

# Elevated microRNA-145-5p increases matrix metalloproteinase-9 by activating the nuclear factor- $\kappa$ B pathway in rheumatoid arthritis

XIAOXUE WANG<sup>1\*</sup>, KE TANG<sup>2\*</sup>, YUANYUAN WANG<sup>3</sup>, YAQING CHEN<sup>2</sup>, MENGCHEN YANG<sup>4</sup>,  
CHUNGANG GU<sup>5</sup>, JING WANG<sup>1</sup>, YI WANG<sup>6</sup> and YUHUA YUAN<sup>1</sup>

<sup>1</sup>Clinical Laboratory Diagnostics, Tianjin Medical University General Hospital Airport Site, Tianjin 300308; <sup>2</sup>School of Laboratory Medicine, Tianjin Medical University, Tianjin 300070; <sup>3</sup>Department of Cardiology, The Second Hospital of Tianjin Medical University, Tianjin 300211; <sup>4</sup>Department of Neurosurgery, Tianjin Medical University General Hospital, Tianjin 300052; <sup>5</sup>Clinical Laboratory Diagnostics, Tianjin Third Center Hospital, Tianjin 300170; <sup>6</sup>Clinical Laboratory Diagnostics, Tianjin Hospital, Tianjin 300211, P.R. China

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**Abstract.** The present study explored whether miR-145-5p can aggravate the development and progression of rheumatoid arthritis (RA) by regulating the expression of matrix metalloproteinases (MMPs). ELISAs, reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and western blotting were used to examine the expression levels of MMP-1, MMP-3, MMP-9, and MMP-13 in fibroblast-like synoviocytes (FLS) from patients with RA. Levels of MMP-1, MMP-3, MMP-9, and MMP-13 were assessed in the right hind ankles of a murine collagen-induced arthritis (CIA) model by RT-qPCR and immunohistochemical (IHC) analysis. The effects of activation or inhibition of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway on MMPs were evaluated by RT-qPCR and western blotting. Subcellular localization of NF- $\kappa$ B p65 was visualized by confocal microscopy. Overexpression of miR-145-5p increased the expression of MMP-3, MMP-9, and MMP-13 in RA-FLS. Moreover, injection of a miR-145-5p

agomir into mice increased MMP-3, MMP-9, and MMP-13, as demonstrated by RT-qPCR and IHC analysis. A chemical inhibitor that selectively targets NF- $\kappa$ B (BAY11-7082) significantly attenuated MMP-9 expression, while it did not influence the levels of MMP-3 and MMP-13. Immunofluorescence analysis revealed that nuclear localization of p65 was significantly enhanced, indicating that miR-145-5p enhances activation of the NF- $\kappa$ B pathway by promoting p65 nuclear translocation. miR-145-5p overexpression also significantly increased phosphorylated p65 levels; however, the levels of I $\kappa$ B- $\alpha$  were reduced in response to this miRNA. Moreover, our results indicated that miR-145-5p aggravated RA progression by activating the NF- $\kappa$ B pathway, which enhanced secretion of MMP-9. In conclusion, modulation of miR-145-5p expression is potentially useful for the treatment of RA inflammation, by regulating the expression of MMPs, and MMP-9 in particular, through inhibition of the NF- $\kappa$ B pathway.

## Introduction

Rheumatoid arthritis (RA), which affects approximately 1% of the population of the world, is characterized by chronic inflammation and the destruction of the cartilage and bone in various joints. RA is usually associated with disability and systemic complications (1). The extracellular matrix (ECM) is an important structural component that maintains the integrity of bone tissue. In patients with RA, degradation of the ECM by matrix metalloproteinases (MMPs) is considered essential to joint destruction (2) and MMPs also have important roles in the erosion of joint bone and cartilage (3).

