MiR-338-5p suppresses rheumatoid arthritis synovial fibroblast proliferation and invasion by targeting ADAMTS-9

Y. Sun, X. Sun, Z. Liu, X. Wang, Y. Li

Department of Rheumatology, the Second Affiliated Hospital of Harbin Medical University, Heilongjiang, China.

Abstract

Objective

We proposed to find out the role of miR-338-5p played in cell proliferation and invasion of rheumatoid arthritis synovial fibroblasts (RASFs) by regulating ADAMTS-9.

Methods

QRT-PCR was performed to quantify the miR-338-5p and ADAMTS-9 mRNA expression in RA sample tissues and normal synovial tissues. Western blot was performed to evaluate the ADAMTS-9 protein levels in transfected RASFs. Luciferase reporter assays were used to demonstrate whether miR338-5p directly targets ADAMTS-9. MTT, Transwell and wound healing assays were respectively used to evaluate the growth and mobility of RASFs. Flow cytometry was applied to detect cell cycle distributions and apoptosis rates in transfected RASFs.

Results

MiR-338-5p was significantly downregulated in rheumatoid arthritis (RA) tissues while ADAMTS-9 was obviously overexpressed ($p<0.001$). Luciferase reporter assays demonstrated that miR-338-5p directly targeted ADAMTS-9. Moreover, overexpression of miR-338-5p suppressed RASFs biological functions and induced G0/G1 arrest and apoptosis of RASFs ($p<0.001$), while all the effects could be efficiently attenuated by the upregulation of ADAMTS-9.

Conclusion

By inhibiting ADAMTS-9, miR-338-5p suppressed the proliferation and metastasis of rheumatoid arthritis synovial fibroblasts. Thus, replenishing miR-338-5p may be a potential therapy for the clinic management of RA.

Key words

MiR-338-5p, ADAMTS-9, rheumatoid arthritis, RASFs

You Sun, PhD
Xuehui Sun, BD
Zixuan Liu, MD
Xiaoxue Wang, MD
Yang Li, PhD

Please address correspondence to:
Dr You Sun,
Department of Rheumatology,
the Second Affiliated Hospital
of Harbin Medical University,
246 Xuefu Road,
Harbin,
Heilongjiang 150086, China.
E-mail: hydfsyy@163.com

Received on January 19, 2017; accepted in revised form on June 8, 2017.

Funding: the work was supported by the National Natural Science Foundation of China (NSFC) (no. 81202339) and the Basic Research of the Science and Technology Project Sponsored by Educational Department of Heilongjiang Province (no. 11511238).

Competing interests: none declared.

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease that progressively destroys the joints and severely jeopardises the normal life of patients (1, 2). Common clinical symptoms include non-septic proliferative synovitis, articular dysfunctions, inflammation around the lung and heart and even lifelong disability (3-5). Clustering in the centre of pathogenic lesion, generating various chemokines and matrix-degrading enzymes to modulate the interaction with adjacent inflammatory tissues, Rheumatoid arthritis synovial fibroblasts (RASFs) actively contributed to the initiation and perpetuation of RA (1, 2). Previous studies indicated that RASFs share similar properties with tumour cells: aberrant cell proliferation, migration and invasion, as well as decreased cell apoptosis (3, 6). Furthermore, RASFs were found to be responsible for the destruction of surrounding tissues and act a pro-inflammatory role in immune responses (7). Therefore, figuring out the potential mechanism regulating the proliferation and metastasis of RASFs in RA pathology was meaningful to provide novel treatment strategies.

MicroRNAs (miRNAs) are a collection of small non-coding RNAs, each consisting of about 20 nucleotides (8). Accumulating evidence manifests the gene expression regulation function of miRNAs (9). It is predicted that human genome contains approximately 1500 miRNAs that may have control over the expression of about one third of our genes (10). The abnormal expression of various miRNAs might be implicated with the pathogenesis of severe disorders, such as cancer and infection (10, 11). Specific to immune-mediated disorders, the potential effect of miRNAs on RA has recently arisen indicating that a miRNA-based therapeutic method is plausible (10). In 2011 Stanczyk *et al*. revealed the puzzling altered-expression of miR-203 at different stages of RA samples (12). By targeting Follistatin-Like Protein 1 and restraining the TLR4/NFB pathway, miR-27a inhibited the migration and invasion of RASFs (13). MiR-338-5p has been reported as tumour-suppressor in colorectal cancer, gastric cancer, hepatocellular carcinoma, etc., inhibiting the tumour progress and metastasis (14-16). However, the effects of miR-338-5p in RASFs have long been overlooked while the microarray assays demonstrated a possible link to the promotion of RASF which thus interested us. Then with a series of experiments, we observed aberrant levels and significant effects of miR-338-5p in RASFs. To explore the regulatory mechanism of miR-338-5p, we combined the analysis of microarray assays and TargetScan database and found the potential downstream gene, ADAMTS-9, which was directly targeted by miR-338-5p. A disintegrin-like and metalloproteinase with thrombospondin type 1 motif (ADAMTS) is a group of genes encoding corresponding enzymes. ADAMTS-9, a member of the family, is located on chromosome 3p14.2 and has been confirmed to be involved in the inhibition of cell-autonomous angiogenesis which lead to the suppression of human cancers (17). However, ADAMTS-9 also functions as aggrecanase gene proteolytically process aggrecan and has a synergistic effect on other pro-inflammatory cytokines, which accompanies a series of cellular responses that contribute to the degradation of human cartilage (18). Thus, we took great interest in the role of ADAMTS-9 in RASFs pathogenesis and its interaction with miR-338-5p.

