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miR-449a inhibits cell proliferation, migration, and inflammation by regulating high-mobility group box protein 1 and forms a mutual inhibition loop with Yin Yang 1 in rheumatoid arthritis fibroblast-like synoviocytes

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Abstract

Background: We previously found that high-mobility group box protein 1 (HMGB1) promoted cell proliferation, migration, invasion, and autophagy in rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS), but little is known about its regulatory mechanism. The aim of this study was to investigate the regulatory mechanism of HMGB1 at the posttranscription level.

Methods: Real-time qPCR, CCK-8 cell proliferation assay, transwell cell migration assay, enzyme-linked immunosorbent assay (ELISA), and western blotting were used in this study. The targeting relationship between miRNA and mRNA was presented by the luciferase reporter assay.

Results: MiR-449a was downregulated in RA synovial tissue and inhibited RA-FLS proliferation, migration, and IL-6 production. MiR-449a directly targeted HMGB1 and inhibited its expression. Yin Yang 1 (YY1) negatively regulated miR-449a expression and formed a mutual inhibition loop in RA-FLS. MiR-449a inhibited TNF α -mediated HMGB1 and YY1 overexpression and IL-6 production.

Conclusions: Our results reveal the regulatory mechanism of HMGB1 in RA and demonstrate that miR-449a is a crucial molecule in RA pathogenesis and a suitable candidate for miRNA replacement therapies in RA.

Keywords: miR-449a, Rheumatoid arthritis, Fibroblast-like synoviocytes, Proliferation, Inflammation, High-mobility group box protein 1

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Background

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease characterized by synovial hyperplasia and joint destruction [1]. The understanding of the pathogenic mechanisms of RA and treatments for RA have been greatly improved in the last decade, but there is still no cure for RA [2]. Accumulating evidence indicates that RA fibroblast-like synoviocytes (RA-FLS) play a key role in the pathogenesis of RA [3].

Studies have found that microRNAs (miRNAs) control various cellular processes and play an important role in different diseases, including RA [4, 5]. Recently, several miRNAs were observed to be abnormally expressed in RA, and some of which affected RA-FLS cell proliferation, inflammation, or cellular signaling pathways [6–8]. MiR-449a, located on chromosome 5q11, has been reported to be dysregulated in some diseases and influences cell apoptosis, proliferation, invasion, migration, and inflammation by regulating the expression of various genes [9–14]. Our previous research found that miR-449a expression was downregulated in RA synovial tissue compared with osteoarthritis (OA) tissue. However, little is known about the function of miR-449a in RA.

High-mobility group box protein 1 (HMGB1) is a highly conserved chromatin-binding nuclear protein that has been confirmed to play important roles in many diseases [15–17]. Our previous studies found that HMGB1 expression was increased in RA synovial tissue and that HMGB1 promoted cell proliferation, migration, invasion, and autophagy in RA-FLS [18, 19]. Therefore, exploring the molecular mechanisms of HMGB1 dysregulation in RA may help us better understand the pathogenesis of RA. HMGB1 has been reported to be regulated by miRNAs in some diseases [20–24], but whether the overexpression of HMGB1 in RA is related to dysregulated miRNAs remains unknown.

In the present study, we showed that miR-449a expression was downregulated in RA synovial tissue. miR-449a inhibited RA-FLS proliferation, migration, and IL-6 production by targeting HMGB1 and Yin Yang1 (YY1). YY1 negatively regulated miR-449a expression and formed a mutual inhibition loop in RA-FLS. We further found that miR-449a inhibited TNF α -mediated HMGB1 and YY1 overexpression and IL-6 production. miR-449a may be a crucial molecule in RA pathogenesis and a suitable candidate for miRNA replacement therapies in RA.

Methods

Patients and tissue collection

Human synovial tissue samples were obtained from 14 OA patients and 14 RA patients undergoing knee arthroplasty surgery (Department of Joint Surgery, Xi'an Hong Hui Hospital, Xi'an, China). This study was performed with the approval of the human research ethics

committee of Xi'an Hong Hui Hospital. All patients fulfilled the American College of Rheumatology (ACR) criteria for the classification of RA or OA and provided informed consent. Information on all patients is summarized in Table 1.

Cell culture

FLS were dissociated from RA synovial tissue specimens, and 3rd to 8th passage cells were used in our study. The cells were grown in DMEM/F12 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco Life Technologies, USA), 100 units/mL penicillin, and 100 μ g/mL streptomycin at 37 °C under 5% CO₂.

Oligonucleotides and vector construction

The miR-449a mimics, YY1-siRNA targeting human YY1, and negative control RNAs for both the miRNA and siRNA were chemically synthesized by GenePharma (GenePharma, Shanghai, China). For the expression vector, HMGB1 and YY1 were cloned into pCMV-Blank (Beyotime, Shanghai, China) between the site BamHI and site EcoRI. We used a bioinformatics analysis to determine that both HMGB1 and YY1 are targets of miR-449a (TargetScan, www.targetscan.org). The linker fragment containing the HMGB1 or YY1 wild-type or the mutant 3'UTR-binding site was synthesized and cloned into the pmirGLO Dual-Luciferase vector (Promega, Madison, WI, USA) between site SacI and site XhoI. All the primer sequence information is given in Additional file 1: Table S1.