MicroRNAs (miRNAs) are small, conserved, non-coding RNA molecules that regulate approximately 30% of human genes and influence several cellular processes, including cell growth, differentiation, proliferation, and death (4). Numerous studies have demonstrated the involvement of miRNAs in RA development (5), and these molecules have emerged as important contributing factors in RA pathophysiology. Some microRNAs, for example, miRNA-203 (6) and miRNA-155 (7),

*Correspondence to:* Professor Yuhua Yuan, Clinical Laboratory Diagnostics, Tianjin Medical University General Hospital Airport Site, 85 Dongliu Road, Tianjin 300308, P.R. China  
E-mail: yhx39@sina.com

\*Contributed equally

**Abbreviations:** CIA, collagen-induced arthritis; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; FLS, fibroblast-like synoviocytes; IHC, immunohistochemical; MAPK, mitogen-activated protein kinase; miRNAs, microRNAs; MMP, matrix metalloproteinase; NF- $\kappa$ B, nuclear factor- $\kappa$ B; OA, osteoarthritis; PBMCs, peripheral blood mononuclear cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; RA, rheumatoid arthritis; STAT, signal transduction and transcriptional activator

**Key words:** rheumatoid arthritis, MMP-9, microRNA, FLS, NF- $\kappa$ B

can affect RA development by regulating the levels of MMPs that participate in the processes of bone and joint erosion and destruction. Previously, we determined that the levels of miR-145-5p are significantly increased in peripheral blood mononuclear cells (PBMCs) and synovial tissue from patients with RA compared with healthy control PBMCs and osteoarthritis (OA) tissue samples. Micro-computed tomography (CT) of the joints, the expression levels of mRNA and histopathological analysis of arthritic joints revealed that the miR-145-5p agomir aggravates cartilage erosion (8); however, whether miR-145-5p is related to the levels of MMPs in RA patients and whether this miRNA is involved in bone erosion remains unknown.

In the present study, it was revealed that overexpression of miR-145-5p increased expression of MMP-3, MMP-9, and MMP-13 in RA-fibroblast-like synoviocytes (FLS) from patient samples. Furthermore, a chemical inhibitor of nuclear factor  $\kappa$ B (NF- $\kappa$ B) signaling, BAY11-7082, significantly attenuated the expression of MMP-9. Overexpression of miR-145-5p increased p65 nuclear localization and levels of phosphorylated p65 (p-p65), while significantly reducing those of I $\kappa$ B- $\alpha$ . These results demonstrated that miR-145 can promote increased MMP-9 levels in patients with RA by targeted activation of the NF- $\kappa$ B pathway.

## Materials and methods

**Isolation and culture of FLS from patients with RA.** RA patients (n=30; female, 22; male, 8; median age, 56.2 years; age range, 21–61 years) who were admitted to the Department of Rheumatology and Immunology, Tianjin Medical University General Hospital (Tianjin, China), between October 2015 and January 2017, were enrolled in the present study. To isolate synovial fibroblasts, synovial tissue specimens obtained immediately after opening the knee-joint capsule were minced and digested with dispase at 37°C for 60 min. After washing, the cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 100 IU/ml penicillin or streptomycin and 10% fetal calf serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA). RA-FLS cultures were maintained at 37°C under humid conditions with 5% CO<sub>2</sub>. All FLS cultures were subjected to experimental procedures between passages 4 and 6.