In this study, we explored the concrete role miR-338-5p in the proliferation, metastasis and apoptosis of RASF and verified the potential regulatory mechanism by targeting ADAMTS-9. The results suggest that restoring miR-338-5p expression might be a new therapy for clinical management of RA.

Materials and methods

Clinical sample collection

A total of 15 cases of synovial tissues were obtained from RA patients during knee joint replacement surgery in the Second Affiliated Hospital of Harbin Medical University between September 2014 and February 2016. All the RA patients recruited were diagnosed according to 1987 revised criteria of the American College of Rheumatology

(ACR) (19). 10 cases of normal synovial tissues were collected from patients undergoing total knee joint replacement or amputation due to serious trauma. All sample tissues were stored in brine ice. None of the patients had received any preoperative treatments. Written informed consents were provided by every patient and the research was supported by the Institutional Review Board of the Second Affiliated Hospital of Harbin Medical University.

Cell culture

The RA synovial tissues obtained were cut into pieces and digested by trypsin for 4h. Then the cells were harvested after centrifugation and incubated in DMEM (Hyclone, USA) containing 20% FBS (Hyclone, USA). The fifth to eighth generations of passaged cells were harvested for further experiments when grown to 90% confluence. HUVEC was purchase from BeNa Culture Collection and maintained in cell culture flasks coated with 0.1% gelatin (Wako Pure Chemical Industries, Ltd) in endothelial cell basal medium (Invitrogen, Grand Island, NY, USA) supplemented with low serum growth supplement (Invitrogen, Grand Island, NY, USA). Confluent cultures of HUVECs were serially passaged and used between passages 3 and 6. All cells are incubated at 37°C in 5% CO_2 in humidified incubator.

RNA isolation and quantitative Real Time-PCR (qRT-PCR)

Total RNA of collected samples was extracted using TRIzol Reagent (Invitrogen, USA). For miRNA detection, cDNAs were transcribed and qRT-PCR analysis was applied using a sequence detector (ABI-Prism, Applied Biosystems). U6 snRNA was used as an internal reference for miR-338-5p while GAPDH was for ADAMTS-9. The relative quantification of gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method. Specific primer sequences set for miR-338-5p and ADAMTS-9 are listed in Table I.

Table I. Specific primer sequences for RT-PCR.

	Forward primers	Reverse primers
MiR-338-5p	5'-AACAAUAUCCUGGUGCUGAGU-3'	
ADAMTS-9	5'-GGACAAGCGAAGGACATCC-3'	5'-ATCCATCCATAATGGCTTCC-3'
U6	5' -CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	5'-GATTTGGTCGTATTGGGC-3'	5'-CGTGTTGTCATACTTCTC-3'

Cell transfection

For transient transfections, RASFs and HUVEC cells were seeded in a 24-well plate. After cell fusion reached more than 70%, cells were subsequently transfected with miR-338-5p mimics, mimics control, miR-338-5p inhibitors or ADAMTS-9 siRNA respectively using Lipofectamine™2000 (Invitrogen, USA) as instructions guided. MiR-338-5p mimics, inhibitors and their controls were synthesised and purified by GenePharma Co, LTD (Shanghai, China).

Luciferase reporter assays

The plasmids with wild or mutated sequence of ADAMTS-9 3'UTR were sub-cloned into pmiRGLO Dual-Luciferase miRNA Target Expression Vector (Promega, USA). When RASFs grew to 80% confluence, cells were then co-transfected with plasmids containing wild or mutant type ADAMTS-9 3'UTR and miR-338-5p mimics or control. After 48h of incubation, a Sirius Luminometer (Berthold Detection Systems, Germany) was used to measure Firefly and Renilla luciferase activities. Data were shown as Firefly/Renilla luciferase ratios.

Western blot

After 48 of incubation, the total protein of frozen RASFs was extracted using RIPA Reagent (Invitrogen, Carlsbad, CA, USA) and its concentration was detected using the bicinchoninic acid protein assay kit (Beyotime, China). A total amount of 100 μg protein sample was separated by SDS-PAGE and electro-transferred to PVDF membranes (Santa Cruz biotechnology, Inc., USA). PVDF membranes were then blocked with 5% non-fat milk and incubated overnight with rat anti-human ADAMTS and GAPDH primary antibodies (Abcam, Cambridge, USA), diluted at 1:1000. The membranes were subsequently incubated with horseradish peroxidase-labeled goat anti-rat secondary antibodies (diluted at 1:4000) for 1 h. Protein levels were visualised using chemiluminescence detection (SignaGen, Rockville, MD, USA).

Endothelial cell tube formation assay

After 48h of transfection, HUVECs (2×10^4 cells/ml) were seeded in a 96-well tissue culture plate (50 μl per well) coated with Growth factor-depleted Matrigel (BD Pharmingen, Bedford, MA, USA). Plate was incubated at 37 °C, 5% CO_2 for 12 h, tube forming condition was observed under Zeiss Observer Z1 microscope (×100). Each experiment was conducted in triplicate.

MTT assay

Twenty-four hours after transfection, transfected cells were harvested and seeded into a 96-well plate at the density of 5×10^3 cells per well. 10 μl MTT reagent (Sigma-Aldrich, St Louise, MO, USA) was added to the 96-well plate once a day within 72h. Additional 100μl DMSO solution was added after centrifugation at 1000 rpm for 5min. The absorbance of tested RASFs was measured at 570 nm under a multi-well spectrophotometer (bio-Tek Instruments, Winooski, VT, USA).