RNA extraction and quantitative reverse transcription PCR

Total RNA was isolated from the synovial tissue samples and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Two micrograms of total RNA and a Transcriptor cDNA

Table 1 Patient characteristics

Clinical data	RA	OA
Number of patients	14	14
Sex		
Male	4	6
Female	10	8
Age [†] (years)	59.4 \pm 8.2	68.9 \pm 4.9
Disease duration [†] (years)	9.3 \pm 2.3	15.1 \pm 3.5
RF [†] (IU/ml)	123.3 \pm 112.2	7.1 \pm 3.5
ESR [†] (mm/h)	41.9 \pm 12.9	22.9 \pm 17.3
CRP [†] (mg/l)	27.6 \pm 15.4	9.6 \pm 9.2
Anti-CCP positive, <i>n</i>	12/14	0/14

RF rheumatoid factor, ESR erythrocyte sedimentation rate, CRP C-reactive protein, CCP cyclic citrullinated peptide

[†]Mean \pm standard deviation

Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) were used to synthesize cDNA, which was subsequently used to detect mRNA expression. A total of 500 ng of total RNA and miR-XmiRNA First-Strand Synthesis Kits (Clontech, Mountain View, CA, USA) were used to synthesize cDNA, which was subsequently used to detect miRNA expression. qPCR using a SYBR Green System (Roche Diagnostics, Mannheim, Germany) was used to detect gene expression. β -actin and U6 expression levels were used as endogenous controls to normalize mRNA and miRNA expression, respectively. The forward primer for miR-449a was purchased from Tiangen Biotech (Tiangen Biotech, Beijing, China), and the reverse primer was obtained from the miR-X miRNA First-Strand Synthesis Kits. The other primers used in this study are listed in Additional file 1: Table S1. The $2^{-\Delta\Delta CT}$ method was used to quantify the relative expression.

Cell proliferation assay

Cell proliferation under different stimulation conditions was assessed using a cell counting kit-8 (Nanjing Key-Gen Biotech, Co., Ltd., Nanjing, China) according to the manufacturer's protocol.

Cell migration assay

Cell migration under different stimulation conditions was performed using a Transwell assay (Sigma Company, St. Louis, Missouri, USA) according to Mu's protocol [25], with some minor modifications. Briefly, after undergoing transfection for 48 h, RA-FLS were starved in serum-free DMEM/F12 medium for another 24 h. Then, then 3×10^4 cells were plated in 200 μ l of serum-free medium in the upper chamber with the noncoated membrane (8 μ m pores), and the lower chamber was filled with 500 μ l of medium containing 10% FBS. After incubating for 24 h at 37 °C, the cells on the upper surface of each membrane were removed with a cotton swab, and those on the bottom side of the membrane were stained with crystal violet and counted under a microscope.

Enzyme-linked immunosorbent assay (ELISA)

After RA-FLS were stimulated for 48 h, the cell culture supernatants were collected and diluted for the measurement of IL-6 secretion by ELISA (BOSTER, Wuhan, China) according to the manufacturer's recommendations.

Cell transfection

RA-FLS were transfected with the miRNA (20 nM), siRNA (20 nM), negative control RNA (20 nM), or expression vectors encoding HMGB1 (1 μ g/ml) or YY1 (1 μ g/ml) using Lipofectamine 2000 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's recommendations.

Luciferase reporter assay

HEK293 cells were cotransfected with 100 ng pmirGLO vectors containing the wild-type or mutant 3'UTR of HMGB1 or YY1 mRNA and 20 nM of miR-449a or 20 nM of the miRNA negative control according to the manufacturer's protocol. After 24 h, luciferase activity was assayed using a Dual-Luciferase Reporter Assay System Kit (Promega, Madison, WI, USA). The relative firefly luciferase activity was normalized to the Renilla luciferase activity.

Western blotting

A 20- μ g protein sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). After blocking in 10% nonfat dry milk in Tris-buffered saline/Tween-20 (TBST) for 2 h, the membranes were then incubated with the following primary antibodies overnight at 4 °C: anti- β -actin monoclonal antibody (Bioss, Beijing, China, 1:1000), anti-HMGB1 monoclonal antibody (Abcam, Cambridge, UK, 1:2000), and anti-YY1 monoclonal antibody (Abcam, Cambridge, UK, 1:1000). After washing three times in TBST for 10 min each time, the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (BOSTER, Wuhan, China, 1:10000) for 1 h at room temperature. After that, the membranes were washed three times with TBST and detected using an electrochemiluminescence (ECL) system (Gene Gnome 5, Synoptics Ltd., UK). The protein levels were normalized to the β -actin protein levels in each sample.

