PU.1 attenuates TNF-α-induced proliferation and cytokine release of rheumatoid arthritis fibroblast-like synoviocytes by regulating miR-155 activity

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Abstract. The present study aimed to determine the role of transcription factor PU.1 (PU.1) in tumor necrosis factor-α (TNF-α)-induced proliferation and cytokine release of rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS). It was determined that TNF-α-induced proliferation of RA-FLS, whereas transfection with PU.1 3′untranslated region (UTR) inhibited this proliferation. Additionally, PU.1 3′UTR attenuated TNF-α-induced production of interleukin (IL)-6 and IL-1β, and downregulated the expression level of micro RNA (miR)-155 in a dose-dependent manner. Furthermore, transfection with PU.1 3′UTR significantly attenuated TNF-α-induced decrease in forkhead box protein O3 (FOXO3) expression level in RA-FLS and these effects were consistent with the effects of miR-155 inhibition. PU.1 and FOXO3 formed a competing endogenous RNA (ceRNA) network that regulated miR-155 activity. In this competing endogenous RNA network, PU.1 3′UTR modulated FOXO3 expression in a miRNA- and 3′UTR-dependent manner. Downregulation of FOXO3 expression reversed the PU.1 3′UTR-mediated protective effects. Therefore, the results of the present study indicate that PU.1 3′UTR attenuates TNF-α-induced proliferation and cytokine release of RA-FLS by acting as a ceRNA for FOXO3 to regulate miR-155 activity.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory polyarthritis characterized by synovial heterogeneity (1). Fibroblast-like synoviocytes (FLS) serve a role in RA, a disease characterized by a continuous inflammatory response in the synovial membrane, leading to cartilage and joint damage (2). FLS, together with other immune cells, including macrophages and neutrophils, constitute an inflammatory environment in the synovium and further recruit numerous immune cells, contributing to joint damage (3). Following stimulation of inflammatory cytokines, including tumor necrosis factor (TNF-α), pro-inflammatory signaling is activated in FLS via release of a number of pro-inflammatory molecules, including interleukin (IL)-6 and IL-1β, which further aggravate the inflammatory processes (4). Consequently, elucidation of the mechanisms underlying the inflammatory process in FLS is necessary for identification of novel targets for treatment of RA.

The competing endogenous RNA (ceRNA) theory suggests that transcripts having common miRNA binding sites may modulate the other's expression by competing for the same miRNAs (5). The ceRNA network has been demonstrated to serve a role in tumors, pulmonary arterial hypertension and autophagy (6-9). However, the role of the ceRNA network in RA remains to be elucidated.

A number of previous studies demonstrated a role of miR-155 in RA (10-13). miR-155 has been identified as a proinflammatory regulator in clinical and experimental arthritis (10). Transcription factor PU.1 (PU.1), which is upregulated in miR-155 deficient B cells, is a target of miR-155 (11). miR-155/PU.1 pathway serves a role in RA, in addition to other diseases (11-13). However, to the best of the authors knowledge, the ceRNA network mediated by miR-155 in RA has not been previously investigated.

Therefore, the present study aimed to determine whether there is a miR-155-mediated ceRNA network that serves a role in the inflammatory process and the TNF-α-induced proliferation and cytokine release of FLS.

Materials and methods

Cells and reagents. Human rheumatoid arthritis FLS (RA-FLS) MH7A cells, human FLS (HFLS) and 293 cells were purchased from the Cell Bank of the Chinese Academy of Sciences of China (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific,
Inc., Waltham, MA, USA), supplemented with 15% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere in an environment containing 5% CO₂. TNF-α was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. To measure the expression level of miR-155, total RNA extracted from the MH7A and HFLS cells with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was reverse-transcribed using miScript II RT kit (Qiagen GMBH, Hilden, Germany). For determination of PU.1 and FOXO3 mRNA expression, iScript™ Advanced cDNA Synthesis kit (cat. no. 1725037; Bio-Rad Laboratories, Inc., Hercules, CA, USA) for RT-qPCR was used with the Prime PCR™ Assay Validation Report primer reagent (cat. no. 1723894; Bio-Rad Laboratories, Inc.). The RT-qPCR experiment was performed using ABI Prism 7500 Sequence Detector (Applied Biosystems; Thermo Fisher Scientific, Inc.). The 2−ΔΔCT method was used to determine the expression of each transcript (14). U6 small nuclear RNA (Guangzhou RiboBio Co., Ltd., Guangzhou, China) and GAPDH were used as control for miR-155 and genes, respectively. Primers are presented in Table I.