Transwell assay

After 36h of transfection, RASFs were harvested and digested with trypsin and re-suspended in DMEM. An amount of 2×10^5 RASFs were grown in the upper Transwell chambers coated with 100μL Matrigel (BD biosciences, Bedford, MA, USA) while 100 μL 10% FBS were added to the lower chambers. The chambers were incubated for 24h, then fixed by paraformaldehyde and subsequently stained by crystal violet for 30 min. The average cell numbers of random five fields were observed, photographed and recorded under an optical microscope. The numbers of RASFs invaded through Matrigel-coated membranes were calculated.

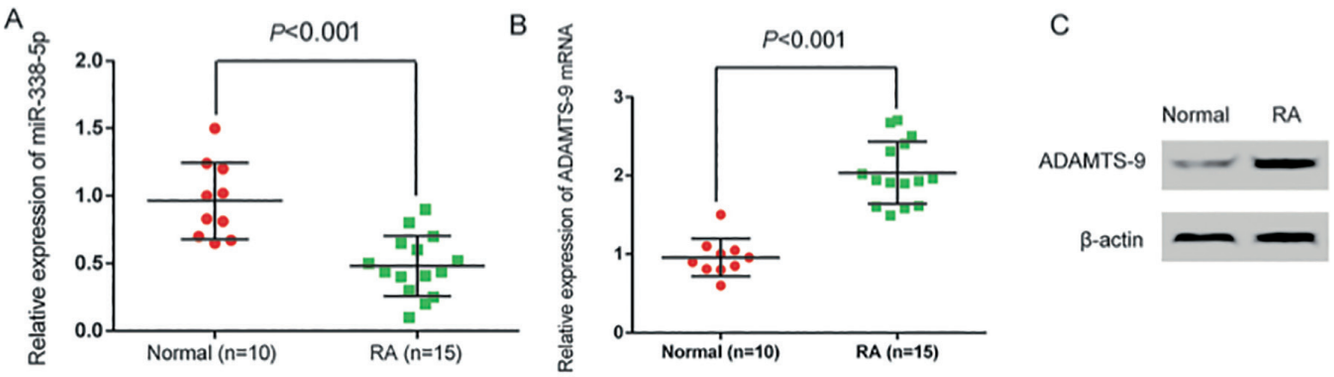

Fig. 1. **A**: MiR-338-5p was downregulated in RA sample tissues while ADAMTS-9 was upregulated. **B**: MiR-338-5p and ADAMTS-9 mRNA expression levels in RA tissues and normal synovial tissues. **C**: The protein levels of ADAMTS-9 in RASFs were detected with Western blot.