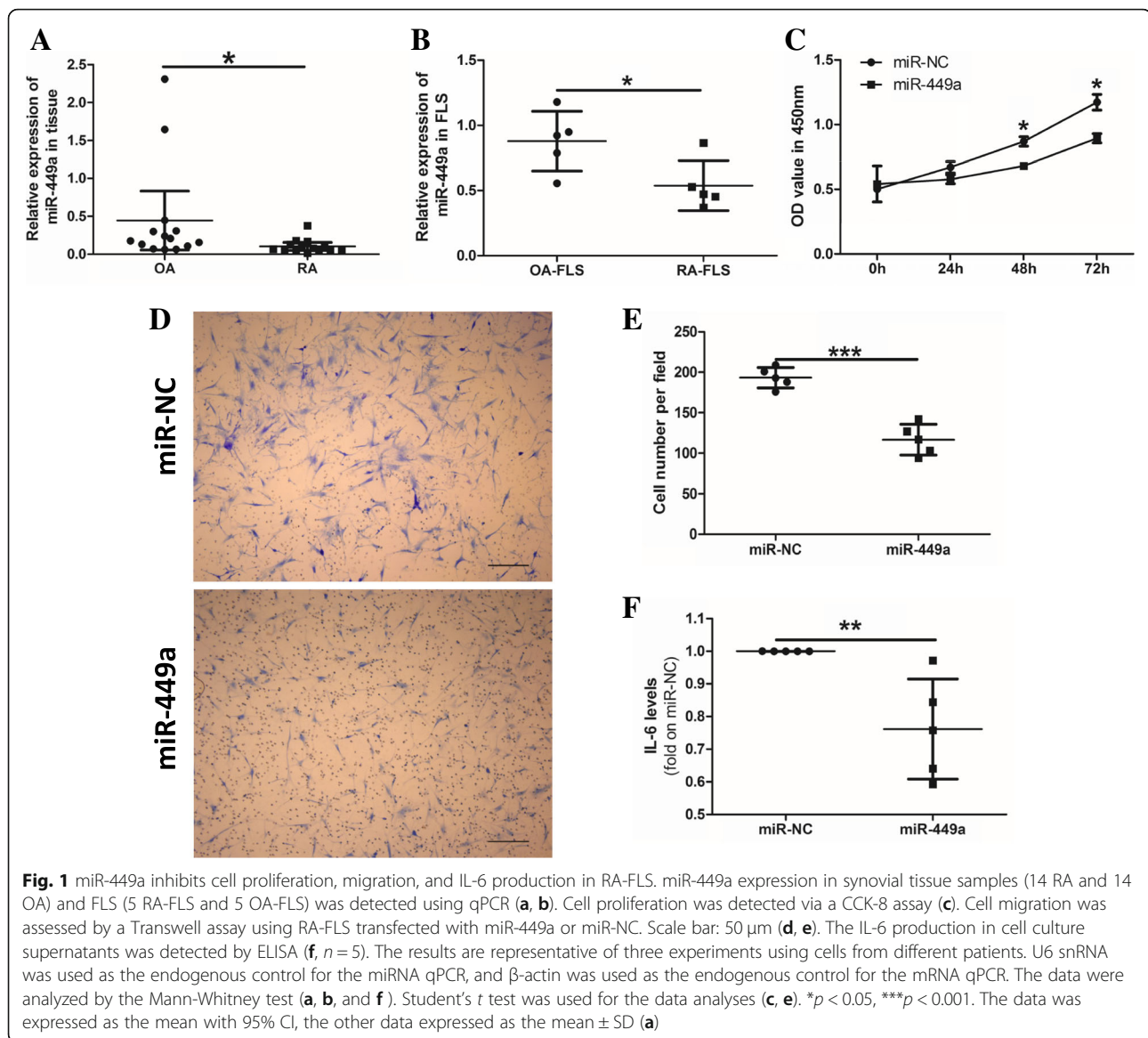
Statistical analysis

Each experiment was repeated three times, and the data were presented as the mean \pm standard deviation (SD) or mean with 95% confidence interval (CI). Differences between two groups were analyzed using Student's *t* test, or the Mann-Whitney *U* test for experiments in which the datasets were not normally distributed. All the results were analyzed with GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). *P* values less than 0.05 were considered statistically significant.

Results

MiR-449a inhibits cell proliferation, migration, and inflammation in RA-FLS

The quantitative PCR (qPCR) results indicated that miR-449a expression was significantly downregulated both in RA synovial tissue and RA-FLS compared with OA (Fig. 1a, b). RA-FLS were transfected with 20 nM miR-449a mimic or 20 nM negative control RNA (miR-NC) for 24 h, 48 h, or 72 h. Cell proliferation was detected via a