Plasmid construction and cell transfection. Lentiviral small hairpin (sh)RNA against human Dicer 1 (Lenti-shDicer), FOXO3 (Lenti-shFOXO3), PU.1 (Lenti-shPU.1) and a scrambled non-targeting shRNA were purchased from Sigma-Aldrich (Merck KGaA), and cloned into pLKO.1 vector (Sigma-Aldrich, USA). The detailed procedure was performed as previously described (16). FOXO3 (Lenti-shFOXO3), PU.1 (Lenti-shPU.1) and a scram hairpin (sh)RNA against human Dicer 1 (Lenti-shDicer), were transfected with Lenti-PU.1-3'UTR and subsequently treated with 50 ng/ml of TNF-α for 1 h (n=5/group). A total of 24, 48 and 72 h following Lenti-PU.1-3'UTR transfection, proliferation of MH7A was detected using an MTT assay kit (Beyotime Institute of Biotechnology, Haimen, China) following the manufacturer's protocol. Acetic acid (30%) was used to dissolve the purple formazan and a 570 nm wavelength was used to measure formazan.

ELISA. To determine the effect of PU.1 3'UTR on TNF-α-induced inflammation of MH7A cells, MH7A cells were transfected with Lenti-PU.1-3’UTR and subsequently treated with 50 ng/ml of TNF-α for 1 h (n=5/group). A total of 48 h following transfection with Lenti-PU.1-3’UTR, the concentration of IL–6 (cat. no. ab46042) and IL-1β (cat. no. ab214025; both Abcam, Cambridge, UK) in cell culture supernatant (obtained by centrifugation at 13,500 x g for 5 min at room temperature) was performed using IL–6 and IL-1β ELISA kits according to the manufacturer's protocol.

Transwell migration and invasion assays. To determine the effects of PU.1 3'UTR on RA-FLS MH7A migration and invasion ability, Transwell migration and invasion assays were carried out using 24-well Millicell Hanging Cell Culture inserts. Polyethylene Terephthalate 8 µm (Merck KGaA, Darmstadt, Germany), which were coated without or with Matrigel matrix gel (BD Biosciences, Franklin Lakes, NJ, USA). The detailed procedure was performed as previously described (16).

Adhesion assay. To determine the effect of PU.1 3'UTR on RA-FLS MH7A adhesion ability, the colorimetric MTT assay was used to determine the number of adherent cells. Acetic acid (30%) was used to dissolve purple formazan and 570 nm wavelength was used to measure formazan. Detailed procedure was conducted as previously described (17).

Luciferase reporter assay. To determine whether PU.1 3'UTR mediated its effect through the 3'UTR, pMIR-Report vector (Promega Corporation, Madison, WI, USA) was used to clone the fragments of PU.1 3'UTR and FOXO3 3'UTR, named Luc-PU.1-3’UTR and Luc-FOXO3-3’UTR, respectively. To investigate whether PU.1-mediated regulation of FOXO3 depends on the 3’UTR, MH7A cells overexpressing PU.1 3’UTR or PU.1 shRNA were co-transfected with Luc-FOXO3-3’UTR or an empty vector and β-gal expression vector (Hunan China Sun Medical Devices, Co., Ltd.) using Lipofectamine® 2000 according to the manufacturer's protocols. Transfection efficiency was determined using a Luciferase Reporter Assay kit (cat. no. K801-200; BioVision, Inc., Milpitas, CA, USA). β-gal activity was determined using a β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer (cat. no. E2000; Promega Corporation) for normalization, according to the manufacturer's protocols.

