Expression and function of miR-155 in rat synovial fibroblast model of rheumatoid arthritis

HEWEI LI1, PING LIU1, YANLIN GONG2, JIALI LIU1 and FENG RUAN1

1Department of Orthopedics, Liyuan Hospital Affiliated to Tongji Medical College of Huazhong University of Science and Technology, Wuhan, Hubei 430077; 2Department of Endocrinology, Wuhan No. 1 Hospital, Wuhan, Hubei 430022, P.R. China

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Abstract. Rheumatoid arthritis (RA) is a common autoimmune disease characterized by joint synovial inflammation and is a challenge for researchers and clinicians. MicroRNAs (miRNAs/miRs) represent a group of small non-coding RNA molecules that post-transcriptionally regulate mRNA expression and are involved in various diseases, including cancer, autoimmune and metabolic diseases, as well as neurological disorders. In the present study, various experiments were performed to investigate the effects and underlying mechanism of miR-155 in RA using rat synoviocytes induced by lipopolysaccharide (LPS) to model rheumatoid arthritis. It was revealed that synovial fibroblasts exhibited significantly higher miR-155 mRNA levels than the control group. Compared with the RA group, the viability of synovial fibroblasts was significantly decreased in the miR-155 mimics + RA group, but markedly increased in the miR-155 inhibitor + RA group. Compared with that in the RA + NC mimic or RA + NC inhibitor groups, the apoptosis of synovial fibroblasts increased significantly in the miR-155 mimics + RA group, but was significantly decreased in the miR-155 inhibitor + RA group. The miR-155 mimics + RA group exhibited higher expression levels of β-catenin, matrix metalloproteinase 7 and cyclin D1 compared with the miR-155 inhibitor + RA group, and the glycogen synthase kinase protein levels was lower compared with the miR-155 inhibitor + RA group. In brief, it was inferred that the Wnt signaling pathway is involved in the miR-155-associated inhibition of RA synovial fibroblast viability and induction of cell apoptosis. Inhibition of miR-155 may be an effective treatment for RA through regulation of the Wnt signaling pathway to reduce cell apoptosis and enhance cell viability.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease mainly characterized by a systemic autoimmune disorder, cartilage degradation and synovial inflammation (1). Several risk factors, including heredity, infection and gonadal hormone contribute to RA (2). Synovial tissue in patients with RA features the production of inflammatory cytokines, including tumor necrosis factor α and interleukin-1β by macrophages, and T and B cells, and inhibiting these cytokines may ameliorate the clinical symptoms of RA (3). However, the mechanisms underlying the genesis and progression of RA remain elusive, and no appropriate therapeutic strategies have been developed to cure this disease (4). Aberrant gene expression in RA is associated with synovial inflammation; however, the precise mechanisms that lead to altered gene expression in RA remain poorly understood.

MicroRNAs (miRNAs/miRs) are small noncoding RNAs of 22-24 nucleotides in length that regulate target gene expression by binding to the 3’-untranslated region, and miRNAs have roles in biological processes, including cell proliferation, apoptosis and differentiation (5). Hundreds of miRNAs have been detected in various organisms and most of them have an essential role in regulating gene expression through targeting mRNA translation or inducing mRNA cleavage (6). A large number of studies have revealed that miRNAs, including miR-155, miR-146, miR-132 and miR-16, are involved in RA. miR-155 is upregulated in the synovial membrane and synovial fluid macrophages from patients with RA (7), miR-146 appears to be associated with the inflammatory response in RA (8), miR-16 was reported to be overexpressed in the peripheral blood mononuclear cells of RA patients (8). However, the effects and mechanisms of miRNA gene expression in RA are not well understood.

The present study evaluated the expression levels of miR-155, miR-203 and miR-146 in rat synovial fibroblasts of control and RA groups. The aim of the present study was to evaluate the effects of miRNA on synovial fibroblast viability, apoptosis and cell cycle, and the underlying mechanisms, including inflammatory signaling pathways in RA, were also investigated.

