, AtI2 knockdown reversed the inhibitory effect of miR-30e-5p knockdown on proliferation and inflammatory response of RA-FLS.

DISCUSSION

The inflammation, proliferation, and invasion of the synovial layer are the main

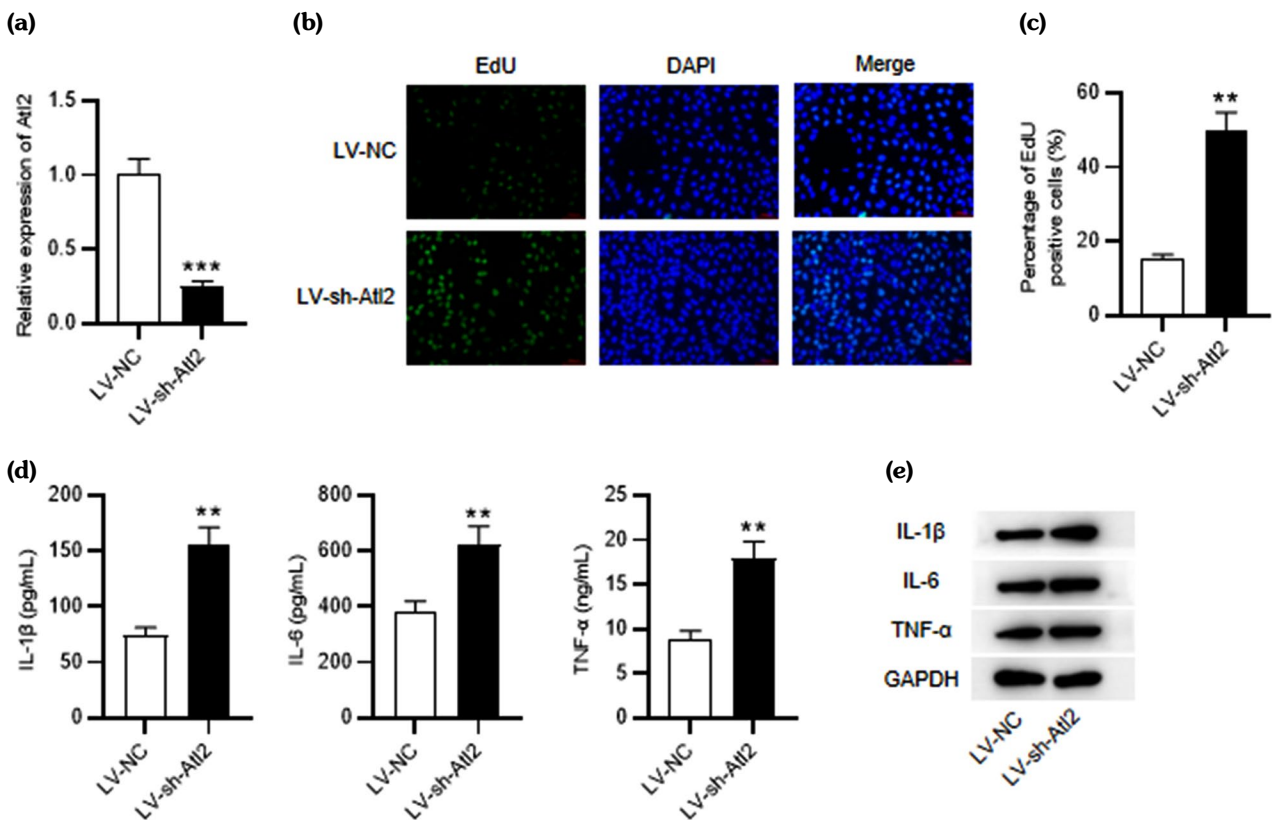


Figure 4. AtI2 knockdown exerts a proinflammatory effect on RA-FLS. **(a)** AtI2 expression in RA-FLS silencing AtI2 was evaluated by RT-qPCR. **(b, c)** The EdU assay showed the number of proliferating cells in RA-FLS silencing AtI2. **(d, e)** The levels of IL-1 β , IL-6, and TNF- α in RA-FLS silencing AtI2 was evaluated by ELISA and western blotting in the cells silencing AtI2.