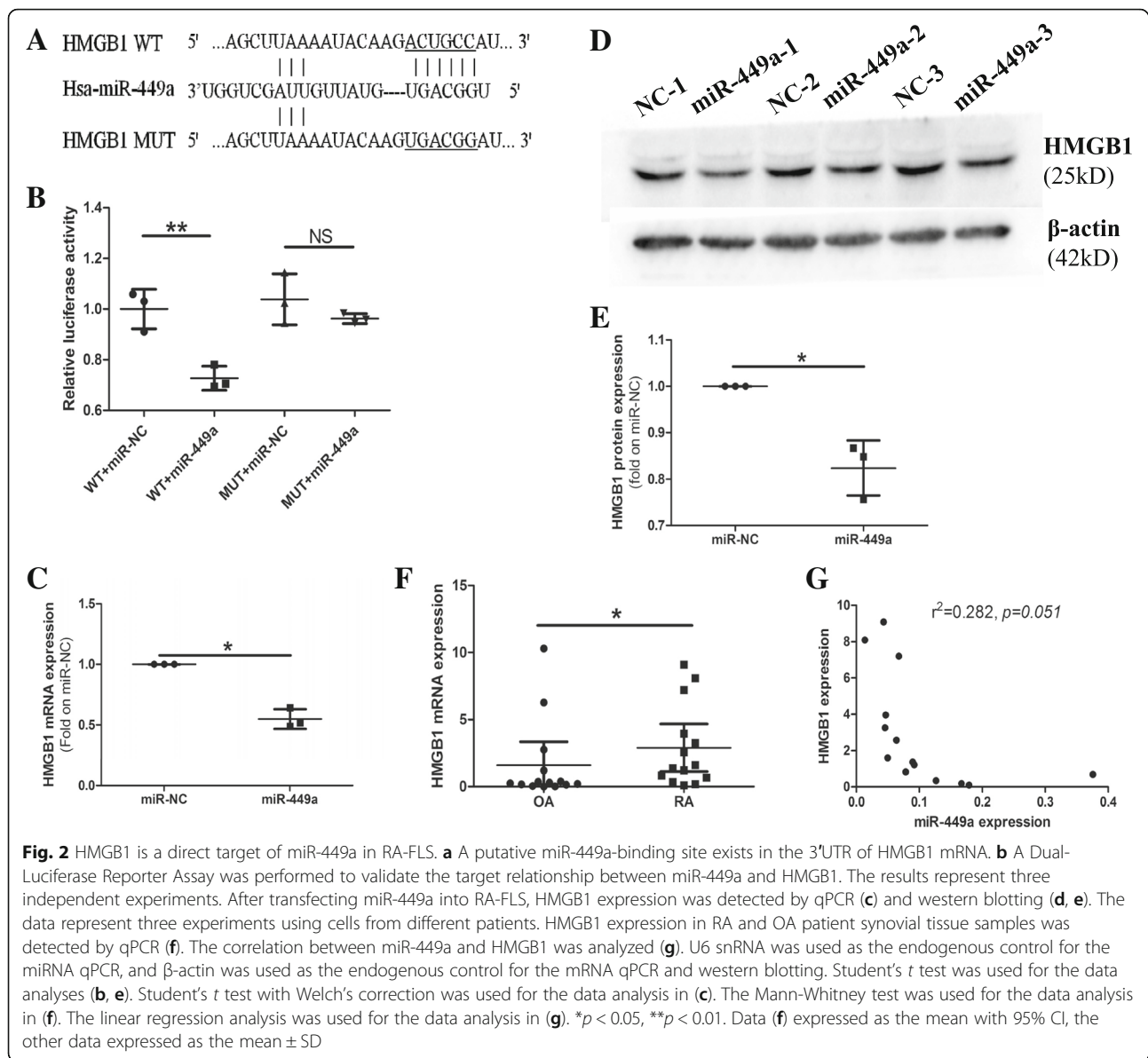
CCK-8 assay. The optical density (OD) value showed that RA-FLS in the miR-449a mimic groups had lower cell proliferation ability than those in the miR-NC groups (Fig. 1c). We also detected the effect of miR-449a on cell migration and IL-6 production in RA-FLS. Both cell migration (Fig. 1d, e) and IL-6 production (Fig. 1f) were significantly inhibited in the RA-FLS transfected with the miR-449a mimic compared with the miR-NC groups. These data demonstrated that miR-449a negatively regulated cell proliferation, migration, and IL-6 production in RA-FLS.

HMGB1 is a direct target of miR-449a in RA

Based on the bioinformatics prediction, we identified a putative binding site for miR-449a in the HMGB1 mRNA 3'UTR (Fig. 2a). The Dual-Luciferase Reporter Assay

results showed that miR-449a inhibited the luciferase activity in the cells containing the miR-449a-binding site of the HMGB1 3'UTR but failed to affect the cells containing a mutated version of the miR-449a-binding site of the HMGB1 3'UTR (Fig. 2b), which indicated that miR-449a bound directly to the 3'UTR of the HMGB1 mRNA to suppress translation. After the transfection of RA-FLS with the miR-449a mimic or miR-NC, the expression of HMGB1 was detected. The results revealed that miR-449a inhibited HMGB1 expression at both the mRNA (Fig. 2c) and protein levels (Fig. 2d, e).

qPCR results showed that the expression of HMGB1 was significantly increased in RA synovial tissue compared with OA tissue (Fig. 2f). Immunohistochemistry results also found that HMGB1 was overexpressed in RA synovial tissue (Additional file 2: Figure S1). Furthermore, the