**Transfection of FLS.** miR-145-5p mimic (cat. no. miR10000437), miR-145-5p inhibitor (cat. no. miR20000437), mimic negative control (cat. no. miR01101), and inhibitor negative control (cat. no. miR02101) were provided by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cells (2x10<sup>6</sup>) were transfected with miR-145-5p inhibitor (final concentration, 100 nM), miR-145-5p mimic (final concentration, 50 nM), and corresponding controls using RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. Western blot analyses of factors in the NF- $\kappa$ B, p53/MDM2, JNK, and mitogen-activated protein kinase (MAPK) pathways were performed using proteins extracted from cells treated with miR-145-5p mimic and control mimic for 24 h. Chemical inhibitors were added to cultures 2 h prior to stimulation with miR-145-5p. Subsequently, the cells were used for functional assays or RNA/protein extraction.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** TRIzol<sup>®</sup> reagent (Takara Bio, Inc., Otsu, Japan) was used to isolate total RNA, including miRNA from FLS. A FastQuant RT Kit (Tiangen Biotech Co., Ltd., Beijing, China) was used to reverse-transcribe RNA into complementary DNA, following the manufacturer's instructions. SYBR-Green PCR Master Mix (Takara Bio, Inc.) was used to perform RT-qPCR, on a Light Cycler 480 (Roche Diagnostics, Indianapolis, IN, USA) with the following program: 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec, 60°C for 30 sec, 95°C for 5 sec, and 60°C for 1 min. Specific primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). A forward primer was designed based on the mature miR-145-5p DNA sequence (5'-GUCCAGUUU UCCAGGAAUCCCU-3') from miRBase (<http://www.mirbase.org>), whereas a universal 3' reverse primer (5'-AGTGCAGGG TCCGAGGTATT-3'; Guangzhou RiboBio Co., Ltd.) was used. Other primers were as follows: MMP-1 forward, 5'-TTCTAC ATGCGCACAAATCC-3' and reverse, 5'-ACCGGACTTCAT CTCTGTTCG-3'; MMP-3 forward, 5'-GCGCCCTGGTCTTG GTGCCAT-3' and reverse, 5'-GAAACCACAATTCTGTCTTTC AC-3'; MMP-9 forward, 5'-CCTTCACTTTCCTGGGTAAG-3' and reverse, 5'-CCATTCACGTCGTCCTTATG-3'; MMP-13 forward, 5'-TCCTGATGTGGGTGAATACAATG-3' and reverse, 5'-GCCATCGTGAAGTCTGGTAAAAT-3'. GAPDH (forward, 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGTTGTGCATACTTCTCATGG-3') and U6 (forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCA CGAATTTGCGT-3') were used as internal controls for mRNA and miRNA, respectively. Relative quantification was performed based on differences in cross-threshold values ( $\Delta$ Cq) between the gene of interest and the endogenous control [i.e., Cq(gene of interest)-Cq(endogenous control)]. Relative expression was calculated using the comparative threshold cycle method (9).

**Enzyme-linked immunosorbent assay (ELISA).** The concentrations of MMP-1 (cat. no. 70-EK1M01-96), MMP-3 (cat. no. 70-EK1M03-96), MMP-9 (cat. no. 70-EK1M09-96) and MMP-13 (cat. no. 70-EK1M132) were assessed in cell supernatants by ELISA using the SensoLyte ELISA kit according to the instructions of the manufacturer (MultiSciences (Lianke) Biotech Co., Ltd., Hangzhou, China).

**Western blot analysis.** Cultured FLS were lysed in radioimmunoprecipitation assay buffer supplemented with a protease inhibitor cocktail (both from Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), and boiled. The protein concentration was measured using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Equal volumes (25  $\mu$ g) of concentrated samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 8–10% gels, before transfer to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Non-fat dried milk powder in Tris buffered saline with 0.1% Tween-20 was used to block membranes at room temperature for 2 h, and membranes were then incubated with primary antibodies at 4°C overnight. Incubation with horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. 7074S; Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 h at room temperature was used to assess the protein levels. Enhanced chemiluminescence (Amersham Life Sciences; GE Healthcare Life Sciences,