RA: Rheumatoid arthritis; FLS: Fibroblast-like synoviocytes; RT-qPCR: Reverse transcription quantitative polymerase chain reaction; IL: Interleukin; TNF: Tumor necrosis factor; ** $p < 0.01$; *** $p < 0.001$.

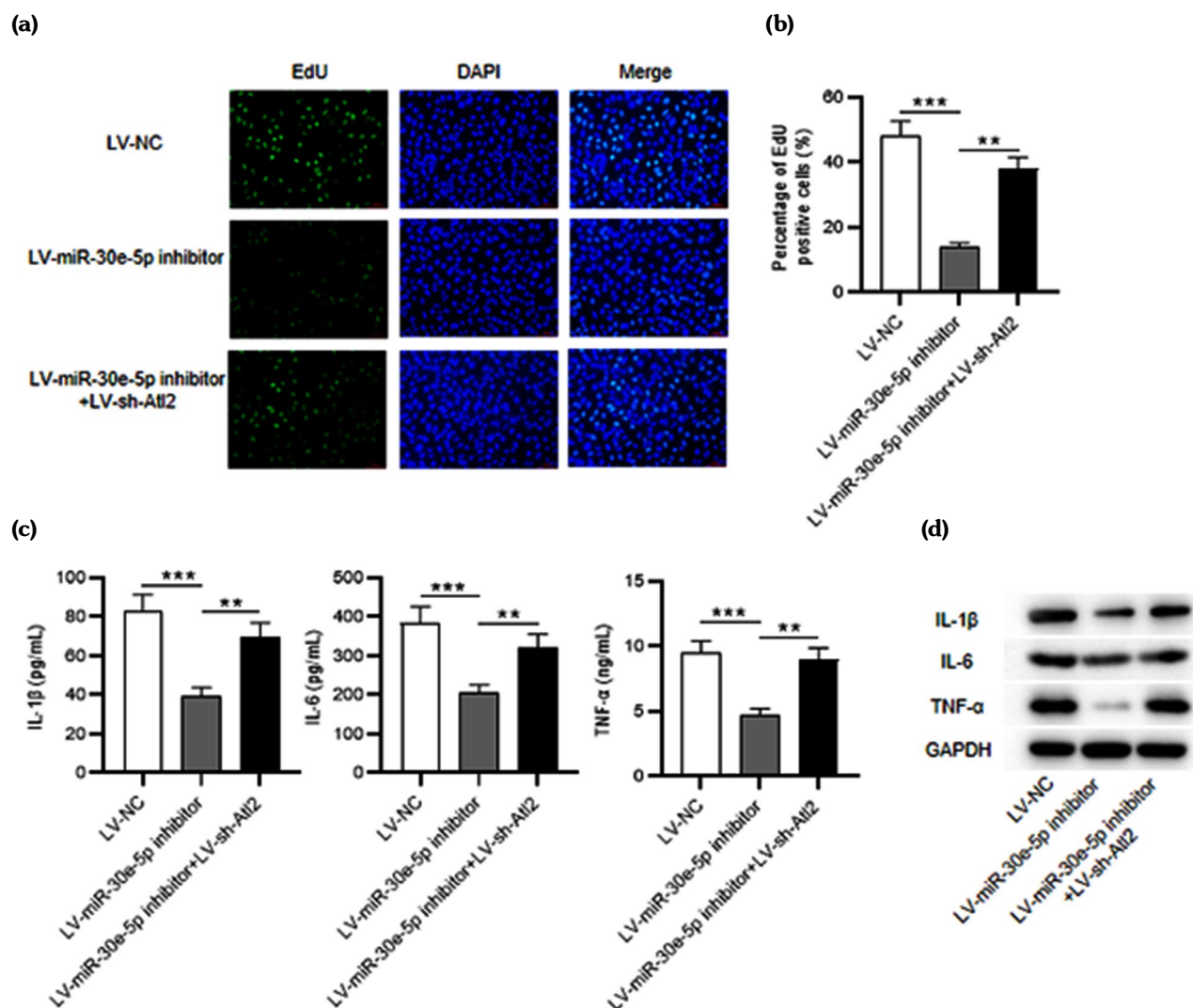


Figure 5. AtI2 knockdown rescues the effect of miR-30e-5p knockdown on inflammatory response of RA-FLS. **(a, b)** EdU assay showed the number of proliferating cells in RA-FLS silencing miR-30e-5p and AtI2. **(c, d)** The levels of IL-1 β , IL-6, and TNF- α in RA-FLS silencing miR-30e-5p and AtI2 were evaluated by ELISA and western blotting in LV-NC, LV-miR-30e-5p inhibitor, LV-miR-30e-5p inhibitor+LV-sh-AtI2 groups.

RA: Rheumatoid arthritis; FLS: Fibroblast-like synoviocytes; IL: Interleukin; TNF: Tumor necrosis factor; miR: microRNA; ** p < 0.01; *** p < 0.001.

features of RA.¹⁸ It has been confirmed that etiology of RA is related to the induction of inflammatory cytokines and chemokines.¹⁹ Of these inflammatory factors, IL-1 β , IL-6, and TNF- α have been emphasized, as high levels of IL-1 β , IL-6, and TNF- α are detected in the synovial fluid and the plasma for RA patients.^{20,21} In addition, as a fundamental regulator of joint impairment, RA-FLS can invade articular cartilage.²² Importantly, increasing evidence have confirmed the significant role of gene

therapy in RA treatment.²³ Thus, in our study, we identified a novel biomarker of RA, and explored its role and mechanism in inflammation of RA mice and RA-FLS.

Many miRNAs including miR-146a,²⁴ miR-22²⁵ and miR-130a²⁶ have been found to participate in the regulation of RA development.²⁷ Notably, GSE115885 data suggested that miR-30e-5p expression was upregulated in the blood samples from early RA patients, compared to the blood samples from healthy controls. In the current