Correspondence to: Dr Feng Ruan, Department of Orthopedics, Liyuan Hospital Affiliated to Tongji Medical College of Huazhong University of Science and Technology, 18 Guangrong Village, Wuhan, Hubei 430077, P.R. China
E-mail: rf001m5@sina.com

Key words: rheumatoid arthritis, microRNA-155, synovial fibroblasts, viability, apoptosis, cell cycle
Materials and methods

Animals. A total of 3 healthy male Sprague Dawley rats (age, 8 weeks; weight, 200±20 g) were purchased from the Laboratory Animal Center of Huazhong University of Science and Technology (Wuhan, China). The experimental rats had been bred under constant conditions, including 55-60% humidity at 23±2°C and were provided with water and food ad libitum. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Isolation and culture of synovial fibroblasts. Rat knee joint synovial tissue was cut into small fragments and digested in Dulbecco's modified Eagle's medium (DMEM) containing 1 mg/ml collagenase II (both Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C for 2 h. The synovial fibroblasts were collected after centrifugation and maintained in DMEM containing 5 mmol/l glucose and 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) in a humidified atmosphere containing 5% CO₂ at 37°C. The cells were passaged when they reached confluency. The fourth generation of cells was used for the experiment. Microscopy images displaying the cell morphology are presented in Fig. 1A. The purity of the cells was tested using immunofluorescence with rabbit anti-vimentin (cat. no. ab45939; 1:100 dilution; Abcam, Cambridge, MA, USA) and mouse anti-CD68 (cat. no. ab125212; 1:50 dilution) antibodies. Following blocking in 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 1 h at room temperature, cells were incubated with anti-vimentin and -CD68 primary antibodies for 1 h at room temperature. Cells were washed with PBS, incubated for 1 h at room temperature with AlexaFluor® 488-conjugated secondary antibodies (cat. no. ab150081; 1:500; Abcam) and incubated for 1 h at room temperature with nuclear DNA was labeled in blue with DAPI.

Cell transfection and reagents. Synovial fibroblasts were divided into a negative control + RA group, miR-155 mimics + RA group and a miR-155 inhibitor + RA group. Cells were cultured until confluency. The fourth generation of cells was used for the experiment. Microscopy images displaying the cell morphology are presented in Fig. 1A. The purity of the cells was tested using immunofluorescence with rabbit anti-vimentin (cat. no. ab45939; 1:100 dilution; Abcam, Cambridge, MA, USA) and mouse anti-CD68 (cat. no. ab125212; 1:50 dilution) antibodies. Following blocking in 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 1 h at room temperature, cells were incubated with anti-vimentin and -CD68 primary antibodies for 1 h at room temperature. Cells were washed with PBS, incubated for 1 h at room temperature with AlexaFluor® 488-conjugated secondary antibodies (cat. no. ab150081; 1:500; Abcam) and incubated for 1 h at room temperature with nuclear DNA was labeled in blue with DAPI.

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Effect of LPS treatment on cell viability. A number of studies have suggested that LPS and its pattern recognition receptor, Toll-like receptor 4, have a critical role in the development of RA (10,11). In the present study, cells were stimulated with LPS at a concentration of 1, 10 or 100 mg/l for 24, 48 or 72 h. The results indicated that the cell viability was reduced in a dose- and time-dependent manner (Fig. 2). In order to ensure that LPS induces an inflammatory response in synovial fibroblasts, while minimizing the adverse impact of LPS in the subsequent experiments, the conditions of 1 mg/l and 24 h were selected to induce the RA model in the present study (12).

miR-155, miR-203 and miR-16 RNA expression in synovial fibroblasts. The mRNA expression levels of miR-155, miR-203 and miR-16 were analyzed by RT-qPCR in control and RA cells. As presented in Fig. 3, miR-155 and miR-16 expression levels were significantly higher in the RA group than in the control group, and miR-155 levels were much higher than miR-16 levels in the RA group. miR-155 was therefore selected for further study.

miR-155 regulates the viability of synovial fibroblasts with RA. The following five groups were set up: Control, RA + mimics

Table I. Primers used for polymerase chain reaction.