RNA immune co-precipitation (RIP) assays. The RIP assays were performed as previously described (16). Total RNA in the RIP assay product was determined via the aforementioned RT-qPCR protocol.
Western blot analysis. Detailed procedure was previously described (7). Antibodies against PU.1 (cat. no. ab76543; 1:5,000), FOXO3 (cat. no. ab12162; 1:4,000), Cyclin D1 (cat. no. ab134175; 1:3,000), c-Myc (cat. no. ab32072; 1:5,000) and Dicer (cat. no. ab14601; 1:2,000) were all purchased from Abcam. β-actin (cat. no. AM1021B; 1:5,000) was purchased from OriGene Technologies, Inc. (Rockville, MD, USA). Membranes were blocked with 5% non-fat milk and incubated with the primary antibodies in 5% bovine serum albumin (cat. no. A8020; Beijing Solarbio Science & Technology Co., Ltd.) at 4°C overnight, followed by incubation with the following secondary antibodies: Horseradish peroxidase (HRP)-conjugated goat-anti-rabbit (cat. no. TA130003; 1:5,000) and HRP-conjugated goat-anti-mouse (cat. no. THRPA140003; 1:5,000; both OriGene Technologies, Inc.). Blots were washed 3 times for 15 min each, and the signals were detected using enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Inc.) followed by exposure and densitometric analysis was performed using the Tanon 5200 automatic chemiluminescence image analysis system (Tanon Science & Technology Co., Ltd., Shanghai, China). β-actin was used as an internal control.

Statistical analysis. All data were obtained from at least three independent experiments, and presented as the mean ± standard deviation. Datasets with only two groups were analyzed using a Student’s t-test. Differences between multiple groups were analyzed using one-way analysis of variance with the Tukey-Kramer post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

PU.1 3’UTR inhibits TNF-α-induced proliferation and inflammation of MH7A cells. To determine the effects of PU.1 3’UTR on RA-FLSMH7A proliferation, MH7A cells were transfected with Lenti-PU.1-3’UTR for 6 h and subsequently treated with 50 ng/ml of TNF-α for 1 h. A total of 24, 48 and 72 h following transfection with Lenti-PU.1-3’UTR, proliferation of MH7A was assessed using an MTT assay. The results indicated that, compared with the control group, TNF-α induced the cell viability of MH7 A cells (Fig. 1A). However, transfection with Lenti-PU.1-3’UTR inhibited the proliferation induced by TNF-α. To determine the effects of PU.1 3’UTR on inflammation, MH7A cells were
transfected with Lenti-PUI.3'-UTR and subsequently treated with TNF-α for 48 h. ELISA analyses were used to detect the concentration of IL-6 and IL-1β in cell culture supernatant. Overexpression of PU.1 3’UTR significantly attenuated TNF-α-induced production of IL-6 and IL-1β (Fig. 1B and C).

PU.1 3’UTR inhibits TNF-α-induced miR-155 expression. miR-155/PUI.1 and miR-155/FOXO3 signaling pathways have been previously demonstrated to serve roles in RA and TNF-α was demonstrated to induce miR-155 expression in RA-FLS (2). The present study aimed to detect expression levels of miR-155 and FOXO3 in RA-FLS and HFLS.

Figure 2. PU.1 3’UTR inhibits TNF-α-induced miR-155 expression. (A) Expression levels of miR-155, PU.1 and FOXO3 were evaluated in HFLS and RA-FLS/MH7A cells. (B) miR-155 level was determined in MH7A cells. (C) FOXO3 mRNA level was detected in MH7A cells. Data are presented as the mean ± standard deviation. n=3. *P<0.05 and **P<0.01 vs. the respective control group. miR, microRNA; PU.1, transcription factor PU.1; TNF-α, tumor necrosis factor-α; FOXO3, forkhead box protein O3; HFLS, human fibroblast-like synoviocytes; RA-FLS, rheumatoid arthritis fibroblast-like synoviocytes.

