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Original Article

MiR-125a-3p inhibits cell proliferation and inflammation responses in fibroblast-like synovial cells in rheumatoid arthritis by mediating the Wnt/β-catenin and NF-κB pathways via targeting MAST3

Yingxue Wang, Zhe Yin , Ni Zhang, Huishu Song, Qiuting Zhang, Xuexi Hao, Zhilun Wang

Department of Rheumatology, The Second Hospital of Shandong University, Shandong Province, China

Abstract

Objectives: To explore the role and mechanism of miR-125a-3p in rheumatoid arthritis (RA) progression. **Methods**: The RA-tissues and fibroblast-like synovial cells in rheumatoid arthritis (RA-FLS) were used in this study. qRT-PCR, western blot and ELISA assay were performed to detect the expression levels of IL-6, IL-β and TNF-α. Dual-luciferase reporter gene assay was used to observe the binding effect of miR-125a-3p and MAST3, and CCK-8 was used to observe the effect of miR-125a-3p on the proliferation of RA-FLS. **Results**: miR-125a-3p was significantly downregulated in the RA-tissues and RA-FLS, and miR-125a-3p could inhibit the proliferation and reduce the inflammation response of RA-FLS. Besides, MAST3 was found as a target of miR-125a-3p, and increased MAST3 could reverse the effects of miR-125a-3p on RA-FLS including decreased proliferation, reduced inflammation level and the inactivation of Wnt/β-catenin and NF-κB pathways. **Conclusions**: This study suggests that miR-125a-3p could inactivate the Wnt/β-catenin and NF-κB pathways to reduce the proliferation and inflammation response of RA-FLS via targeting MAST3.

Keywords: MAST3, miR-125a-3p, NF-κB, Rheumatoid Arthritis, Wnt/β-catenin

Introduction

A better understanding of how the pathological mechanisms drive the deterioration of RA progress in individuals is urgently required in order to develop therapies that will effectively treat patients at each stage of the disease progress.

Rheumatoid arthritis (RA) is a chronic inflammatory disorder that mainly affects the lining of the synovial joints and is associated with progressive disability. It is the most frequent inflammatory arthropathy[1,2]. RA affects adults of any age, and the prevalence rate is approximately 1%[3]. At present, the intervention of RA focuses on relieving joint pain, slowing progression of the disease and improving the patients' quality of life[4]. Treatment methods include anti-rheumatic drugs and agents targeting immunological factors such as tumor necrosis (TNF) and interleukin-1 (IL-1)[5,6]. which play dominant role in preventing or slowing down joint damage, reducing inflammation and disability[7,8]. However, there is no cure for RA. An increasing number of studies show that the phenotypic changes of RA fibroblast-like synoviocytes (RA-FLS) contribute to the development of RA[9].

MicroRNAs (miRNAs), small non-coding RNAs containing about 19-21 nucleotides, play a crucial role in regulating various cellular processes[10,11]. The regulations of targeting with 3'-UTRs cause the cleavaging or translational inhibition of target mRNAs, which result in decreased expression level of related proteins[12,13]. Dysregulation of miRNAs is related with many diseases, ranging from several inflammatory diseases to cancers[14,15]. In RA, it has been found that the significant difference exists in the miRNA profiles of the serum and tissues between the patients and health people. Moreover, modulating the abundance of miRNAs is considered as promising therapeutic strategies[16,17]. A study has shown that miR-125a-3p was upregulated in serum of patients with RA, while few studies have illustrated the regulation mechanism of miR-125a-3p on RA[18]. In this study, we explored the role and mechanism of the miR-125a-3p on RA.

The authors have no conflict of interest.

Corresponding author: Zhilun Wang, Department of Rheumatology, The Second Hospital of Shandong University, No. 247 Beiyuan Street, Tianqiao District, Jinan 250033, Shandong Province, China
E-mail: funanbi6453@163.com

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Table 1. Primer sequence of miR-382-3p and U6.