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
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<tbody>
<tr>
<td>miR-155</td>
<td>TTAATGCTAATCGTG</td>
<td>CTCAACTGGTGCTGGAATGCCTCTGAGAGGACCCCTAT</td>
</tr>
<tr>
<td>miR-203</td>
<td>AGTGGTTCTAAACAGTT</td>
<td>CTCAACTGGTGCTGGAATGCCTCTGAGAGGACCCCTAT</td>
</tr>
<tr>
<td>miR-16</td>
<td>TAGCAGCAGTAAA</td>
<td>CTCAACTGGTGCTGGAATGCCTCTGAGAGGACCCCTAT</td>
</tr>
<tr>
<td>β-catenin</td>
<td>AATGGCTTGGAATTGAGA</td>
<td>AGTGAATAAGAACGTTAG</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>CCGGATCCGCAACCATGTCG</td>
<td>CCCCTCCAGGAATTCTCAGTGAATGGAGGCTGA</td>
</tr>
<tr>
<td>MMP-7</td>
<td>CCAAAATAGCCAAAAATGGACTTC</td>
<td>TGTAAATATGGTAAGTCTCAGTATATG</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>TGGCTGCCCCCTGAAAGATGAAG</td>
<td>GGAAGTGGTTCGATGAAATCGTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCCATCAATGACCCCTTCATTG</td>
<td>CATGGGTGAATCATATGGAAC</td>
</tr>
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GSK, glycogen synthase kinase; MMP, matrix metalloproteinase; miR, microRNA.
NC (LPS stimulation and transfection of mimics NC), RA + inhibitor NC (LPS stimulation and transfection of inhibitor NC), RA + mimics 155 (LPS stimulation and transfection of miR-155 mimics) and RA + inhibitor 155 (LPS stimulation and transfection of miR-155 inhibitor). The results indicated that the cell viability in the RA + mimics NC and RA + inhibitor NC groups was lower than that in the control group (Fig. 4). Compared with that in the RA + mimics NC and RA + inhibitor NC groups, the RA + mimics 155 group had a reduced cell viability, but the RA + inhibitor 155 group had a significantly elevated cell viability (Fig. 4). Taken together, miR-155 inhibits the viability of synovial fibroblasts with RA.

miR-155 regulates apoptosis of synovial fibroblasts with RA. Cell apoptosis was measured by Annexin V-FITC/PI staining and flow cytometric analysis. As presented in Fig. 5, the percentages of apoptotic cells in the RA + mimics NC and RA + inhibitor NC groups were significantly higher than those in the control group. The RA + mimics 155 group had a markedly elevated percentage of apoptotic cells but the RA + inhibitor 155 group had a significantly decreased apoptotic rate (Fig. 5). These results suggest that miR-155 increased the apoptosis of synovial fibroblasts with RA.

miR-155 regulates the cell cycle of synovial fibroblasts with RA. The cell cycle was measured by flow cytometry following PI staining. As presented in Fig. 6, a significant cell cycle arrest in G<sub>1</sub> phase was detected in the RA + mimics NC, RA + inhibitor NC and RA + mimics 155 groups, while this arrest was abrogated in the RA + inhibitor 155 group and the cell cycle progression was restored partially compared to the RA + mimics 155 group. These results indicated that miR-155 induced cycle arrest in G<sub>1</sub> phase in synovial fibroblasts with RA.

miR-155 regulates Wnt effector protein expression in synovial fibroblasts with RA. The effector proteins of the Wnt signaling pathway were quantified by RT-qPCR. As presented in Fig. 7, the expression levels of β-catenin, matrix metalloproteinase (MMP)7 and cyclin D1 in the RA + mimics NC and RA + inhibitor NC groups were higher than those in the control group, while the glycogen synthase kinase (GSK)-3β expression in the RA + mimics NC and RA + inhibitor NC groups was lower than that in the control group. In the RA + mimics 155 group, the expression levels of β-catenin, MMP7 and cyclin D1 were significantly elevated compared with those in the NC + RA groups, but those in the RA + inhibitor 155 group were reduced. Conversely, the expression of GSK-3β was significantly decreased in the RA + mimics 155 group, but increased in the RA + inhibitor 155 group compared with that in the NC + RA groups.

