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

ZIKANG XIE[1], YUXING QU[2], PENGFEI SHEN[2], BIN WANG[2], KANG WEI[2] and BIN DU[3]

[1]Nanjing University of Traditional Chinese Medicine, Nanjing, Jiangsu 210023; [2]Department of Orthopedics, Changzhou Traditional Chinese Medicine Hospital, Changzhou, Jiangsu 213003; [3]Department of Orthopedics, Affiliated Hospital of Nanjing University of Traditional Chinese Medicine, Nanjing, Jiangsu 210000, P.R. China

Received July 18, 2017; Accepted November 11, 2017

DOI: 10.3892/mmr.2018.8920

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,

Correspondence to: Dr Bin Du, Department of Orthopedics, Affiliated Hospital of Nanjing University of Traditional Chinese Medicine, 155 Hanzhong Road, Nanjing, Jiangsu 210000, P.R. China
E-mail: bin_du@126.com

Key words: competitive endogenous RNA, forkhead box protein O3, microRNA-155, transcription factor PU.1, rheumatoid arthritis

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% CO_2. 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-PCR 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 $2^{-\Delta\Delta Cq}$ 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, Merck KGaA). PU.1 3'UTR or coding sequences of PU.1 were amplified by polymerase chain reaction (PCR) and PU.1 3'UTR with mutant-type of miR-155 binding site (mut) was synthesized using Fast Multi Site Mutagenesis System (Beijing Transgen Biotech Co., Ltd., Beijing, China). PCR-amplified sequences of PU.1 3'UTR, PU.1 3'UTR with mutant-type of miR-155 binding site or coding sequences of PU.1 were cloned using *SpeI* and *Xbal* sites of pLVX-IRES-ZsGreen1 vector (Hunan China Sun Medical Devices, Co., Ltd., Hunan, China), referred to as Lenti-PU.1-3'UTR, Lenti-PU.1-3'UTR-mut and Lenti-PU.1-coding sequence (CDS). The temperature protocol for PCR was as follows: Initial degeneration at 95˚C for 5 min, followed by 35 cycles of degeneration at 95˚C for 40 sec, annealing at 58˚C for 30 sec, extension at 72˚C for 2 min and a final extension step at 72˚C for 10 min. The primers used for PCR using 2xTaq PCR MasterMix (cat. no. PC1120; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) are presented in Table I. The cDNA reverse transcribed from total RNA, according to the aforementioned protocol, was used as the template. Lentiviral particles were packaged in 293 cells, as previously described (15).

miR-155 mimic/inhibitor (mimics, 5'-UUAAUGCUAAUG UGUAGGGGU-3'; inhibitor, 5'-AAUUACGAUUAUACA UCCUC-3') and negative control (NC; 5'-GGCGCUAGCGUC ACGUUUA-3') were synthesized by Guangzhou RiboBio Co., Ltd.. Lipofectamine® 2000 Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect miR-155 mimic, inhibitor and NC (all 5 nM).

Cell viability assay. To determine the effects of PU.1 3'UTR on RA-FLS proliferation, MH7A cells were transfected with Lenti-PU.1-3'UTR and subsequently treated with 50 ng/ml of TNF-α for 1 h (5 wells/group). Cells that were untreated served as the control 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.

Table I. Primer sequences used for polymerase chain reaction.

Gene	Sequence (5'→3')	
	Forward	Reverse
Cyclin D1	GCCCGCAGCCCCCGCCGGGCCCCGC	GCGGGGCCCGGCGGGGGCTGCGGGC
c-Myc	TTTTGAGAGGTGGAGAAAGAGATG	AAAGAAGTGCAGAAATAATGAGCG
FOXO3	AATAAAGCAGTCAAAAAGAAGTCC	ACAGCAAGAAAAATAAAACCAGAG
Dicer1	ACTGTGGAAGTAGTAGGAAAGGGG	ACAAAGCAGAAGTGAGGAAAGAAG
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
PU.1-CDS	CTAGACTAGTATGTTACAGGC GTGCAAAATGGAAG	CTAGTCTAGATCAGTGGGGCG GGTGGCGCCGCTCG
PU.1 3'UTR	CTAGACTAGTCCAGGCCT CCCCGCTGGCCATAGCA	CTAGTCTAGAGATGGATT GAGAATAACTTTACTTG
miR-155	TTAATGCTAATGTGTAGGGGT	ACCCCTACACATTAGCATTAA

c-Myc, Myc proto-oncogene protein; FOXO3, forkhead box protein O3; CDS, coding sequence; UTR, untranslated region; miR, microRNA.