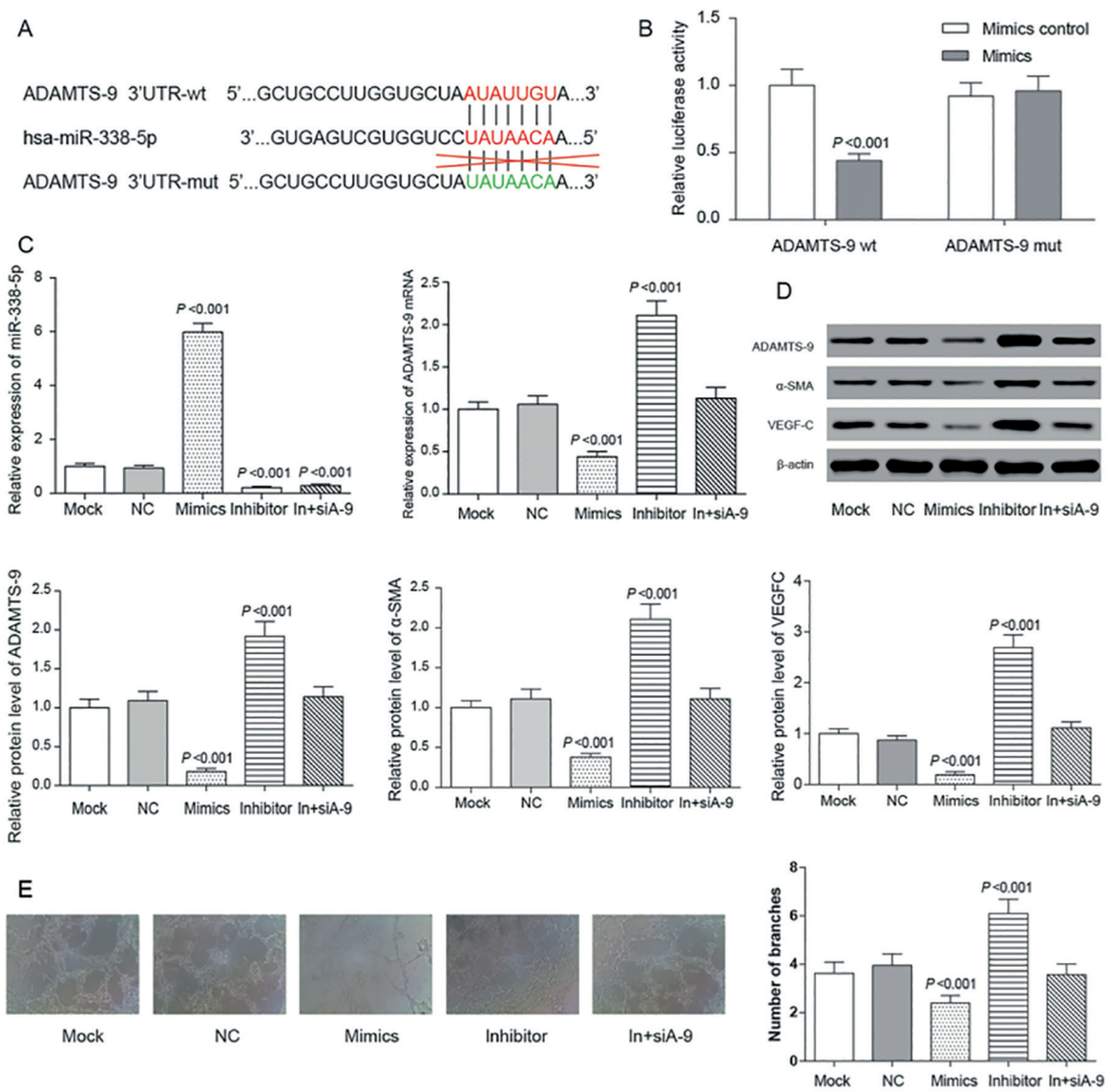

Fig. 2. ADAMTS-9 was a direct target for miR-338-5p. **A**: Predicted target sequence of miR-338-5p in the 3'UTR of ADAMTS-9 and the mutated sequence of the 3'UTR. **B**: Luciferase reporter assays in RASFs co-transfected with wild or mutant ADAMTS-9 vectors and miR-338-5p mimics or mimics control. **C**: qRT-PCR was utilised to determine the miR-338-5p and ADAMTS-9 expression at 48h after transfection. **D**: Western blot was used to detect the protein level of ADAMTS-9, VEGF-C and α-SMA at 48h after transfection. Semi-quantitative analysis was performed using Image J. **E**: Tube formation of transfected HUVEC cells was performed to study the angiogenesis effect of miR-338-5p/ADAMTS-9. Data was presented as mean ± SD from three independent experiments. $p<0.001$ compared with the Mock group. Data was presented as mean±SD from three independent experiments. $p<0.001$ compared with the Mock group.

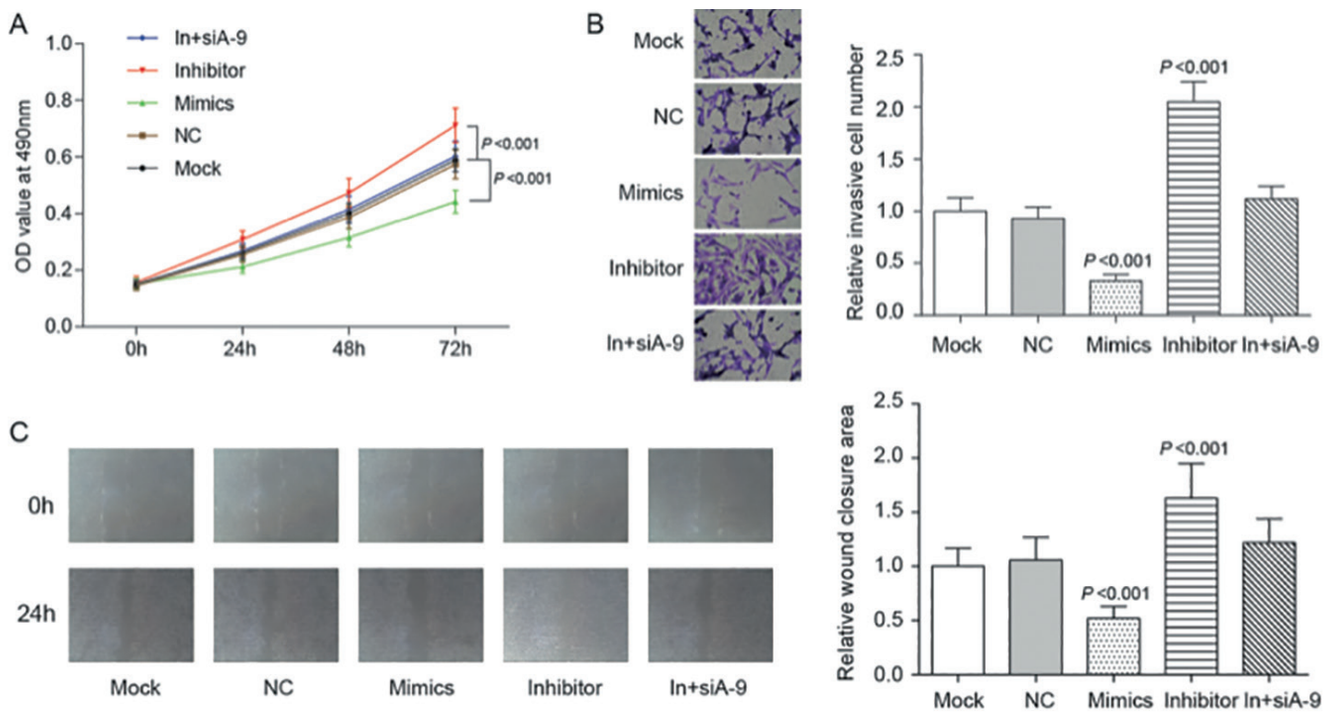

Fig. 3. MiR-338-5p suppressed RASFs proliferation and mobility of RASFs. **A**: The effect of miR-338-5p on RASFs proliferation measured by MTT assay. **B**: The numbers of RASFs that invaded through Matrigel-coated membrane were calculated 72 hours after transfection. **C**: Wound healing assay was performed to evaluate the effect of miR-338-5p on RASFs migration. Data was presented as mean±SD from three independent experiments. $p<0.001$ compared with the Mock group.

Wound healing assay

After 36h of transfection, cells were harvested and seeded into a 6-well plate at a density of 1×10^5 cells/well. When grown to 90% confluence, wound were created using a 200 µl pipette tip and washed three times by PBS to remove the debris. Wound healing was observed and photographed after 24h of incubation within the scrape line using an optical microscope.