Name of primer	Sequences
miR-125a-3p-F	5'-ACACTCCAGCTGGGACAGGTGAGGT TCTTG-3'
miR-125a-3p-R	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGCTC CCA-3'
U6-F	5'-CTCGCTTCGGCAGCACA-3'
U6-R	5'-AACGCTTCACGAATTTGCGT-3'

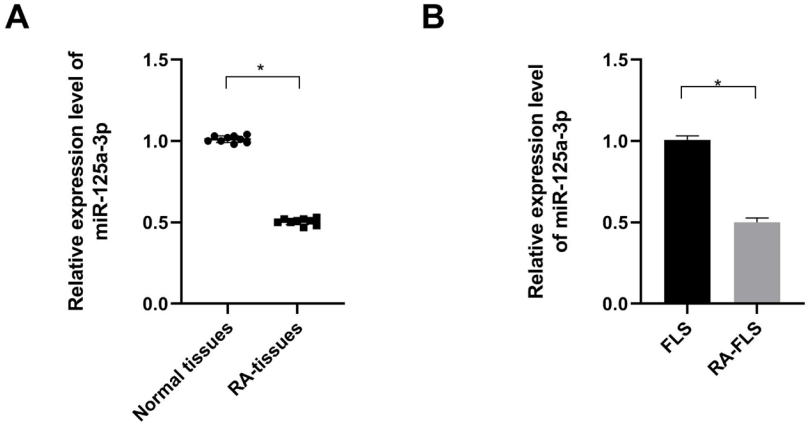

Figure 1. MIR-125a-3p was downregulated in RA-tissues and RA-FLS. (A) and (B) Relative expression level of miR-125a-3p in RA-tissues and RA-FLS, respectively.

Materials and methods

Cell culture

In this study, we used RA fibroblast-like synoviocytes (RA-FLS) and normal fibroblast-like synoviocytes (FLS) which were isolated from the specimens of RA tissues and normal tissues, respectively. DMRM/F12 (Thermo Fisher Scientific, Waltham, UAS) including 10% fetal bovine serum (Gibco Life technologies, USA), 100 units/mL penicillin and 100 μg/mL streptomycin were used to culture the cells. Besides, all cells were cultured at 37°C under 5% CO_2.

Cell transfection

The MAST3 were subcloned into pcDNA3.1 vectors, and miR-125a-3p mimics and miR-NC were purchased from Genomeditech Co.,Ltd. When the cells were at 70% confluence, the miR-NC, miR-125a-3p mimics, MAST3 overexpressed vectors or empty vectors were transfected into the cells of corresponding wells with lipofectamine 2000 reagant (Invitrogen, USA).

Quantitative reverse transcription polymerase reaction (qRT-PCR)

The total RNAs, extracted by Trizol reagent from the cells or tissues, were used to transcribed into cDNA with a PrimeScript® RT reagent Kit (Thermo Fisher, Massachusetts, USA). The primers synthesized by Synbio Technology (Suzhou, China) were used to the qRT-PCR reaction. The reaction systems (10 μL) were prepared following the operational instruction of KAPA qRT-PCR kit (Sigma-Aldrich, Missouri, USA). The reaction condition included pre-denaturation at 95°C for 30s, 95°C for 5s, and 60°C for 30s for a total of 40 cycles. Moreover, U6 was used as the control in qRT-PCR. The primer sequences of miR-125a-3p and U6 have been listed in Table 1. qRT-PCR was used to detected the expression level of miR-125a-3p in RA-tissues and RA-FLS.