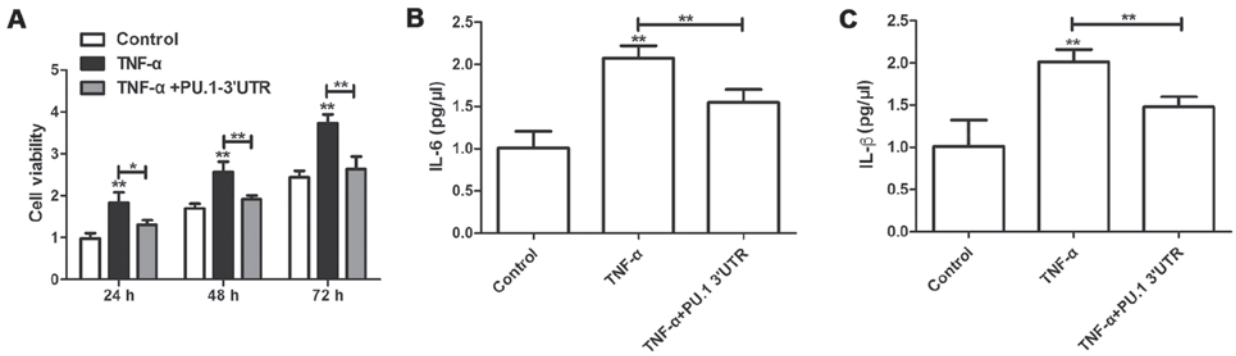

Figure 1. PU.1 3'UTR inhibits TNF-α-induced proliferation and inflammation of MH7A cells. (A) Viability of wild type MH7A cells and MH7A cells transfected with Lenti-PU.1-3'UTR prior to TNF-α treatment was evaluated by MTT assay. Compared with the control, TNF-α induced proliferation of wild type MH7A cells. Levels of (B) IL-6 and (C) IL-1β in cell culture supernatants detected by ELISA. Data are presented as the mean ± standard deviation. n=3. *P<0.05 and **P<0.01 vs. the control. IL, interleukin; UTR, untranslated region; TNF-α, tumor necrosis factor-α; PU.1, transcription factor PU.1.

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-FLSMH7Aproliferation, 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 MH7A 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