Figure 3. PU.1 3’UTR promotes FOXO3 expression by acting as a ceRNA for FOXO3. Levels of (A) mRNA of FOXO3 and Dicer1 and (B) protein expression of FOXO3 detected in MH7A cells transfected with Lenti-shPU.1 with or without Lenti-shDicer. Downregulation of FOXO3 mRNA and protein levels induced by transfection with Lenti-shPU.1 was reversed by co-infection with Lenti-shDicer. (C) Luciferase activity of Luc-FOXO3-3’UTR. Absolute numbers of (D) miR-155, (E) PU.1 and (F) FOXO3 transcripts were detected by RT-qPCR combined with an internal standard curve. RT-qPCR was used to measure the abundance of (G) FOXO3 mRNA and (H) miR-155 levels in the Ago2-IP materials following the RIP assay in MH7A cells transfected with PU.1 3’UTR. (I) mRNA levels of FOXO3. (J) Western blot analysis of FOXO3 protein expression. (K) Quantitative analysis of FOXO3 and PU.1 protein expression. Data are presented as the mean ± standard deviation. n=3. **P<0.01 vs. the control group. Ns, not significantly different; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ago2-IP, protein argonaute-2; miR, microRNA; FOXO3, forkhead box protein O3; PU.1, transcription factor PU.1; CDS, coding sequence; Cq, results of 2-△△Cq method of quantification.
miR-155 level was upregulated in RA-FLS, however PU.1 and FOXO3 expression decreased, compared with the HFLS cells (Fig. 2A). Furthermore, transfection with Lenti-PU.1-3'UTR inhibited the TNF-α-mediated upregulation of miR-155 (Fig. 2B). Furthermore, transfection with Lenti-PU.1-3'UTR reversed the TNF-α-mediated inhibition of FOXO3 (Fig. 2C). Therefore, the results of the present study demonstrated that PU.1 3'UTR may regulate RA and be associated with miR-155 and FOXO3 activities.

PU.1 3'UTR promotes FOXO3 expression by acting as a ceRNA for FOXO3. Since PU.1 3'UTR and FOXO3 3'UTR both bind miR-155, PU.1 3'UTR may serve a role of a ceRNA for FOXO3 in MH7A cells. It was hypothesized in the present study that the effect of PU.1 3'UTR on FOXO3 expression is mediated by miRNAs. Cell lines with Dicer 1 knockdown, a ribonuclease critical for miRNA biogenesis, were used in the present study. Dicer 1 deficiency significantly reduces levels of mature miRNAs (6). Transfection with Lenti-shDicer1 demonstrated 60% knockdown efficiency and knockdown of PU.1 decreased FOXO3 expression in Dicer 1-proficient cells, however not that in isogenic Dicer 1 knockdown cells (Fig. 3A and B). In order to determine whether PU.1 3'UTR mediated its effect through its 3'UTR, FOXO3 3'UTR-luciferase reporter (Luc-FOXO3-3'UTR) was co-expressed in Dicer 1-proficient and -deficient MH7A cells with PU.1 knockdown. Knockdown of PU.1 decreased the activity of Luc-FOXO3-3'UTR, which was reversed in Dicer 1-knockdown cells (Fig. 3C). The absolute number of PU.1, FOXO3 and miR-155 transcripts was determined by RT-qPCR combined with an internal standard curve to determine their abundance (18). The results revealed that miR-155, PU.1 and FOXO3 were expressed at 8.66x10^{10}, 1.16x10^{10} and 5.22x10^{10} copies/cell multiplicity of infection in MH7A cells, respectively (Fig. 3D-F). RIP assay demonstrated that PU.1 3'UTR overexpression facilitated the association between protein argonaute-2 (ago2) and FOXO3 mRNA (Fig. 3G) and the association between ago2 and miR-155 (Fig. 3H). The results indicate that miR-155 directly binds PU.1 and FOXO3. Additionally, ectopic expression of PU.1-coding sequence (CDS) increased PU.1 protein level, however did not affect the protein level of FOXO3 (Fig. 3I-K).

The aforementioned results suggest that PU.1 3'UTR promotes FOXO3 expression by acting as a ceRNA for FOXO3. PU.1 3'UTR inhibition of TNF-α-induced proliferation and inflammation of MH7A cells is dependent on FOXO3 expression. Following elucidation of the interaction between PU.1 and FOXO3, potential relevance of this phenotype for TNF-α-induced proliferation and inflammation was investigated in RA-FLS MH7A cells. MH7A cells were transfected with Lenti-PU.1-3'UTR and Lenti-shFOXO3 for 6 h, and treated with 50 ng/ml TNF-α for 1 h. Following 24, 48 and 72 h of treatment, proliferation of MH7A was assessed using an MTT assay. The results indicated that knockdown of FOXO3 attenuated the inhibitory effect of Lenti-PU.1-3'UTR on TNF-α-induced proliferation of MH7A cells (Fig. 4A). Expression levels of cell proliferation markers cyclin D1 and c-Myc were significantly inhibited in TNF-α activated MH7A cells following PU.1 3'UTR overexpression (Fig. 4B-D). However, these effects were reversed in MH7A cells co-transfected with
Lenti-shFOXO3. Similarly, knockdown of FOXO3 reversed the anti-inflammatory effects of PU.1 3'UTR and resulted in upregulation of IL-6 and IL-1β levels, compared with the TNF-α+Lenti-PU.1 3'UTR group (Fig. 4E and F).