Western blot

The total protein from the cells were extracted by RIPA reagent, and then were added to 1% Phenylmethylsulfonyl fluoride (Absin Bioscience Inc, Shanghai, China) so as to keep the stability of the proteins. The concentrations of the

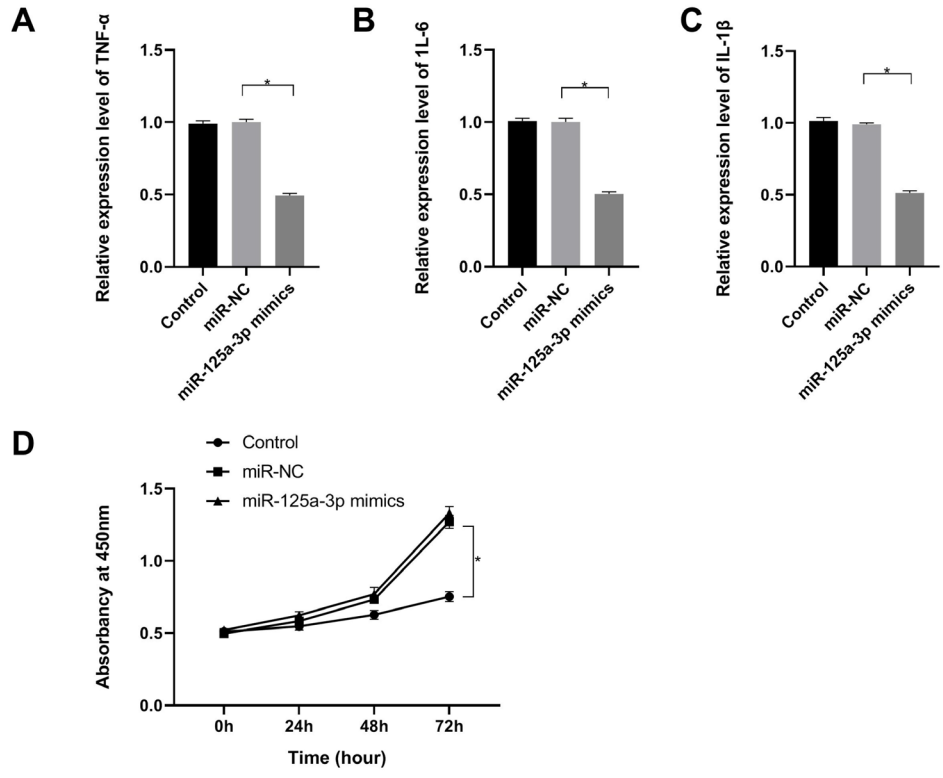

Figure 2. MiR-125a-3p inhibited the proliferation and reduce inflammation response of RA-FLS. (A) , (B) and (C) The effects of miR-125a-3p on the relative expression levels of TNF-α, IL-6 and IL-1β in RA-FLS. (D) The effect of miR-125a-3p on the proliferation of RA-FLS.

extracts were measured by BCA protein assay kit (Beyotime, Shanghai, China). After that, the total proteins were separated by 10% SDS-PAGE gel and then transferred on the PVDF membranes. All membranes were blocked with 5% fat-free milk for 1 hour, and the first antibody (1:2000) was used to combine with target proteins on the membranes at 4°C overnight. After that, the membranes were washed with TBST buffer for 3 times and then were incubated with the second antibody for 1 hours at 25°C. Finally, the expression levels of target proteins were observed by a chemiluminescence detection system.

ELISA assay

The cells were cultured and centrifuged at 2000rpm for 10 min, and the expression level of IL6, IL-8 and TNF-α in the cells were measured by an ELISA kits (Ji Ning Industrial Co., Ltd, Shanghai, China).

CCK-8 assay

The cells were seed into 96-well plates, and the transfectants were transfected into the cells in corresponding wells. The cells were added with CCk-8 solution (Amyjet, Wuhan, China) and incubate for 4 hours, and the blank wells only with CCk-8 solution were set as controls. After that, the absorbance value was measured at 450 nm by a microplate reader (Molecular device, Shanghai, China).

To explore the functions of miR-125a-3p in the development of RA, the miR-125a-3p mimics or miR-NC (miRNA negative control) were transfected into RA-FLS, and CCK-8 assay and ELISA were used to observe the proliferation and levels of imflamatory factors of the cells, respectively.