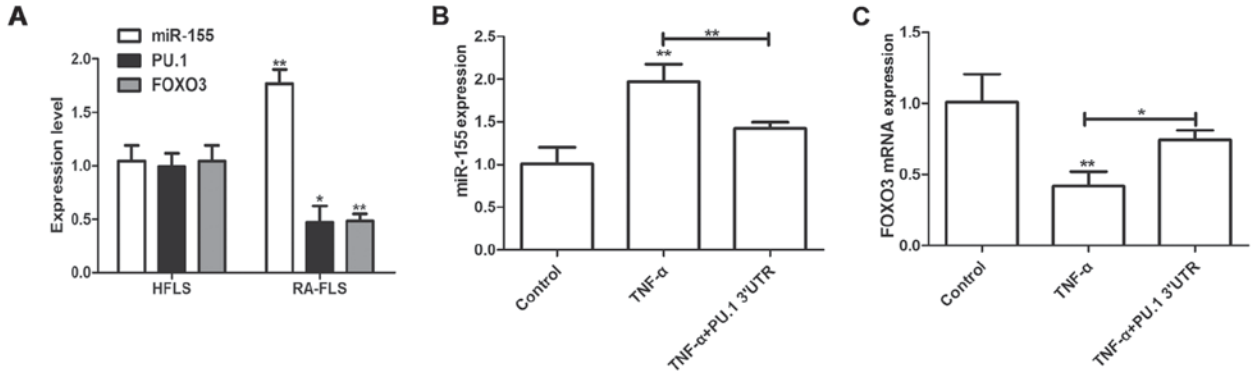

Figure 2. PU.1 3'UTR inhibits TNF-α-induced miR-155 expression. (A) Expression levels of miR-155, PU.1 and FOXO3 were evaluated in HFLS and RA-FLSMH7A cells. (B) miR-155 level was determined in MH7A cells. (C) FOXO3 mRNA level was detected in MH7A cells. Data are presented as the mean ± standard deviation. 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.

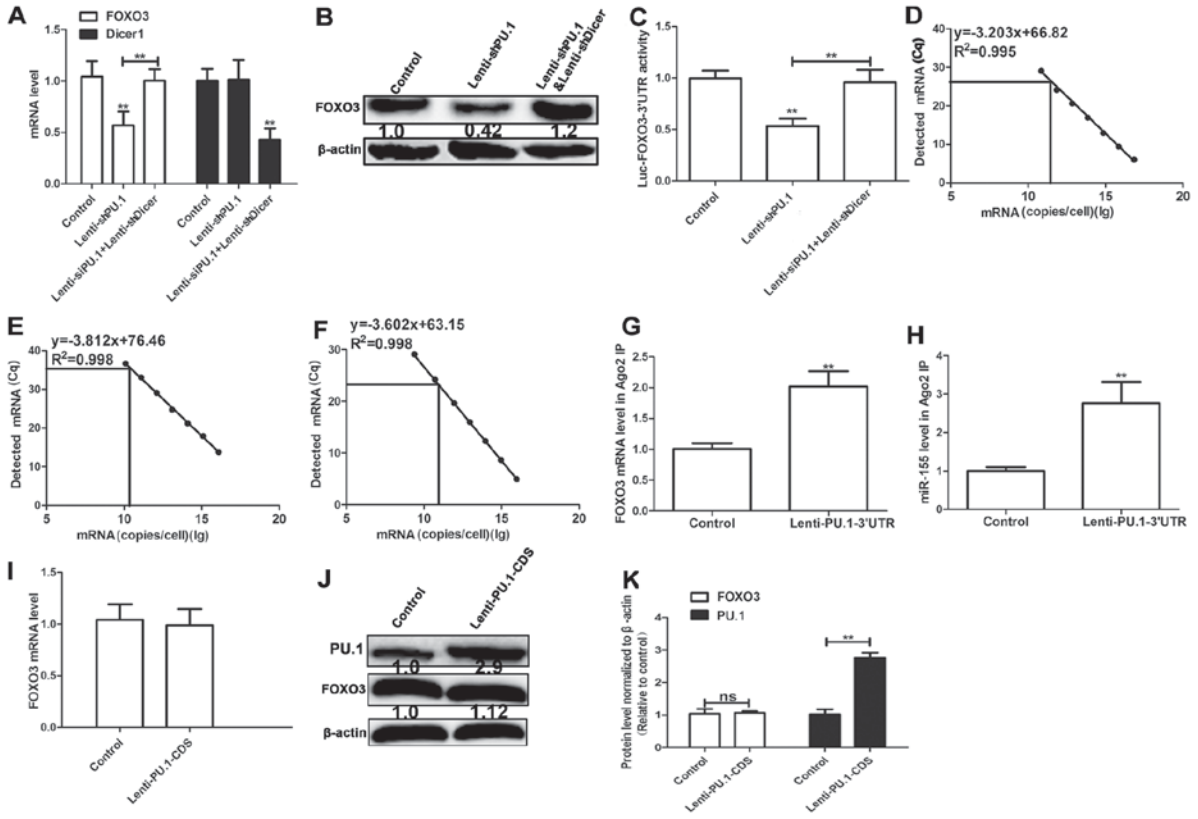

Figure 3. PU.1 3'UTR promotes FOXO3 expression by acting as a ceRNA for FOXO3. Levels of (A) mRNA of FOXO3 and Dicer1 and (B) protein expression of FOXO3 detected in MH7A cells transfected with Lenti-shPU.1 with or without Lenti-shDicer1. Downregulation of FOXO3 mRNA and protein levels induced by transfection 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 $2^{-\Delta\Delta Cq}$ method of quantification.

transfected with Lenti-PU.1-3'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/PU.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.