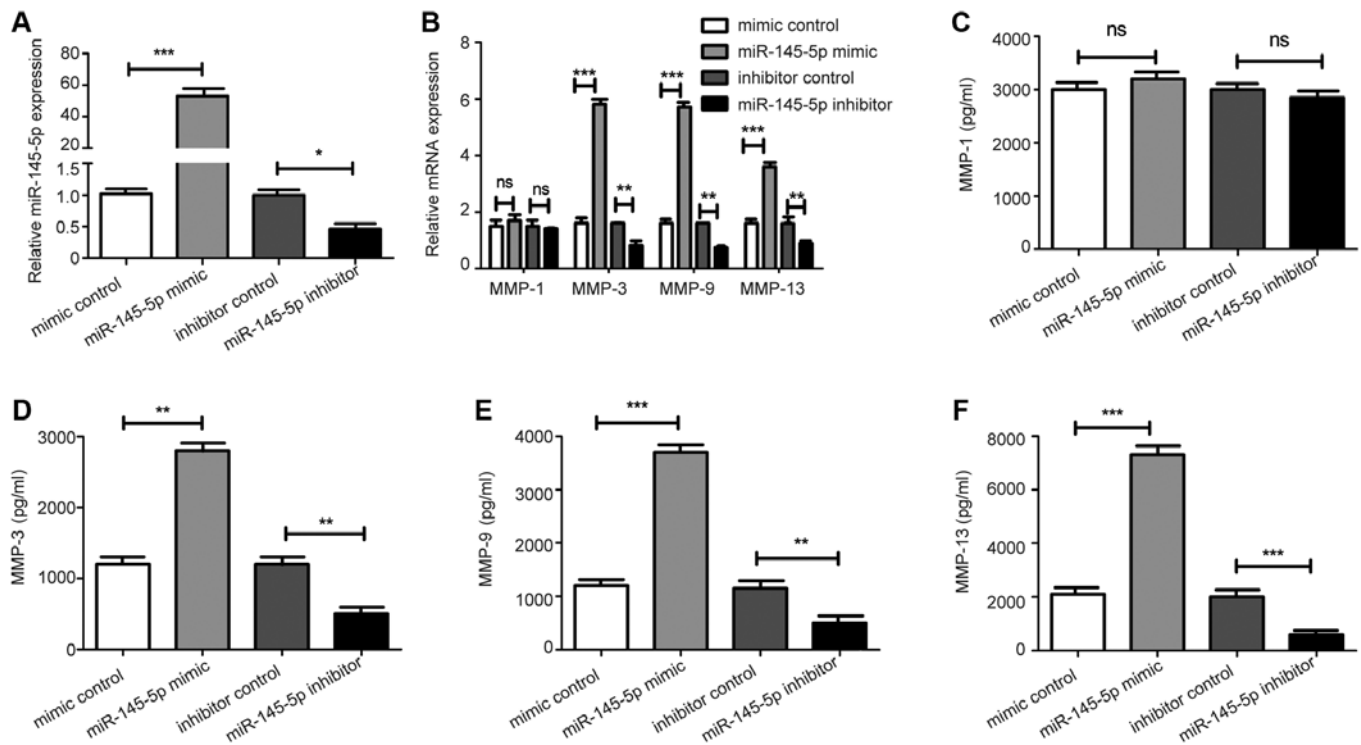


Figure 1. miR-145-5p regulates MMPs. (A) After transfection with the miR-145-5p mimic, miR-145-5p inhibitor, and respective controls, levels of miR-145-5p in RA-FLS were assessed by RT-qPCR. (B) Assessment of intracellular levels of MMPs in RA-FLS after transfection with miR-145-5p mimic, miR-145-5p inhibitor, and respective controls by RT-qPCR and (C-F) ELISA. This experiment was repeated at least three times with similar results; one set of representative results is presented. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  vs. control. Values are expressed as the mean  $\pm$  SD. MMPs, matrix metalloproteinases; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocytes; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

Little Chalfont, UK) reagent was used to visualize signals, and individual protein band intensities were quantified using ImageJ software (version 1.6.0; National Institutes of Health, Bethesda, MD, USA). Primary antibodies used were as follows: MMP-1 (1:1,000; cat. no. ab137332), MMP-3 (1:1,000; cat. no. ab52915), MMP-9 (1:1,000; cat. no. ab38898), MMP-13 (1:3,000; cat. no. ab39012), I $\kappa$ B $\alpha$  (1:2,000; cat. no. ab32518), p65 (1:3,000; cat. no. ab32536), and p-p65 (1:700; Ser536; cat. no. ab28856) were all purchased from Abcam (Cambridge, MA, USA).  $\beta$ -actin antibody (1:2,000; cat. no. ab8227; Abcam) was applied as a loading control.

**Induction of CIA.** Male DBA/1 mice (15-20 g; 8 weeks old) were used in the present study. A total of 30 animals were housed at  $24\pm1^{\circ}\text{C}$  with a 12:12-h light/dark cycle and a relative humidity of  $56\pm5\%$ . Animals were provided access to food and water *ad libitum*, and were fed a commercial diet according to the guidelines of the Animal Ethical and Welfare Committee. All mice were housed individually. Lyophilized bovine type II collagen (Chondrex, Inc., Redmond, WA, USA) was dissolved overnight at  $4^{\circ}\text{C}$  in 0.05 M acetic acid under constant stirring. Next, the collagen was emulsified using Freund's complete adjuvant (Chondrex, Inc.) to provide a final concentration of 2 mg/ml. Mice were injected in the tail with 0.1 ml of emulsion on day 1 and booted intraperitoneally on day 21 with collagen. On day 30, an equal volume of mir-145-5p agomir or scrambled miRNA agomir (15 nM; Guangzhou RiboBio Co., Ltd.) was injected