Bioinformatic analysis and dual-luciferase reporter gene assay

MiRwalk, an online database for prediction of miRNAs targets, was used to search the downstream factors of miR-125a-3p. The pGL3 vectors containing with normal and mutant 3'-UTR sequences of MAST3 were named as MAST3-wt and MAST3-mut, respectively. After that, the vectors of MAST3-wt and MAST3-mut were respectively co-transfected with miR-382-3p mimics or miR-NC. The luciferase activities in the cells were observed by a dual-luciferase reporter assay system.

Statistical analysis

All experiments were repeated independently for three times. The data were analyzed by SPSS 20.0, and Chi-squared test or ANOVA with Tukey's post hoc-test were used

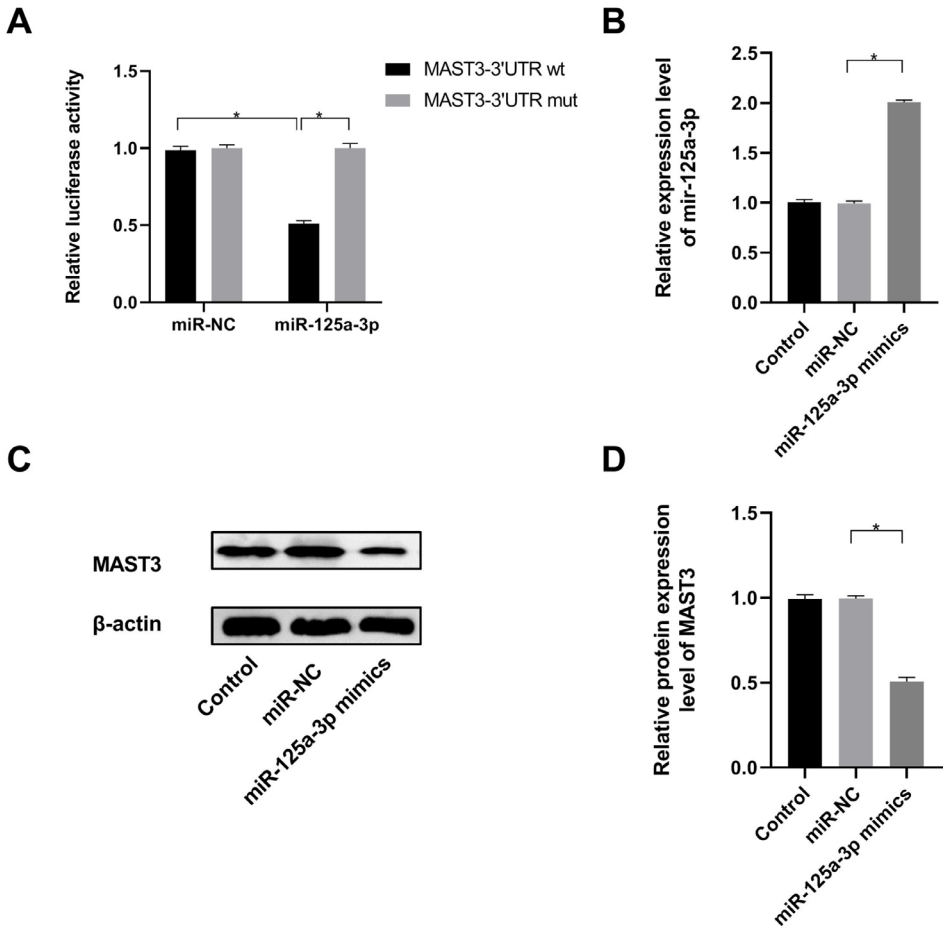

Figure 3. MAST3 was a target of iR-125a-3p, and miR-125a-3p could inhibit the expression level of MAST3. (A) The effect of miR-125a-3p on the relative Luciferase activity of MAST3-wt. (B) The relative expression level of miR-125a-3p. (C) and (D) The effect of miR-125a-3p on expression level of MAST3.

to test the difference of the groups. P<0.05 was defined as statistically significant. The figures were created with Graphpad Prism 8.0.