Effects of PU.1 3'UTR with a mutant type of miR-155 binding site on TNF-α-induced proliferation, cytokine release and FOXO3 expression were investigated in RA-FLSMH7A cells. It was determined that PU.1 3'UTR-mut exhibited no effect on TNF-α-induced proliferation (Fig. 6A) and FOXO3 expression (Fig. 6D) in RA-FLSMH7A cells. The aforementioned results suggest that PU.1 3'UTR inhibits TNF-α-induced RA-FLS proliferation.
by upregulation of FOXO3 via competitive binding of miR-155.

**PU.1 3'UTR inhibits MH7A cell migration, invasion and adhesion.** Following determination that RA-FLS exhibit an enhanced invasion phenotype, the present study further investigated whether PU.1 3'UTR modulated RA-FLS/MH7A migration, invasion and adhesion abilities. Infection with Lenti-PU.1-3'UTR markedly reduced migration and invasion abilities of MH7A cells (Fig. 6A and B). Additionally, overexpression of PU.1 3'UTR significantly attenuated the adhesive ability of MH7A cells (Fig. 6C). Therefore, the results of the present study demonstrated that ectopic expression of PU.1 3'UTR significantly inhibited migration, invasion and adhesion of MH7A cells.

**Discussion**

RA-FLS cells serve a role in RA and have been hypothesized to be associated with inflammation and joint damage in RA (1,3). Currently, although the immune system is the target for treatment of RA, developing novel drugs is necessary (19). Therefore, RA-FLS maybe targeted in novel methods of treatment of RA.

In the present study, the effects of PU.1 3'UTR on TNF-α-induced proliferation of RA-FLS and the associated release of pro-inflammatory cytokines were investigated. It was determined that PU.1 3'UTR protected against TNF-α-induced RA-FLS proliferation, decreased the expression of cell proliferation markers cyclin D1 and c-Myc, and reduced the levels of inflammatory factors IL-6 and IL-1β. ceRNA network between RNA transcripts mediated by shared miRNAs represents a novel method of gene regulation, which confers novel functions to non-coding RNAs (ncRNAs) or non-coding parts of mRNAs, for example 3'UTRs (6). ceRNA network has been demonstrated to serve a role in development (20). However, the roles of the ceRNA network in RA remain to be elucidated.

To determine whether PU.1 3'UTR exerts its functions by acting as a ceRNA in RA, FOXO3 has been investigated in the present study based on the observation that PU.1 and FOXO3 are potential targets of miR-155 in inflammation (11-13). miRNA-155 is a proinflammatory regulator in clinical and experimental arthritis (10), these results are consistent with the fact that ceRNAs and the shared miRNAs primarily hold the opposite effects (7). The present study demonstrated that PU.1 3'UTR-mediated upregulation of FOXO3 expression was dependent on the activity of miRNA. The aforementioned observation indicated that a ceRNA network is involved in the development of RA. However, multiple miRNAs may interact with a single 3'UTR. Future studies should therefore aim to investigate other ceRNAs, including ncRNAs, pseudogenes and circular RNAs, which maybe associated with the ceRNA network and therefore modulate RA pathogenesis.

In conclusion, the present study demonstrated that ceRNA interactions may mediate the crosstalk between different signaling pathways during RA development. Further analysis of ceRNA interactions between PU.1 and FOXO3 during RA development may provide insights into gene regulatory networks and their clinical implications for RA.

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**Availability of data and materials**

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

**Authors' contributions**

KW and BD designed the research included in the present study; ZX, YQ and PS analyzed the data; ZX, YQ, PS and BW performed the research; and ZX and YQ wrote the manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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