in the tail vein of mice at 1-week intervals. Four weeks after the first injection, the mice were sacrificed by injecting pentobarbital sodium at a dose of 100 mg/kg, then various indications such as respiration, heartbeat, pupil reflection, and nerve reflex were observed in the mice, and when all the aforementioned reactions disappeared, death was judged. Next, the right hind limb was dissected and synovial tissue was collected for subsequent experiments.

**Immunohistochemistry (IHC).** IHC analysis of MMPs was conducted using 3- $\mu\text{m}$ -thick sections from 8-week-old male DBA/1 mice, obtained from Hua Fukang Co., Ltd. (Beijing, China). Sections were fixed in 4% methanol at  $4^{\circ}\text{C}$  for 24 h, washed in PBS and treated with retrieval solution (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 20 min at  $90^{\circ}\text{C}$ . Following incubation in 5% BSA (cat. no. SW3015; Beijing Solarbio Science & Technology Co., Ltd.) at  $4^{\circ}\text{C}$  for 30 min, the sections were treated with MMP-1 (1:1,000; cat. no. ab137332), MMP-3 (1:1,000; cat. no. ab52915), MMP-9 (1:1,000; cat. no. ab38898) and MMP-13 (1:3,000; cat. no. ab39012) primary antibodies overnight at  $4^{\circ}\text{C}$ . Sections incubated with Alexa Fluor<sup>®</sup> 568-conjugated rabbit anti-goat immunoglobulin G (1:1,000; cat. no. A-11079; Molecular Probes; Thermo Fisher Scientific, Inc.) as a secondary antibody for 1 h at room temperature. Antibodies were diluted with 5% BSA. DAPI solution was applied for 5 min at room temperature for nuclear staining. Negative controls were prepared in the same manner, but without primary antibody. Images were