Results

MiR-125-3p was downregulated in RA-tissues and RA-FLS

It was found that miR-125a-3p was significantly downregulated in RA-tissues and RA-FLS, indicating that decreased miR-125a-3p had related with RA (Figure 1 A and B, P<0.01).

MiR-125a-3p inhibited the proliferation and reduced inflammation responses of RA-FLS

The results showed that the viability of the cells with overexpressed miR-125a-3p significantly decreased at 24, 48, 72 and 96 h post-transfection (Figure 2 D, P<0.01). Moreover, the inflammatory factors including TNF-a, IL-1β and IL-6 in RA-FLS transfected with miR-125a-3p mimics decreased, significantly (Figure 2 A, B and C, all P<0.01).

MiR-125a-3p directly target the 3'-UTR of MAST3

To explore the mechanisms of miR-125a-3p on the regulation of the Wnt/β-catenin and NF-κB signaling, miRWalk was used to search the potential targets of miR-125a-3p. The results showed that MAST3 was an ideal candidate, which has low binding energy with miR-125a-3p. To further confirm the correlation between miR-125a-3p and MAST3, the MAST3-wt and MAST3-mut were transfected into 293T cells together with miR-125a-3p mimics or miR-NC, respectively. It was found that miR-125a-3p significantly decreased the luciferase activities of the MAST3-wt compared with MAST3-mut in the cells (Figure 3 A, P<0.01). Moreover, the expression level of MAST3 in RA-FLS significantly decreased after transfection with miR-125a-3p mimics, which suggests that miR-125a-3p could directly target the 3'-UTR of MAST3 so as to reduce the expression level of MAST3 (Figure 3 B, C and D, P<0.01).