Cell cycle and apoptosis detection

RASFs seeded in plates were harvested after transfection for 48 h, then digested by trypsin and fixed by ice ethanol. For cell cycle detection, RASFs were washed twice by ice PBS followed with an additional of 100 µl PI dye and 100 µl RNaseA in a dark condition. Subsequently, propidium iodide (PI) was added for colouring. Flow cytometry was then performed to quantify the proportions of RASFs in each cycle phase. For cell apoptosis detection, RASFs were centrifuged at 1000 rpm for 5 mins, which was followed by the addition of 100 µl AnnexinV-FITC and 100 µl PI in a dark condition. Flow cytometry was performed again to analyse cell apoptosis in early or advanced stage and necrosis.

Statistical analysis

All data were analysed with GraphPad Prism 6.0. Continuous data were displayed as mean ± standard deviation ($\bar{x}$ ± SD), in which the differences within two different groups were analysed by Student's *t*-test while one-way ANOVA were performed to analyse the difference among multiple groups. *p*-value <0.05 was regarded as statistically significant.

Results

MiR-338-5p was downregulated in RA synovial tissues while ADAMTS-9 was upregulated

To investigate whether miR-338-5p was involved in the pathogenesis of RA, we detected the expression in RA sample tissues which was compared with normal synovial tissues. QRT-PCR revealed that miR-338-5p was significantly downregulated in RA tissues in comparison with normal synovial tissues (Fig. 1A, $p<0.001$). Meanwhile, the ADAMTS-9 mRNA expression level was quantified in passaged RA synovial tissues and corresponding normal synovial tissues. Interestingly, qRT-PCR revealed that the expression of ADAMTS-9 was obviously downregulated in RA synovial tissues (Fig. 1B, $p<0.001$). Western blot was further performed to investigate the protein expression of ADAMTS-9 in both RA and normal synovial tissues. Results indicated that ADAMST-9 was frequently downregulated in RA synovial tissues, which was consistent with previous data (Fig. 1C, $p<0.001$).

ADAMTS-9 was a direct target for miR-338-5p

For further detection, a biological prediction database, TargetScan was used to find potential targets of miR-338-5p. ADAMTS-9 was selected for its 3'-UTR sequence contained binding sites that were complementary with miR-338-5p (Fig. 2A). Luciferase reporter assays were applied to validate the prediction. It was verified that the overexpression of miR-338-5p in RASFs significantly inhibited the luciferase