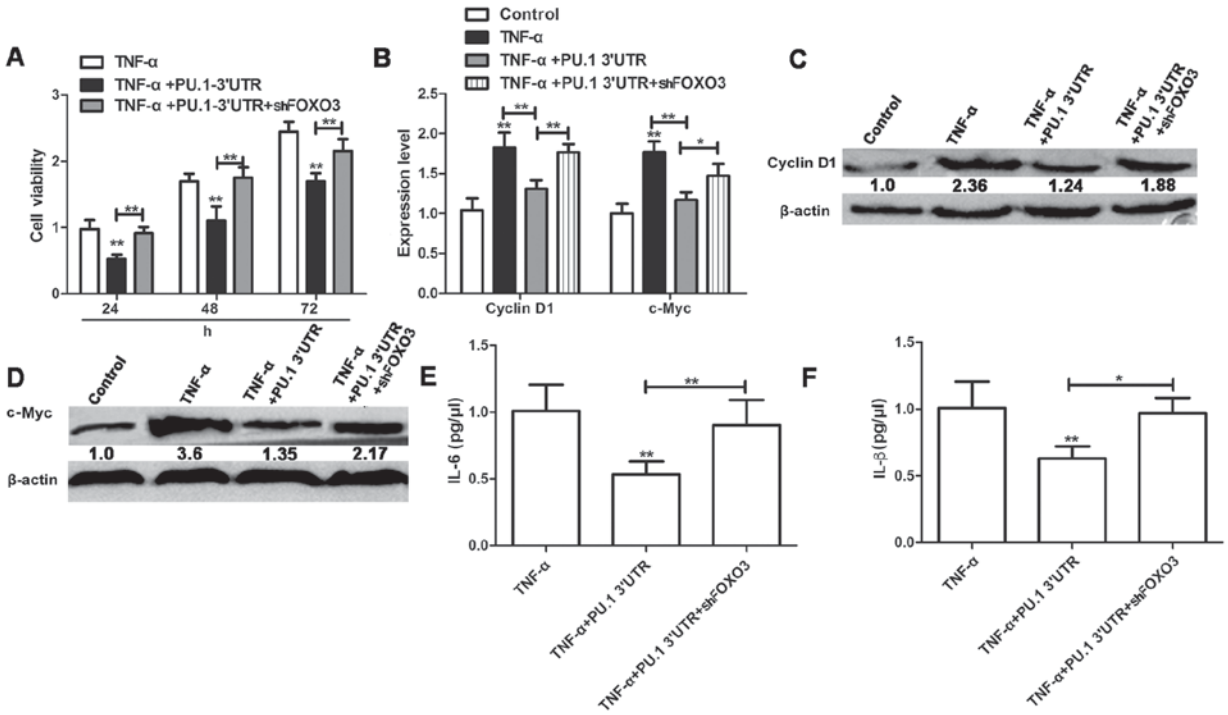

Figure 4. PU.1 3'UTR inhibits TNF-α-induced proliferation and inflammation of MH7A cells dependent on FOXO3 expression. (A) Viability of MH7A cells transfected with Lenti-PU.1-3'UTR and Lenti-shFOXO3 or untreated prior to incubation with TNF-α, was evaluated by MTT assay. (B) mRNA expression level of cyclin D1 and c-Myc. Expression of (C) cyclin D1, (D) c-Myc, (E) IL-6 and (F) IL-1β were detected in cells transfected or not transfected with Lenti-PU.1-3'UTR+Lenti-shFOXO3 prior to TNF-α treatment. Data are presented as the mean ± standard deviation. n=3. *P<0.05 and **P<0.01 vs. the respective control group. PU.1, transcription factor PU.1; UTR, untranslated region; TNF-α, tumor necrosis factor-α; FOXO3, forkhead box protein O3; sh, small hairpin RNA; c-Myc, Myc proto-oncogene protein; IL, interleukin.