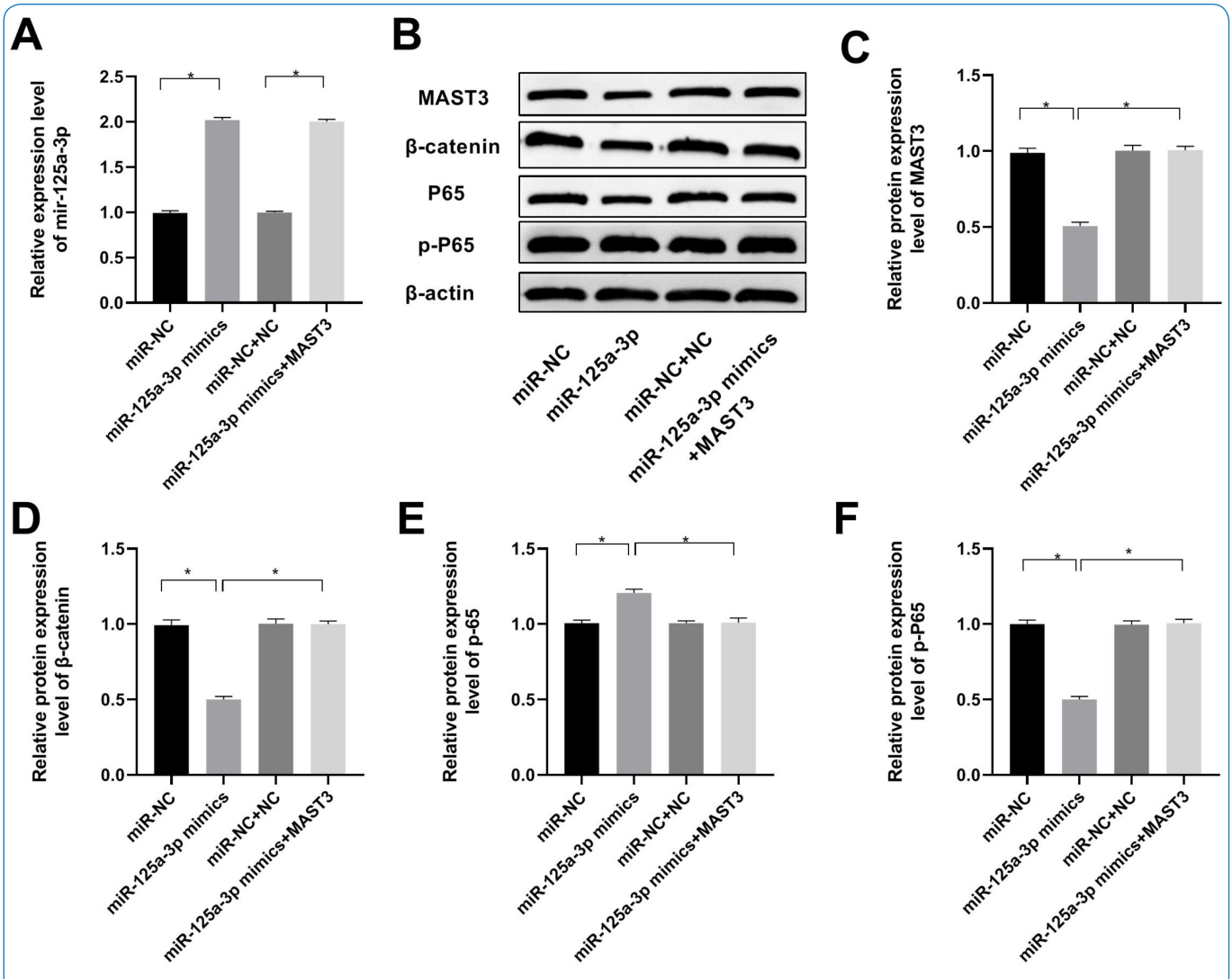

Figure 4. MAST3 reversed the decreased proliferation, reduced inflammatory response and the inactivation of Wnt/β-catenin and NF-κB pathways of RA-FLS induced by miR-125a-3p mimics. (A) Relative expression level of miR-125a-3p. (B), (C), (D), (E) and (F) The expression levels of MAST3, β-catenin, P65 and p-P65, respectively.

MAST3 reversed the inactivated Wnt/β-catenin and NF-κB pathways induced by increased miR-125a-3p expression

To define the mechanism of miR-125a-3p in suppressing the proliferation and levels of inflammatory factors of the cells, the overexpressed vectors of MAST3 and miR-125a-3p mimics were co-transfected into RA-FLS. The expression levels of β-catenin, P65 and p-P65 were determined by western blot to observe the effects of miR-125a-3p and MAST3 on Wnt/β-catenin and NF-κB pathways. The results showed that upregulated miR-125a-3p significantly decreased the expression level of β-catenin, indicating that miR-125a-3p involved in inactivation of Wnt/β-catenin pathway (Figure 4 B, C and D, P<0.01). Besides, p-P65 was also downregulated in the cells after transfecting with miR-125a-3p mimics, which suggests that miR-125a-3p take part in regulation of NF-κB pathway (Figure 4 E and F, P<0.01). Besides, it was observed that miR-125a-3p could significantly inhibit the expression of MAST3, and the downregulated Wnt, β-catenin and p-NF-κB in RA-FLS induced by increased miR-125a-3p expression could be reversed by MAST3 (Figure 4 A- F, P<0.01).

MAST3 rescued the decreased proliferation ability and low inflammatory reflection of RA-FLS induced by increased miR-125a-3p expression

The overexpressed vectors of MAST3 and miR-125a-3p mimics were co-transfected into RA-FLS to further investigate the functions of MAST3 in RA-FLS, and CCK-