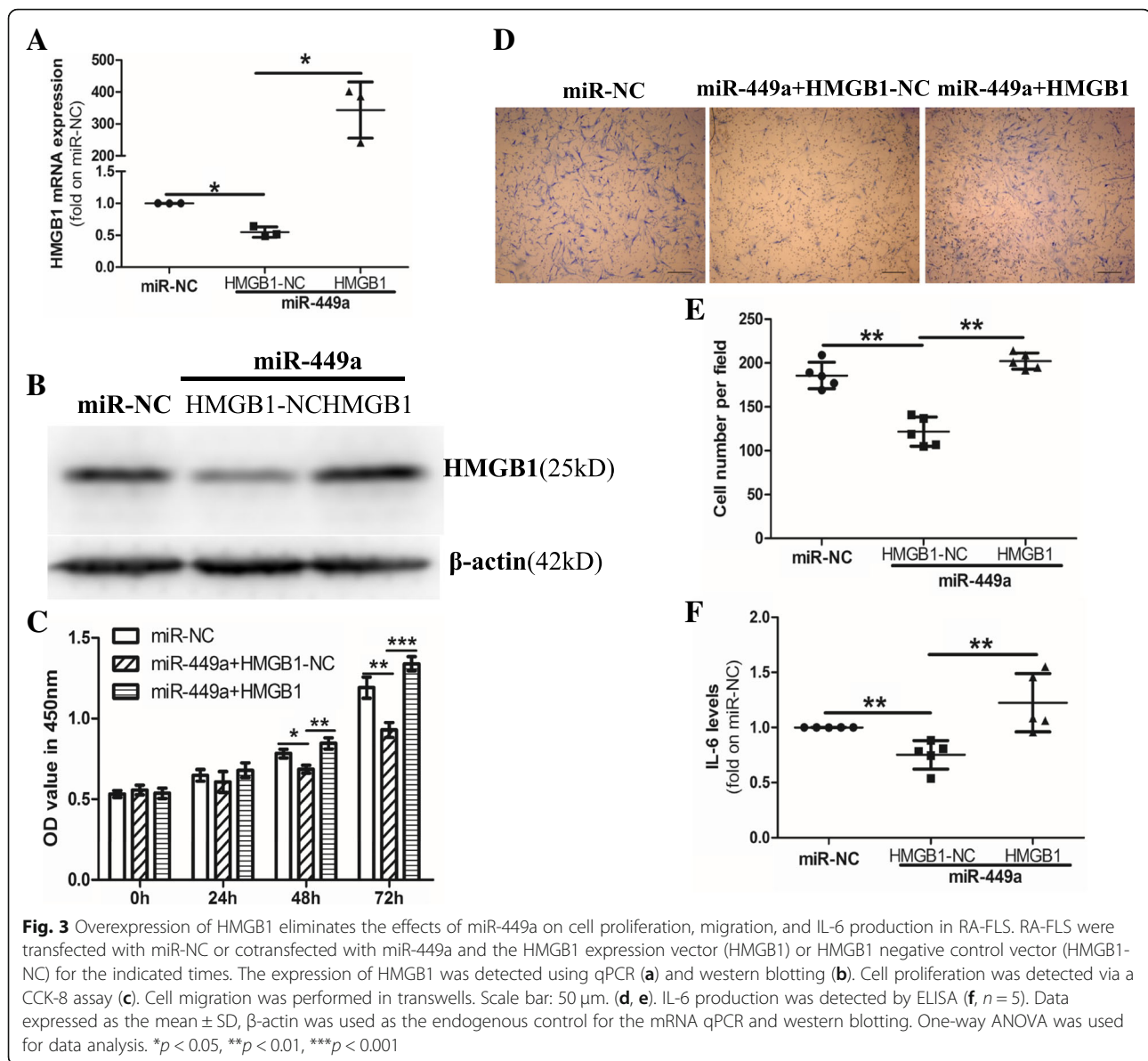


analysis of the expression of miR-449a and HMGB1 mRNA in RA synovial tissue indicated an inverse correlation between the two (Fig. 2g), which suggested that miR-449a might be an important regulator of HMGB1 expression in RA. All the results indicated that HMGB1 was a direct target of miR-449a in RA-FLS.

Overexpression of HMGB1 eliminates the effects of miR-449a in RA-FLS

Given that HMGB1 promotes cell proliferation, migration, invasion, and autophagy in RA-FLS, we wanted to demonstrate whether miR-449a inhibits cell proliferation, migration, and IL-6 production in RA-FLS by inhibiting the expression of HMGB1. After the transfection of RA-FLS with the HMGB1 expression vector

(pCMV-HMGB1), the HMGB1 levels were markedly increased at both the mRNA and protein levels (Fig. 3a, b). After the transfection of RA-FLS with the miR-NC or cotransfection of RA-FLS with the miR-449a and pCMV-HMGB1 (miR-449a + HMGB1) or pCMV blank vector (miR-449a + HMGB1-NC), cell proliferation, migration, and IL-6 production were detected. Compared with the miR-NC group, the miR-449a + HMGB1-NC group inhibited RA-FLS proliferation, migration, and IL-6 production, but the overexpression of HMGB1 reduced the effects of miR-449a in RA-FLS (Fig. 3c–f). These data indicated that the miR-449a-mediated inhibition of RA-FLS proliferation, migration, and IL-6 production might be achieved by inhibiting HMGB1 expression.