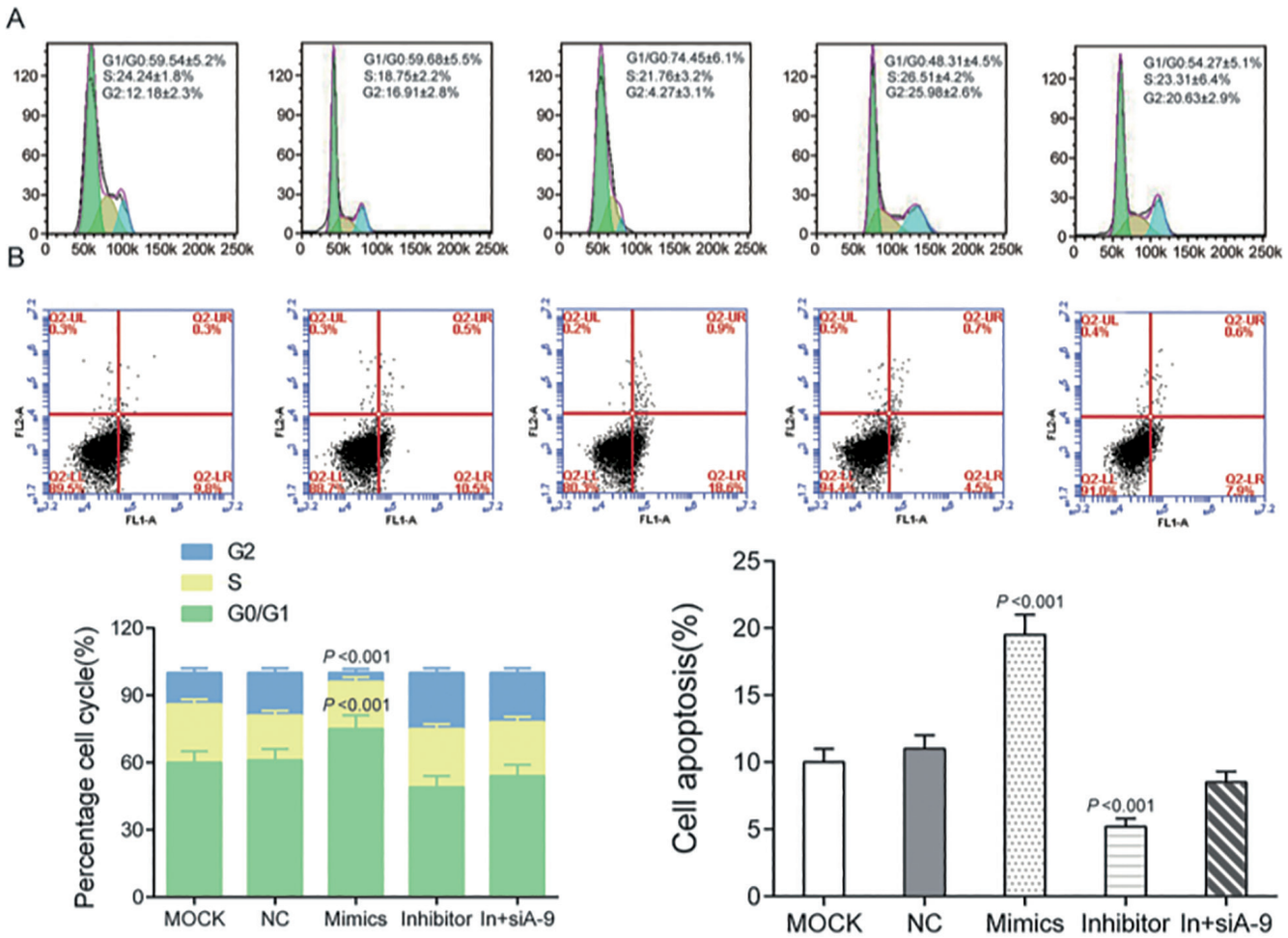

Fig. 4. MiR-338-5p induced G0/G1 arrest and induced apoptosis in RASFs. **A**: The cell cycle distribution of RASFs transfected with miR-338-5p mimics, inhibitor or ADAMTS-9 siRNA was detected using flow cytometry 48 hours after transfection. **B**: Cell apoptosis rate of RASFs was detected using flow cytometry and Annexin V-FITC/PI staining 48 hours after transfection. Data was presented as mean±SD from three independent experiments. $p<0.001$ compared with the Mock group.

activity of ADAMTS-9 site in the wild type, while it was rarely affected in the mutated type (Fig. 2B, $p<0.001$). The results demonstrated that ADAMTS-9 was a direct target for miR-338-5p. Subsequently, we performed transfection to mediate the expression of miR-338-5p and set the following five groups as: Mock group (only treated with reagents), NC group (transfected with miR-338-5p mimics control), Mimics group (transfected with miR-338-5p mimics), Inhibitor group (transfected with miR-338-5p inhibitor) and In+siA-9 group (co-transfected with miR-338-5p inhibitor and ADAMTS-9 siRNA). QRT-PCR was performed to determine the expression of miR-338-5p while Western blot was applied to investigate whether miR-338-5p regulated ADAMTS-9 protein expression. As shown in Figure 2C, miR-338-5p efficiently increased in the Mimics group and decreased in the Inhibitor and In-siA9 group ($p<0.001$). Considering few differences were observed between Inhibitor and In-siA9 group, siADAMTS-9 was not able to affect the level of miR-338-5p. Also miR-338-5p mimics obviously suppressed the ADAMTS-9 mRNA levels compared with Mock and NC groups and miR-338-5p inhibitors obviously enhanced its expression while the enhancement of miR-338-5p was totally attenuated by ADAMT-9 siRNA ($p<0.001$). Protein level of ADAMTS-9 was also measured with Western blot assay (Fig. 2C) and all groups showed the similar expression trends with ADAMTS-9 mRNA. Taken together, miR-338-5p directly targeted ASAMTS-9 in RASFs.

MiR-338-5p inhibited angiogenesis in HUVEC

The protein level of α-SMA and VEGF-C was also detected after manipulation of miR-338-5p and ADAMTS-9 in RAFLs. Semiquantitative analysis was performed with Image J. As shown in Figure 2D, the expression of α-SMA and VEGF-C decreased in the Mimics group and increased in the Inhibitor group. Also, cotransfection of ADAMTS-9 siRNA could attenuate the promoting effect of miR-338-5p. To further explore the effect of miR-338-5p/ADAMTS-9 on angiogenesis, tube formation was performed. Endothelial cells (HUVEC) form tube like structure when these cells were seeded on matrigel. The average number of branches was calculated (Fig. 2E). Compared with the branch num-

ber in the Mock group, reduction was observed in the Mimics group while increasing was obtained in the Inhibitor group. Taken together, miR-338-5p functions as an anti-angiogenesis role via suppressing ADAMTS-9.

The role of miR-338-5p played in RASFs' biological functions

To detect the biological functions of miR-338-5p in RASFs, we restored or reduced its expression respectively through transfection of miR-338-5p mimics or inhibitors. MTT assay indicated that miR-338-5p mimics significantly suppressed the growth of RASFs compared with control and blank control groups while its inhibitors played a completely opposite role (Fig. 3A, $p<0.001$). Transwell assay showed that obviously fewer cells transferred through Matrigel with overexpression of miR-338-5p in comparison with control and blank control groups, suggesting that miR-338-5p could promote the growth of RASFs. Meanwhile, the downregulation of miR-338-5p obviously strengthened the RASFs' invasiveness (Fig. 3B, $p<0.001$). Wound healing assay revealed that miR-338-5p mimics frequently inhibited the RASFs migration (Fig. 3C, $p<0.001$). In all previous experiments, ADAMTS-9 siRNA could efficiently abrogate the promoting effect of miR-338-5p inhibitor on proliferation, invasion and migration.

To further figure out the role of miR-338-5p on RASFs biological functions, low cytometry was applied to investigate RASFs cell cycle distribution and apoptosis rates. As shown in Figure 4A, down-regulation of miR-338-5p significantly increased the number of cells in the S phase and G2/M phase while overexpression of miR-338-5p induced G0/G1 phase arrest compared with control and blank control groups ($p<0.001$). Meanwhile, the overexpression of miR-338-5p rarely induced RASFs apoptosis and miR-338-5p inhibitor efficiently suppressed apoptosis (Fig. 4B, $p<0.001$). Similarly, ADAMTS-9 attenuated the functions of miR-338-5p on RASFs' cell cycle and apoptosis.

All the data above indicated that by down-regulating ADAMTS-9, miR-338-5p suppressed the proliferation, invasion and migration of RASFs, efficiently induced G0/G1 phase arrest and cell apoptosis.

Discussion

In a microenvironment, the over proliferation of RASFs has been directly linked to the initiation and progression of RA (6, 10). Interestingly, RASFs can be expanded in cell culture and also escape contact inhibition which are the features of tumour transformation (20). Considering the behavior of RASF is in some way similar to that of human cancer cells, it inspired us to speculate its passive regulation under miRNAs (1).