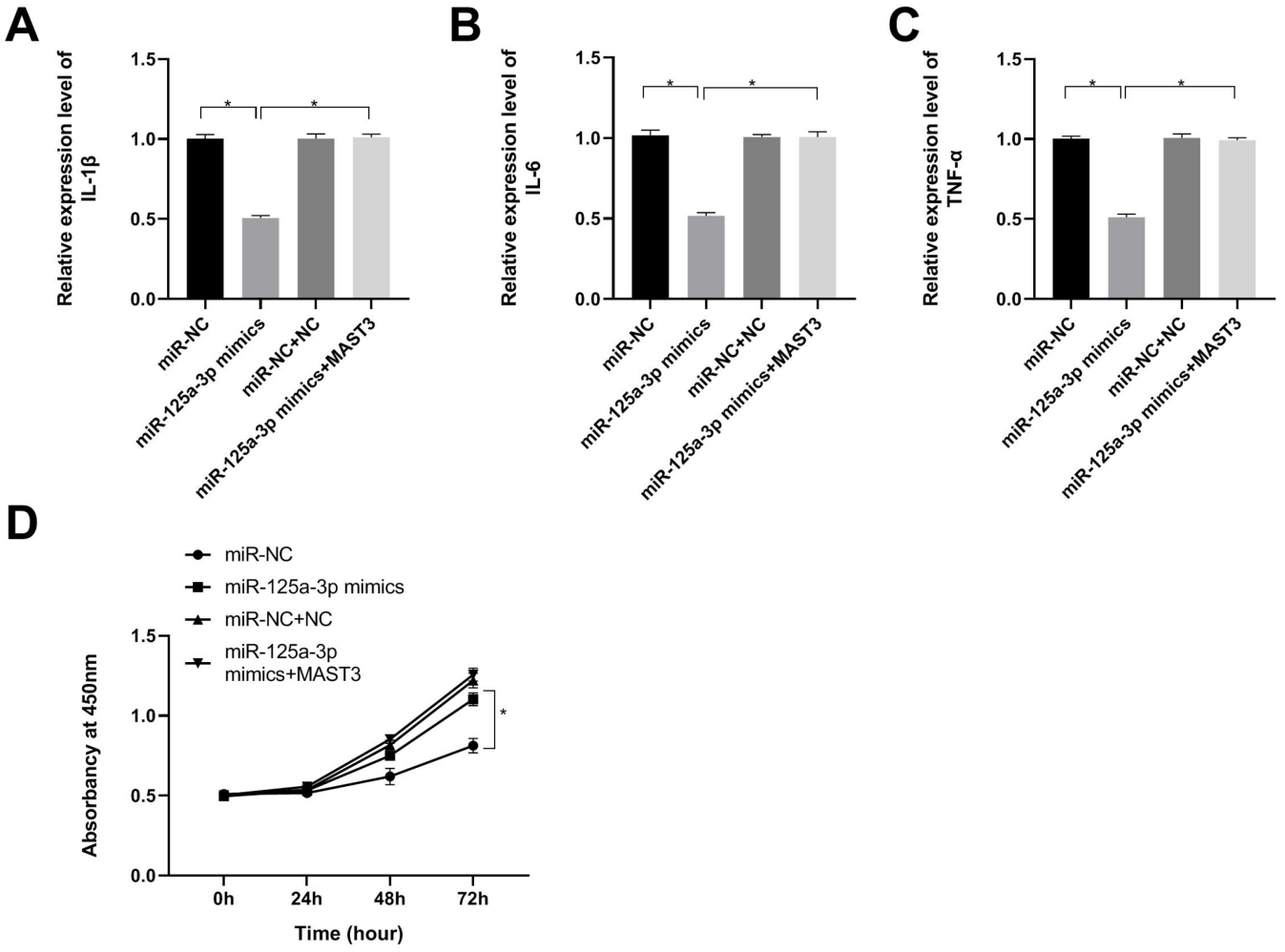

Figure 5. MAST3 can reverse the inhibited the proliferation and reduced inflammation response of RA-FLS induced by MIR-125a-3p. (A), (B) and (C) The effects of MAST3 on reduced expression levels of TNF-α, IL-6 and IL-1β in RA-FLS induced by miR-125a-3p. (D) The effect of MAST3 on decreased proliferation of RA-FLS induced by miR-125a-3p.