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

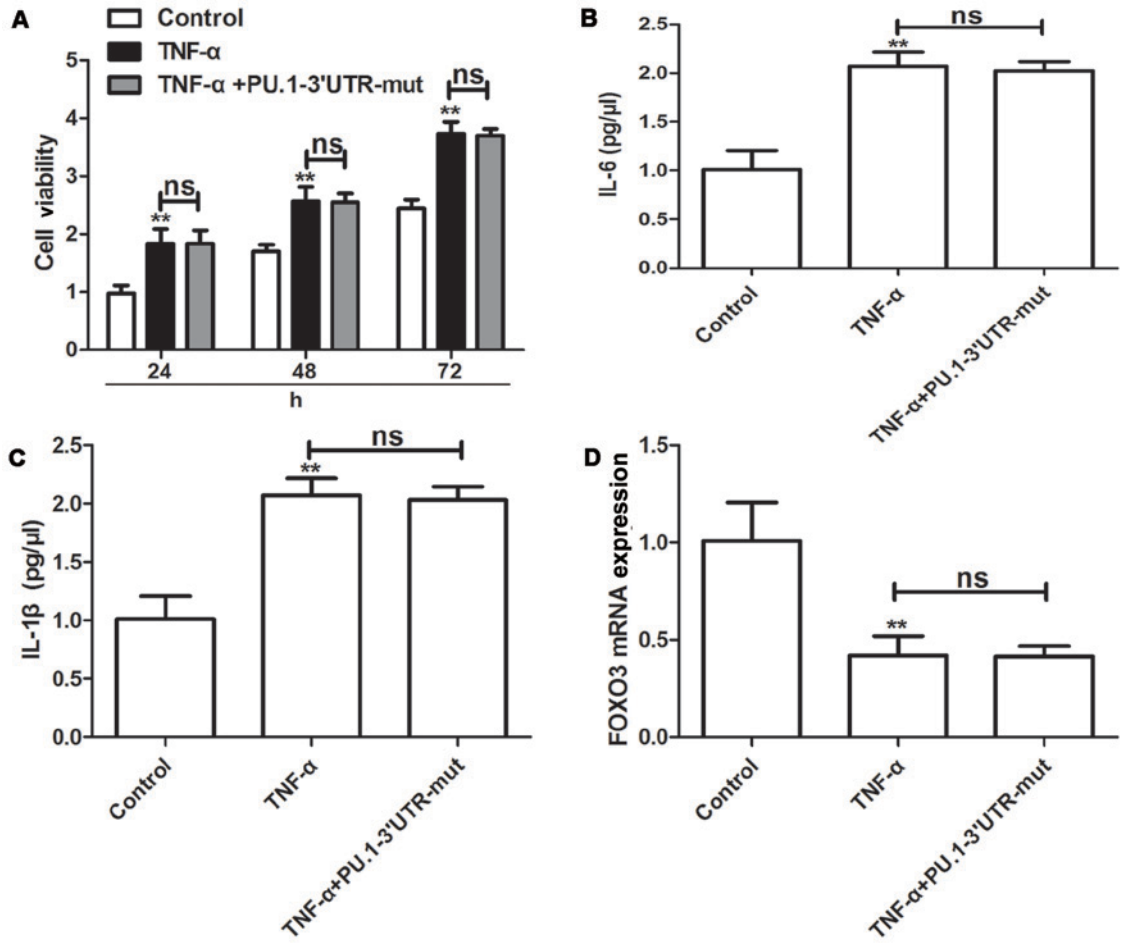

Figure 5. PU.1 3'UTR-mut has no effect on TNF-α-induced proliferation, cytokine release and FOXO3 expression in RA-FLS. (A) Viability of MH7A cells transfected with Lenti-PU.1-3'UTR-mut or untreated prior to TNF-α treatment was evaluated by MTT assays. Levels of (B) IL-6 and (C) IL-1β in cell culture supernatants were detected by ELISA. (D) FOXO3 mRNA expression level. Data are presented as the mean ± standard deviation. n=3. **P<0.01 vs. the respective control group. Mut, mutant type of miR-155 binding site; PU.1, transcription factor PU.1; UTR, untranslated region; TNF-α, tumor necrosis factor-α; ns, not significantly different; miR, microRNA.

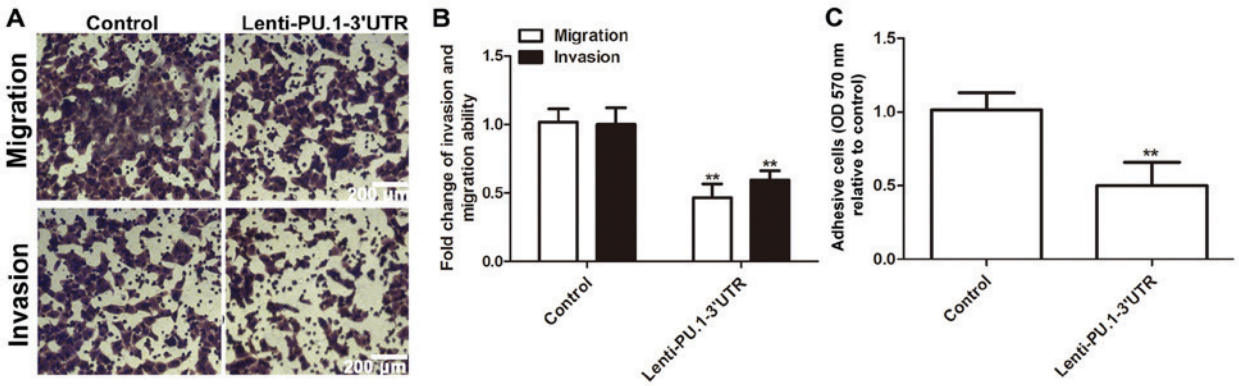

Figure 6. PU.1 3'UTR inhibits MH7A cell migration, invasion and adhesion. (A) Images of invasive and migratory cells. (B) Quantitative analysis of migration and invasion abilities of control cells and cells transfected with Lenti-PU.1 3'UTR. (C) MH7A cell adhesion ability. Data are presented as the mean ± standard deviation. n=3. **P<0.01 vs. the control group. PU.1, transcription factor PU.1; UTR, untranslated region.

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-β 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. 5A), cytokine release (Fig. 5B and C) and FOXO3 expression (Fig. 5D) 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-FLSMH7A 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.

Acknowledgements

Not applicable.

Funding

No funding was received.

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.