MiRNAs are short non-coding RNAs interfering with the post-transcription process of proteins by binding to the 3' UTR of certain mRNAs (21, 22). Accumulating evidence has made the biologists and medical scientists believe that miRNAs are predominant in the tumourigenesis and immune inflammation of humans and have the potential to act as efficient biomarkers in various diseases (23-27). Altered expressions of different miRNAs have been observed in immune cells derived from RA patients (28-30). For instance, it is revealed that miR-155 is a positive regulator while miR-146a is a negative one of inflammation in RA (31-33). In our study, by examining 15 RA synovial tissues samples and 10 normal samples with qRT-PCR test, miRNA-338-5p was found downregulated in RA synovial tissues compared to the normal samples. For the time being, the detailed relationship between miRNAs and RA is yet investigated. MiRNAs are embraced as novel potential markers of various disease including RA, and miRNA-based breakthrough in RA diagnosis and treatment is therefore expected (10). In our study, miRNA-338-5p was found aberrantly downregulated in RA synovial tissues compared with the normal tissues and inhibited the viability, proliferation, migration and invasion of RASFs, which makes it a promising biomarker for RA diagnosis.

Aggrecan is regarded as one of the most common proteoglycans in articular cartilage, enabling articular cartilage to bear great physical forces and move smoothly. The damage to aggrecan can lead to the inflammation of cartilages and induce progression of various types of arthritis (34). During the development of RA, the cartilage matrix is invaded by fibroblasts originating from the synovial tissues (35), and the degradation of aggrecan is required in this process. Belonging to metalloproteinases, both MMPs and ADAMTSs were overexpressed in RASFs and contributed to the damage of joint tissues by impairing the extracellular aggrecan in both RA and osteoarthritis (36, 37). Experiments also revealed that ADAMTS-9 is involved in inflammatory responses associated with cartilage damage (38, 39). Our study confirmed that the up-regulation of miR-338-5p directly suppressed the expression of ADAMTS-9 according to dual-luciferase assay. Afterwards, co-transfection and associated experiments were performed which confirmed the suppressing effects of miR-338-5p on RASFs proliferation and metastasis were efficiently attenuated by ADAMTS-9 siRNA, verifying miR-338-5p functions via the regulation of ADAMTS-9.

For the first time, the role of miR-338-5p in RA was studied. Admittedly, flaws exist in our findings. To enrich our study, we intended to conduct *in vivo* experiments to comprehensively rationalise the assertions we have made. Besides, it is acknowledged that the targets of a single miRNA are usually multiple (40), which leaves us wonder if there exist other genes targeted by miR-338-5p to make a difference in the behaviours of RASFs. There is still a long way to go before we eventually figure out the interactions of miRNAs and genes during RA pathogenesis and development.

References

1. HUBER LC, DISTLER O, TARNER I, GAY RE, GAY S, PAP T: Synovial fibroblasts: key players in rheumatoid arthritis. *Rheumatology* (Oxford) 2006; 45: 669-75.
2. MCINNES IB, SCHETT G: The pathogenesis of rheumatoid arthritis. *N Engl J Med* 2011; 365: 2205-19.
3. BOTTINI N, FIRESTEIN GS: Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors. *Nat Rev Rheumatol* 2013; 9: 24-33.
4. SCOTT DL, WOLFE F, HUIZINGA TW: Rheumatoid arthritis. *Lancet* 2010; 376: 1094-108.
5. DONG L, WANG X, TAN J *et al*.: Decreased expression of microRNA-21 correlates with