**Discussion**

Accumulating evidence indicates that synovial inflammation contributes to the progression of RA (13,14). The presence of synovitis in a significant proportion of patients with primary RA has been increasingly recognized. Based on this observation, further studies have implicated joint inflammation and synovitis in the pathogenesis of RA (15,16). By regulating various cellular processes, including cell proliferation, immune responses, inflammation, apoptosis and cell signaling,
miRNAs have an important role in the development and progression of various diseases. Diverse miRNA expression profiles (such as the deregulation of miRNA expression) were identified as crucial small-molecular regulators in severe joint diseases, including osteoarthritis and RA (7,14,17). The present study mainly investigated the role of miR-155 in RA.

RA synoviocytes are considered as the effector cells of cartilage and bone destruction (18). Joint synovioid cells are divided into two cell types: Macrophage-like synoviocytes (MLS) and fibroblast-like synoviocytes (FLS). Among them, MLS are negative for vimentin and positive for CD68, while FLS are negative for CD68 and positive for vimentin (19). Synovial fibroblasts have been reported to have a crucial role in RA pathogenesis (20). In the present study, synovial fibroblasts were isolated and identified as FLS, not MLS. LPS initiates the signaling cascades that cause inflammation and finally result in RA (21). Therefore, the present study used LPS-stimulated cells as the RA model to evaluate the effect of miR-155 on RA development.

miR-155 and miR-16 have been reported to be associated with RA (22,23). Jin et al (24) reported that overexpression of miR-155 in synovial fluid mononuclear cells leads enhanced proinflammatory factors. The results of the present study indicated that miR-155 expression exhibited a marked difference between the control and RA groups, indicating the crucial role of miR-155 in RA. In a previous study, miR-155 was induced in response to inflammatory stimuli and acted as a positive regulator of inflammation in RA in vivo, indicating
a role in clinical and experimental arthritis. Furthermore, the present results suggested that miR-155 inhibits the viability of synovial fibroblasts and induces cell apoptosis and cell cycle arrest. In addition, miRNA elevated the expression of β-catenin by lowering GSK-3β expression, which activated the Wnt pathway, and enhanced the expression of the target genes cyclin D1 and MMP7. The Wnt signaling pathway regulates cell proliferation, differentiation, adhesion, morphology and motility, as well as inflammation (25). Thus, the present results suggested that miR-155 contributes to inflammation in RA and may be a promising therapeutic target. In order to confirm the hypothesis that miR-155 is a target for RA treatment, miR-155 was inhibited in synovial fibroblasts induced with LPS, and the results indicated that the cell viability was significantly increased, while the apoptotic rate was significantly decreased compared with those in the RA + NC groups. Deregulation of miR-155 in RA monocytes may contribute to the production of pro-inflammatory chemokines by these cells and to their accumulation at sites of inflammation (26). Of note, the present study indicated that inhibition of miR-155 blocks the Wnt signaling pathway in synovial fibroblasts induced with LPS by reducing the expression of β-catenin and increasing the expression of GSK-3β.

In conclusion, the present study demonstrated that miR-155 inhibits RA synovial fibroblast viability and induces apoptosis and cell cycle arrest by regulating the Wnt signaling pathway. It was inferred that miR-155 may be a potential target for RA treatment.
Acknowledgements
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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
FR designed the experiment and was responsible for the acquisition of funding. HL cultured the cells, performed vector transfection and was a major contributor in writing the manuscript. PL assessed the expression of mRNA using reverse transcription-quantitative polymerase chain reaction analyses. YG analyzed cell apoptosis and cell cycle distribution using flow cytometry. JL contributed to the design of the present study and measured the cell viability using the MTT assay. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All experimental procedures on animals were approved by the Ethics approval and consent to participate. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

References