References

1. Dennis G Jr, Holweg CT, Kummerfeld SK, Choy DF, Setiadi AF, Hackney JA, Haverty PM, Gilbert H, Lin WY, Diehl L, *et al*: Synovial phenotypes in rheumatoid arthritis correlate with response to biologic therapeutics. Arthritis Res Ther 16: R90, 2014.
2. Liu N, Feng X, Wang W, Zhao X and Li X: Paeonol protects against TNF-α-induced proliferation and cytokine release of rheumatoid arthritis fibroblast-like synoviocytes by upregulating FOXO3 through inhibition of miR-155 expression. Inflamm Res 66: 603-610, 2017.
3. Chang SK, Gu Z and Brenner MB: Fibroblast-like synoviocytes in inflammatory arthritis pathology: the emerging role of cadherin-11. Immunol Rev 233: 256-266, 2010.
4. Bartok B and Firestein GS: Fibroblast-like synoviocytes: Key effector cells in rheumatoid arthritis. Immunol Rev 233: 233-255, 2010.
5. Salmena L, Poliseno L, Tay Y, Kats L and Pandolfi PP: A ceRNA hypothesis: The rosetta stone of a hidden RNA language? Cell 146: 353-358, 2011.
6. Li X, Zheng L, Zhang F, Hu J, Chou J, Liu Y, Xing Y and Xi T: STARD13-correlated ceRNA network inhibits EMT and metastasis of breast cancer. Oncotarget 7: 23197-23211, 2016.
7. Zheng L, Li X, Gu Y, Lv X and Xi T: The 3'UTR of the pseudogene CYP4Z2P promotes tumor angiogenesis in breast cancer by acting as a ceRNA for CYP4Z1. Breast Cancer Res Treat 150: 105-118, 2015.
8. Ma K, Zhao Q and Li S: Competing endogenous RNA network in pulmonary arterial hypertension. Int J Cardiol 172: e527-e528, 2014.
9. Ge D, Han L, Huang S, Peng N, Wang P, Jiang Z, Zhao J, Su L, Zhang S, Zhang Y, *et al*: Identification of a novel MTOR activator and discovery of a competing endogenous RNA regulating autophagy in vascular endothelial cells. Autophagy 10: 957-971, 2014.

10. Kurowska-Stolarska M, Alivernini S, Ballantine LE, Asquith DL, Millar NL, Gilchrist DS, Reilly J, Ierna M, Fraser AR, Stolarski B, *et al*: MicroRNA-155 as a proinflammatory regulator in clinical and experimental arthritis. Proc Natl Acad Sci USA 108: 11193-11198, 2011.
11. Alivernini S, Kurowska-Stolarska M, Tolusso B, Benvenuto R, Elmesmari A, Canestri S, Petricca L, Mangoni A, Fedele AL, Di Mario C, *et al*: MicroRNA-155 influences B-cell function through PU.1 in rheumatoid arthritis. Nat Commun 7: 12970, 2016.
12. Lu D, Nakagawa R, Lazzaro S, Staudacher P, Abreu-Goodger C, Henley T, Boiani S, Leyland R, Galloway A, Andrews S, *et al*: The miR-155-PU.1 axis acts on Pax5 to enable efficient terminal B cell differentiation. J Exp Med 211: 2183-2198, 2014.
13. Huskova H, Korecka K, Karban J, Vargova J, Vargova K, Dusilkova N, Trneny M and Stopka T: Oncogenic microRNA-155 and its target PU.1: An integrative gene expression study in six of the most prevalent lymphomas. Int J Hematol 102: 441-450, 2015.
14. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
15. Zhang H, Wang F and Hu Y: STARD13 promotes hepatocellular carcinoma apoptosis by acting as a ceRNA for Fas. Biotechnol Lett 39: 207-217, 2017.
16. Wang Y and Lin G: TP53INP1 3'-UTR functions as a ceRNA in repressing the metastasis of glioma cells by regulating miRNA activity. Biotechnol Lett 38: 1699-1707, 2016.
17. Yang J, Li T, Gao C, Lv X, Liu K, Song H, Xing Y and Xi T: FOXO1 3'UTR functions as a ceRNA in repressing the metastases of breast cancer cells via regulating miRNA activity. FEBS Lett 588: 3218-3224, 2014.
18. Denzler R, Agarwal V, Stefano J, Bartel DP and Stoffel M: Assessing the ceRNA hypothesis with quantitative measurements of miRNA and target abundance. Mol Cell 54: 766-776, 2014.
19. Zhu J, Wang H, Chen J and Wei W: Inhibition of plasma kallikrein-kinin system to alleviate renal injury and arthritis symptoms in rats with adjuvant-induced arthritis. Immunopharmacol Immunotoxicol 40: 134-148, 2018.
20. Xu J, Feng L, Han Z, Li Y, Wu A, Shao T, Ding N, Li L, Deng W, Di X, *et al*: Extensive ceRNA-ceRNA interaction networks mediated by miRNAs regulate development in multiple rhesus tissues. Nucleic Acids Res 44: 9438-9451, 2016.