study, miR-30e-5p expression was higher in the synovial tissues of RA mice. More importantly, miR-30e-5p knockdown significantly reduced the arthritis score and paw thickness, as well as the levels of IL-1 β , IL-6, and TNF- α both in RA mice and RA-FLS.

In general, miRNAs mediated the biological processes through regulating the expression of its downstream targeting mRNA.²⁸⁻³⁰ We bioinformatically predicted and confirmed that *AtI2* was a target gene of miR-30e-5p. However, the role and mechanism of *AtI2* were merely reported in any diseases. We, for the first time, revealed that miR-30e-5p expression was negatively correlated with *AtI2* expression. After transfecting RA-FLS with LV-sh-*AtI2*, we surprisingly found that *AtI2* knockdown increased the number of proliferating cells and the levels of IL-1 β , IL-6, and TNF- α . In rescue assay, *AtI2* knockdown also reversed the inhibitory effect of miR-30e-5p knockdown and promoted proliferation and inflammatory response of RA-FLS. However, we still wonder whether certain signaling pathway are activated to contribute to this mechanism, which would be further verified in the future.

In conclusion, we found that miR-30e-5p knockdown repressed the inflammatory response in RA mice and RA-FLS by regulating *AtI2* expression, providing a new research strategy for further studying the development of RA.

Ethics Committee Approval: The study protocol was approved by the Wuhan Hualian ke Biology Technology Co., Ltd. Ethics Review Committee (date: 05.05.2021, no: 202105006). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Idea/concept: S.L.; Design, critical review: S.L.; K.W.; Control/supervision, data collection and/or processing: J.L.; Analysis and/or interpretation: Y.L.; Literature review, writing the article: Z.Z., D.M.; References and fundings, materials: J.L., Y.L.

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