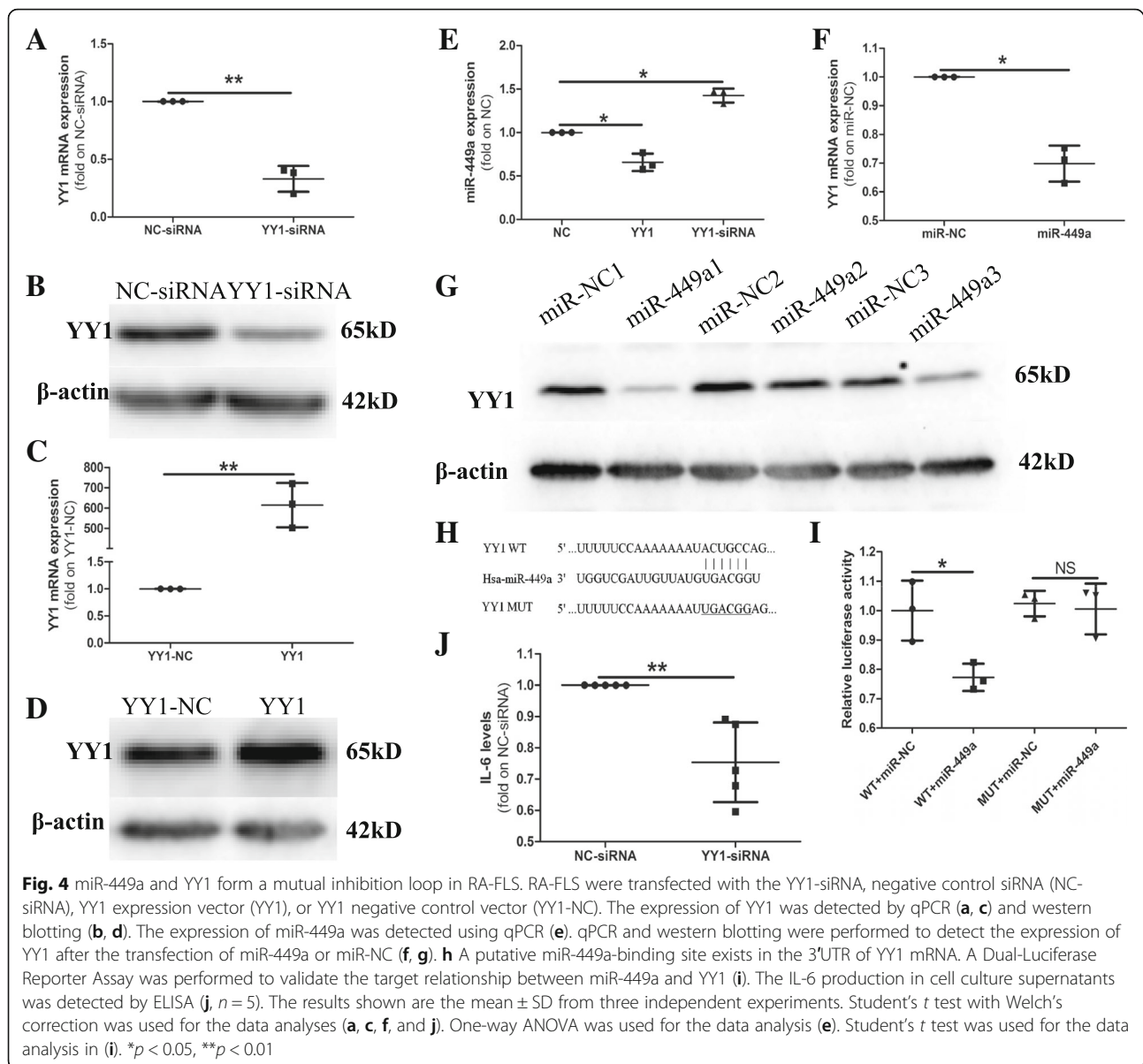


miR-449a and YY1 form a mutual inhibition loop in RA-FLS

Previous studies found that the transcription factor YY1 was closely related to RA inflammation [25, 26]. YY1 regulates IL-6 transcription in RA and inhibits miR-10a expression, which contributes to the excessive secretion of inflammatory cytokines and the migration and proliferation of RA-FLS [25, 26]. Therefore, we sought to determine whether the dysregulation of miR-449a in RA was also related to YY1. The results showed that the YY1 expression markedly decreased at both the mRNA and protein levels (Fig. 4a, b) after the transfection of RA-FLS with the YY1-siRNA. We also induced YY1 expression using the pCMV-YY1 vector (Fig. 4c, d). After the induction or inhibition of YY1 expression, we detected the levels of miR-449a. As shown in Fig. 4e,

compared with the negative control, the YY1-siRNA promoted the expression of miR-449a, and pCMV-YY1 (YY1) inhibited miR-449a expression, indicating that YY1 negatively regulates the expression of miR-449a.

Furthermore, we discovered that the overexpression of miR-449a decreased the level of YY1 at both the mRNA and protein levels (Fig. 4f, g). A luciferase activity assay showed that miR-449a inhibited the luciferase activity in cells containing the miR-449a-binding site of the YY1 3' UTR but failed to inhibit the luciferase activity in cells containing a mutated version of the miR-449a-binding site of the YY1 3' UTR (Fig. 4h, i), indicating that YY1 was a direct target gene of miR-449a. These results indicated that miR-449a and YY1 formed a mutual inhibition loop in RA-FLS. We also detected the effect of YY1



on IL-6 production in RA-FLS. As shown in Fig. 4j, the YY1-siRNA inhibited IL-6 production in RA-FLS.