the imbalance of Th17 and Treg cells in patients with rheumatoid arthritis. *J Cell Mol Med* 2014; 18: 2213-24.
6. KAROUZAKIS E, GAY RE, GAY S, NEIDHART M: Epigenetic control in rheumatoid arthritis synovial fibroblasts. *Nat Rev Rheumatol* 2009; 5: 266-72.
7. MORINOBU A, TANAKA S, NISHIMURA K *et al*.: Expression and functions of immediate early response gene X-1 (IEX-1) in rheumatoid arthritis synovial fibroblasts. *PLoS One* 2016; 11: e0164350.
8. KRIEGSMANN M, RANDAU TM, GRAVIUS S *et al*.: Expression of miR-146a, miR-155, and miR-223 in formalin-fixed paraffin-embedded synovial tissues of patients with rheumatoid arthritis and osteoarthritis. *Virchows Arch* 2016; 469: 93-100.
9. NAKASA T, MIYAKI S, OKUBO A *et al*.: Expression of microRNA-146 in rheumatoid arthritis synovial tissue. *Arthritis Rheum* 2008; 58: 1284-92.
10. DUROUX-RICHARD I, PRESUMEY J, COURTIES G *et al*.: MicroRNAs as new player in rheumatoid arthritis. *Joint Bone Spine* 2011; 78: 17-22.
11. ZHOU Q, HAUPT S, KREUZER JT *et al*.: Decreased expression of miR-146a and miR-155 contributes to an abnormal Treg phenotype in patients with rheumatoid arthritis. *Ann Rheum Dis* 2015; 74: 1265-74.
12. STANCZYK J, OSPELT C, KAROUZAKIS E *et al*.: Altered expression of microRNA-203 in rheumatoid arthritis synovial fibroblasts and its role in fibroblast activation. *Arthritis Rheum* 2011 ;63: 373-81.
13. SHI DL, SHI GR, XIE J, DU XZ, YANG H: MicroRNA-27a Inhibits Cell Migration and Invasion of Fibroblast-Like Synoviocytes by Targeting Follistatin-Like Protein 1 in Rheumatoid Arthritis. *Mol Cells* 2016; 39: 611-8.
14. YONG FL, LAW CW, WANG CW: Potentiality of a triple microRNA classifier: miR-193a-3p, miR-23a and miR-338-5p for early detection of colorectal cancer. *BMC Cancer* 2013; 13: 280.
15. TONG D, ZHAO L, HE K *et al*.: MECP2 promotes the growth of gastric cancer cells by suppressing miR-338-mediated antiproliferative effect. *Oncotarget* 2016; 7: 34845-59.
16. CHEN Y, CHEN J, LIU Y, LI S, HUANG P: Plasma miR-15b-5p, miR-338-5p, and miR-764 as biomarkers for hepatocellular carcinoma. *Med Sci Monit* 2015; 21: 1864-71.
17. LO PH, LUNG HL, CHEUNG AK *et al*.: Extracellular protease ADAMTS9 suppresses esophageal and nasopharyngeal carcinoma tumor formation by inhibiting angiogenesis. *Cancer Res* 2010; 70: 5567-76.
18. DEMIRCAN K, HIROHATA S, NISHIDA K *et al*.: ADAMTS-9 is synergistically induced by interleukin-1beta and tumor necrosis factor alpha in OUMS-27 chondrosarcoma cells and in human chondrocytes. *Arthritis Rheum* 2005; 52: 1451-60.
19. ARNETT FC, EDWORTHY SM, BLOCH DA *et al*.: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
20. FASSBENDER HG: Histomorphological basis of articular cartilage destruction in rheumatoid arthritis. *Coll Relat Res* 1983; 3: 141-55.
21. FILKOVA M, ARADI B, SENOLT L *et al*.: Association of circulating miR-223 and miR-16 with disease activity in patients with early rheumatoid arthritis. *Ann Rheum Dis* 2014; 73: 1898-904.
22. DENLI AM, TOPS BB, PLASTERK RH, KETTING RF, HANNON GJ: Processing of primary microRNAs by the Microprocessor complex. *Nature* 2004; 432: 231-5.
23. CHEN X, BA Y, MA L *et al*.: Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* 2008; 18: 997-1006.
24. WEBER JA, BAXTER DH, ZHANG S *et al*.: The microRNA spectrum in 12 body fluids. *Clin Chem* 2010; 56: 1733-41.
25. MENDELL JT: MicroRNAs: critical regulators of development, cellular physiology and malignancy. *Cell Cycle* 2005; 4: 1179-84.
26. BROOKS WH, LE DANTEC C, PERS JO, YOUINOU P, RENAUDINEAU Y: Epigenetics and autoimmunity. *J Autoimmun* 2010; 34: J207-19.
27. CARLSEN AL, SCHETTER AJ, NIELSEN CT *et al*.: Circulating microRNA expression profiles associated with systemic lupus erythematosus. *Arthritis Rheum* 2013; 65: 1324-34.
28. FILKOVA M, JUNGEL A, GAY RE, GAY S: MicroRNAs in rheumatoid arthritis: potential role in diagnosis and therapy. *BioDrugs* 2012; 26: 131-41.
29. PAULEY KM, SATOH M, CHAN AL, BUBB MR, REEVES WH, CHAN EK: Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients. *Arthritis Res Ther* 2008; 10: R101.
30. LI J, WAN Y, GUO Q *et al*.: Altered microRNA expression profile with miR-146a upregulation in $CD4^+$ T cells from patients with rheumatoid arthritis. *Arthritis Res Ther* 2010; 12: R81.
31. KUROWSKA-STOLARSKA M, ALIVERNINI S, BALLANTINE LE *et al*.: MicroRNA-155 as a proinflammatory regulator in clinical and experimental arthritis. *Proc Natl Acad Sci USA* 2011; 108: 11193-8.
32. BLUML S, BONELLI M, NIEDERREITER B *et al*.: Essential role of microRNA-155 in the pathogenesis of autoimmune arthritis in mice. *Arthritis Rheum* 2011; 63: 1281-8.
33. NAKASA T, SHIBUYA H, NAGATA Y, NIIMOTO T, OCHI M: The inhibitory effect of microRNA-146a expression on bone destruction in collagen-induced arthritis. *Arthritis Rheum* 2011; 63: 1582-90.
34. LOHMANDER LS, NEAME PJ, SANDY JD: The structure of aggrecan fragments in human synovial fluid. Evidence that aggrecanase mediates cartilage degradation in inflammatory joint disease, joint injury, and osteoarthritis. *Arthritis Rheum* 1993; 36: 1214-22.
35. OSPELT C, NEIDHART M, GAY RE, GAY S: Synovial activation in rheumatoid arthritis. *Front Biosci* 2004; 9: 2323-34.
36. BURRAGE PS, MIX KS, BRINCKERHOFF CE: Matrix metalloproteinases: role in arthritis. *Front Biosci* 2006; 11: 529-43.
37. MURPHY G, NAGASE H: Reappraising metalloproteinases in rheumatoid arthritis and osteoarthritis: destruction or repair? *Nat Clin Pract Rheumatol* 2008; 4: 128-35.
38. DEMIRCAN K, YONEZAWA T, TAKIGAWA T *et al*.: ADAMTS1, ADAMTS5, ADAMTS9 and aggrecanase-generated proteoglycan fragments are induced following spinal cord injury in mouse. *Neurosci Lett* 2013; 544: 25-30.
39. KEVORKIAN L, YOUNG DA, DARRAH C *et al*.: Expression profiling of metalloproteinases and their inhibitors in cartilage. *Arthritis Rheum* 2004; 50: 131-41.
40. GAO J, ZHOU XL, KONG RN, JI LM, HE LL, ZHAO DB: microRNA-126 targeting PIK3R2 promotes rheumatoid arthritis synovial fibroblasts proliferation and resistance to apoptosis by regulating PI3K/AKT pathway. *Exp Mol Pathol* 2016; 100: 192-8.