8 asssay and ELISA were used to reflect the changes in proliferation and inflammatory responses in RA-FLS (Figure 5 A, B, C and D, P<0.01). In results, the phenomenon that inhibited the proliferation and decreased the inflammation in RA-FLS induced by miR-125a-3p could be rescued by RA-FLS upregulation were observed (Figure 5 A, B, C and D, P<0.01).

Discussion

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterized by joint synovitis. Previous studies have indicated that miRNAs in cytoplasm could affect cellular biological phenotypes via targeting some key mRNAs to further take part in the development and progression of RA[19,20]. Therefore, in this study, we showed a protector role of miR-125a-3p in inhibiting the progression of RA-FLS. We suggest that miR-125a-3p was downregulated in RA-FLS, and upregulated miR-125a-3p could inhibit the proliferation ability and reduce the inflammatory response of RA-FLS. We have also showed that MAST3 is a downstream of miR-125a-3p, and MAST3 can rescue the effects of increased miR-125a-3p on RA-FLS to promote the progression of RA. Those observations suggests that miR-125a-3p could inactivate Wnt/β-catenin and NF-κB pathways and inhibit the progression of RA via targeting MAST3.

The relationship between the dysfunction of the miRNAs and the pathogenesis of various diseases has been widely accepted in recent years. The functions of miRNAs in impacting the progressions of diseases via modulating the activation or inactivation of several signaling pathways have also been reported by several studies[21]. Although the pathogenesis of RA remains unclear, the increasing evidence have indicated

that the dysregulation of some miRNAs is closely associated with the development and progression of RA. For instance, miR-338-3p was significantly upregulated in RA tissues, and miR-338-3p could enhance the inflammatory response via targeting SPRY1[22]. One study has found that downregulated miR-125a-3p has been previously identified as a landmark event in RA tissues[18]. In this study, we have illustrated the regulation mechanism involved mediating the anti-inflammatory effects of miR-125a-3p in RA-FLS. MiR-125a-3p has been confirmed as a tumor inhibitor to take part in the limitation of multiple cancers, and miR-125a-3p deficiency has also been related with inflammatory reflection of some diseases. For instance, miR-125a-3p has been proved as tumor inhibitor to involve in the negative regulation of lung adenocarcinoma progression[23]. Moreover, one study has confirmed that miR-125a-3p is significantly downregulated in the patients with systemic lupus erythematosus which has similar pathogenesis with RA[24]. in this study, the results suggests that the invention in expression level of miR-125a-3p could effectively inhibit the proliferation ability and reduce the expression level of some inflammatory factors of RA-FLS.

The inflammatory response and promotion of proliferation mediated by MAST3 have been identified in various diseases. MAST3 is abundant in the tissues of ulcerative colitis patients, and has been suggested as an inflammatory inducer to promote the inflammatory response in colonic mucosa[25]. Being overexpressed in the synovial tissue, MAST3 can promote the progression of RA via modulating the inflammatory response and proliferation of RA-FLS[26]. In this study, we determined that MAST3 is a downstream target of miR-125a-3p, and MAST3 could reverse the effects of upregulated miR-125a-3p in inflammatory response and cell proliferation of RA-FLS. The formation and development of RA involve in the changes of the multiple signal pathways including Wnt/β-catenin, NF-κB, JAK/STAT and so on[27]. For instance, the inhibition of Wnt signal pathway induced by increased MALAT1 can impair the proliferation ability of RA-FLS and decrease the inflammation level of RA[28]. Several studies have found that the increased inflammation level of the focus is notable character of RA, which has relationship with abnormal activation of NF-κB[29]. We also confirmed that the effect exerted by miR-125a-3p in reducing MAST3 expression directly involved the Wnt/β-catenin and NF-κB pathways, which further affects the proliferation and inflammatory response of RA-FLS. The data in this study suggests that miR-125a-3p plays a great role in inactivating the Wnt/β-catenin and NF-κB pathways via targeting MAST3.

To conclude, miR-125a-3p could inhibit the Wnt/β-catenin and NF-κB signaling to inhibit the progression of RAFLS via targeting MAST3.

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