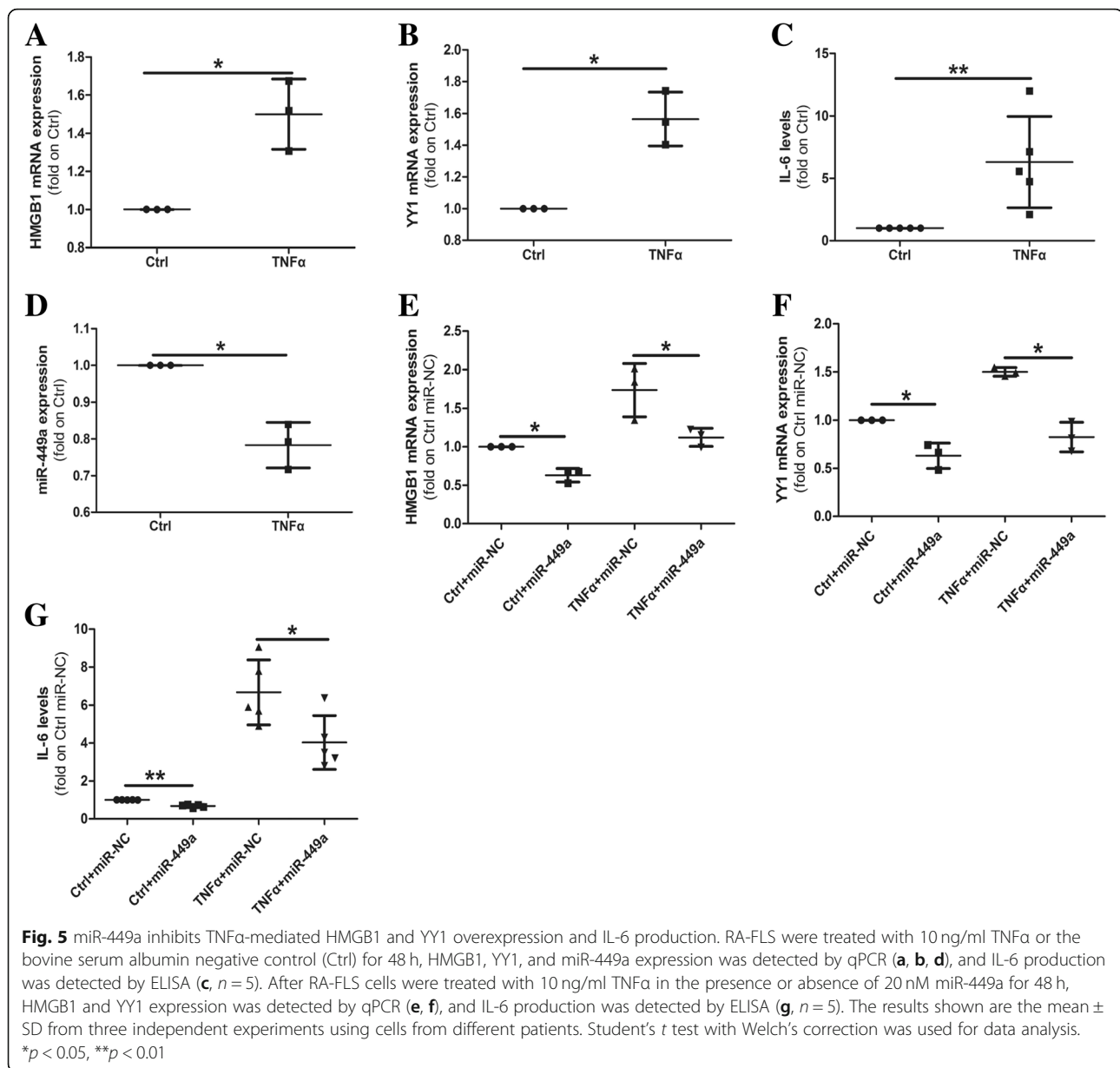
miR-449a inhibits TNF α -mediated HMGB1 and YY1 overexpression and IL-6 production

TNF α plays a crucial role in RA pathogenesis. Therefore, we investigated whether TNF α affected HMGB1, YY1 and miR-449a expression and IL-6 production, and the role of miR-449a in this process. After RA-FLS were treated with 10 ng/ml TNF α for 48 h, both the expression of HMGB1 (Fig. 5a) and YY1 (Fig. 5b) and IL-6 production (Fig. 5c) were significantly increased compared with the control-treated RA-FLS, and the expression of miR-449a was decreased (Fig. 5d). We then detected the effects of TNF α in the presence or absence of miR-449a. Interestingly,

miR-449a expression weakened TNF α -induced HMGB1 (Fig. 5e) and YY1 (Fig. 5f) expression and IL-6 production (Fig. 5g). These results indicated that miR-449a inhibited TNF α -mediated HMGB1 and YY1 overexpression and IL-6 production.

Discussion

In the present study, we determined that the expression of miR-449a was significantly decreased in RA synovial tissue compared with OA tissue. miR-449a inhibited cell proliferation, migration, and IL-6 production by regulating HMGB1 and YY1 expression in RA-FLS. Further study indicated that YY1 negatively regulated miR-449a expression and formed a mutual inhibition loop with miR-449a in RA-FLS. In addition, we also found



that TNF α promoted HMGB1 and YY1 expression and IL-6 production, and miR-449a weakened these effects of TNF α .

Although RA is the most common chronic inflammatory autoimmune joint disease, the pathogenesis of RA remains unclear. RA-FLS, located in the synovial lining, have been proven to play a key role in the RA process [27]. Similar to tumor cells, RA-FLS are a type of special tumor-like cells that are hyperplastic, invasive, and migratory, and these features are closely related to synovial hyperplasia, joint inflammation, and joint destruction [3, 28, 29]. Therefore, we can treat RA as a special tumor-like disease. miRNAs have been proven to be key regulators that target multiple genes in various disease

development and progression processes [30, 31]. Several miRNAs were observed to be abnormally expressed in RA, some of which affect RA-FLS cell proliferation, inflammation, or cellular signaling pathways [6–8]. In this study, we detected miR-449a expression and found that it was decreased in RA synovial tissue, which was consistent with the results of some studies in tumors [10, 13, 14, 32, 33]. We further studied the effect of miR-449a on RA-FLS and found that miR-449a inhibited RA-FLS cell proliferation, migration, and IL-6 production, which indicated that the dysregulation of miR-449a expression may be related to RA progression.

Previous studies found that HMGB1 was overexpressed in RA synovial tissue promoted RA-FLS

proliferation, migration, and invasion, and HMGB1-mediated autophagy decreased the sensitivity of RA-FLS to methotrexate (MTX) [18, 19]. It seems that HMGB1 plays a nonnegligible role in the pathogenesis of RA. In our study, we found that HMGB1 was regulated by miR-449a and was a direct target of miR-449a in RA-FLS, which was consistent with the study by Wu et al. [24]. They found that miR-449a inhibited cell proliferation, migration, and invasion in non-small cell lung cancer by directly targeting HMGB1. The results indicated that decreased miR-449a expression may be the main cause of HMGB1 overexpression in RA synovial tissue.

YY1 is a transcription factor with different biological functions that can either activate or repress gene expression by directly binding to the target promoters and recruiting histones and DNA modifiers [26, 34]. Recent studies have found that YY1 is overexpressed in RA patients and CIA mice [25, 26]. Mu et al. [25] identified that miR-10a, a YY1 target gene, was inhibited by YY1, which contributed to excessive NF- κ B-mediated inflammatory cytokine secretion and RA-FLS migration and proliferation. Lin et al. [26] also found that YY1 promoted IL-6 transcription by directly binding to the IL-6 promoter, which contributed to RA inflammation. These studies indicated that YY1 plays an important role in RA pathogenesis. In this study, when we inhibited the expression of YY1 using an siRNA, we found that miR-449a expression was increased, and when we overexpressed YY1 in RA-FLS, the expression of miR-449a was decreased. The results indicated that YY1 negatively regulated miR-449a expression. Further study found that YY1 was also a direct target of miR-449a. Taken together, the results indicated that miR-449a and YY1 formed a mutual inhibition loop in RA-FLS. We also found that YY1 inhibited IL-6 production in RA-FLS, which was consistent with Lin's research [26].

TNF α is a key inflammatory cytokine implicated in RA pathogenesis, and it plays a crucial role in joint destruction [35]. In recent years, the biological disease-modifying antirheumatic drugs targeting TNF α (infliximab, adalimumab) have achieved good results in clinical application, but more than half of RA patients still have no responses to the drug or unwanted side effects and eventually have to discontinue the treatment [36, 37]. Therefore, exploring the mechanism of TNF α in RA may contribute to overcoming these limitations. Mu et al. [25] found that an NF- κ B/YY1/miR-10a/NF- κ B regulatory circuit, which promotes excessive NF- κ B-mediated inflammatory cytokine secretion and RA-FLS cell proliferation and migration, existed. In this study, we observed that TNF α promoted HMGB1 and YY1 mRNA expression and IL-6 production. After the transfection of miR-449a, the expression of HMGB1 and YY1 and the production of IL-6 were decreased. These results

indicated that miR-449a acted as a regulator to inhibit TNF α -mediated HMGB1 and YY1 overexpression and IL-6 production.

Conclusions

We first demonstrated that miR-449a inhibited RA-FLS proliferation, migration, and IL-6 production by directly targeting HMGB1 and YY1. miR-449a inhibited TNF α -mediated YY1 and HMGB1 overexpression and IL-6 production and formed a mutual inhibition loop with YY1 in RA-FLS, which indicated that miR-449a was a crucial molecule in RA pathogenesis and acted as a switch to control TNF α -mediated inflammation. As a result, miR-449a-based therapeutic strategies may offer a new treatment option for RA. Further study is needed to confirm the new option in *in vivo* experiments.

Additional files

Additional file 1: Table S1. Sequences of oligonucleotides. (DOCX 12 kb)

Additional file 2: Figure S1. HMGB1 was overexpressed in RA synovial tissue compared with OA tissue. (DOCX 488 kb)

Abbreviations

ACR: American College of Rheumatology; CI: Confidence interval; ELISA: Enzyme-linked immunosorbent assay; HMGB1: High-mobility group box protein 1; miRNAs: MicroRNAs; MTX: Methotrexate; OA: Osteoarthritis; RA: Rheumatoid arthritis; RA-FLS: Rheumatoid arthritis fibroblast-like synoviocytes; SD: Standard deviation; YY1: Yin Yang 1

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Authors' contributions

PX and YQ conceived of the study and analyzed the data. YC and CJ carried out the experiments, analyzed the data, participated in the study design, and drafted the article. JZ, KX, XR, PH, and LX collected the clinical patients and partly performed cell culture and miR-expression experiment. BW, QY, YG, and JS participated in the analysis and interpretation of data. All authors have read and approved the final manuscript.

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Availability of data and materials

All the data generated and analyzed during this study are included in this published article and are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was performed with the approval of the human research ethics committee of Xi'an Hong Hui Hospital. All patients fulfilled the ACR criteria for the classification of RA or OA and provided informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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