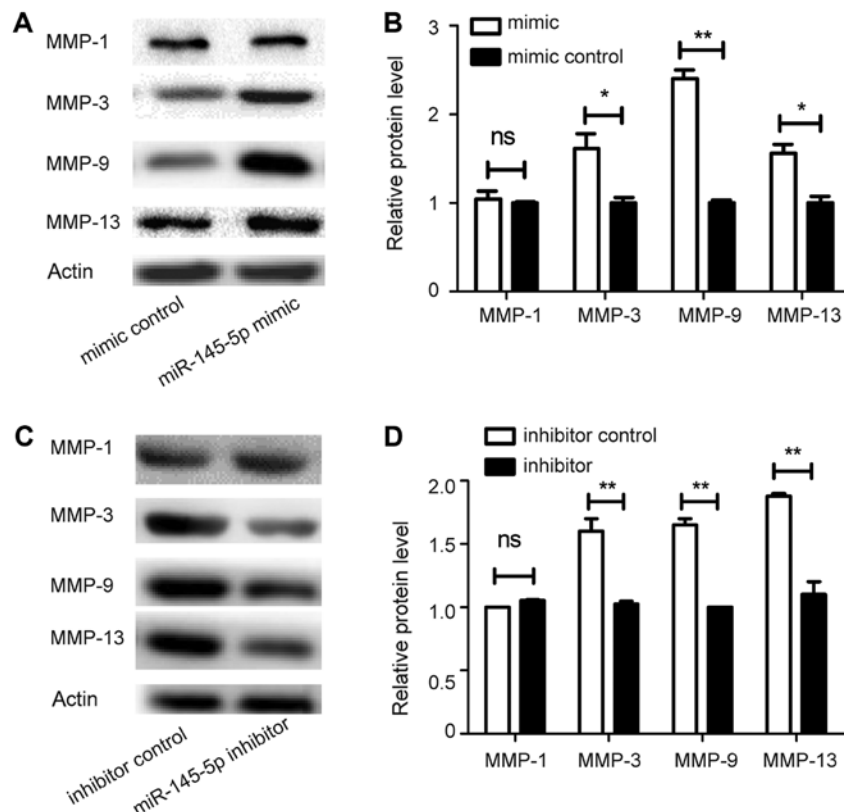


Figure 2. Effect of miR-145-5p on MMPs. (A and C) After transfection with the miR-145-5p mimic, miR-145-5p inhibitor, and respective controls, levels of miR-145-5p in RA-FLS were assessed by western blot analysis. (B and D) Quantification data are presented for the corresponding groups. The relative protein levels were normalized to the level of  $\beta$ -actin as an internal control. \* $P$ <0.05, \*\* $P$ <0.01 vs. control. Values are expressed as the mean  $\pm$  SD. MMPs, matrix metalloproteinases; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocytes.

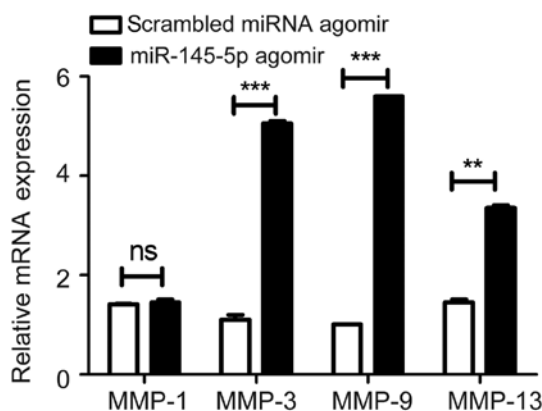


Figure 3. MMP-3, MMP-9, and MMP-13 are upregulated in the ankles of CIA mice. Real-time PCR analysis of MMP-1, MMP-3, MMP-9, and MMP-13 in the ankle synovial tissues from mice with CIA ( $n=4$ ) after injecting miR-145-5p agomir or scrambled miRNA agomir. \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs. scrambled miRNA agomir. Values are expressed as the mean  $\pm$  SD. MMP, matrix metalloproteinase; CIA, collagen-induced arthritis.

captured using an Olympus IX2-UCB microscope (magnification,  $\times 40$ ; Olympus Corporation, Tokyo, Japan).

**Investigation of signaling pathways.** Specific inhibitors of various signaling pathways were purchased from Beijing Solarbio Science & Technology Co., Ltd. and used to analyze the effects of these pathways on levels of MMP-3, MMP-9, and MMP-13. Briefly, specific inhibitors of NF- $\kappa$ B (20  $\mu$ M,

BAY11-7082), p53/MDM2 (10  $\mu$ M, Nutlin-3), JNK (10  $\mu$ M, SP600125), and MAPK (10  $\mu$ M, SB203580) pathways (all from Beyotime Institute of Biotechnology, Haimen, China) were added to FLS cultures 2 h prior to miR-145-5p stimulation, following the manufacturer's instructions. RT-qPCR and western blotting were used to assess the expression levels of MMP-3, MMP-9, and MMP-13 as aforementioned.

**Immunofluorescence.** FLS cells were fixed in 4% methanol at 4°C for 15 min, washed three times (5 min each) with PBS, permeabilized with 0.1% Triton X-100 for 15 min, then, incubated overnight at 4°C with anti-NF- $\kappa$ B p65 primary antibody (1:100; cat. no. ab32536; Abcam), followed by incubation with FITC-labeled secondary antibody (cat. no. ab7086; Abcam) at room temperature for 1 h. Then, the cells were counterstained with DAPI for 5 min. Images were captured using a Leica TCS SP5 confocal fluorescence microscope (magnification,  $\times 80$ ). The negative control was prepared in the same manner without the primary antibody.

**Target sequence prediction.** TargetScan (version 7.2; [http://www.targetscan.org/vert\\_72/docs/help.html](http://www.targetscan.org/vert_72/docs/help.html)) and mirDB (version 6.0; <http://www.mirdb.org/download.html>) were employed to search for putative target sequences of miR-145-5p in the NF- $\kappa$ B pathway.

**Statistical analysis.** Data were expressed as the mean  $\pm$  standard error of the mean (SEM) from at least three independent experiments. The difference among groups was determined by

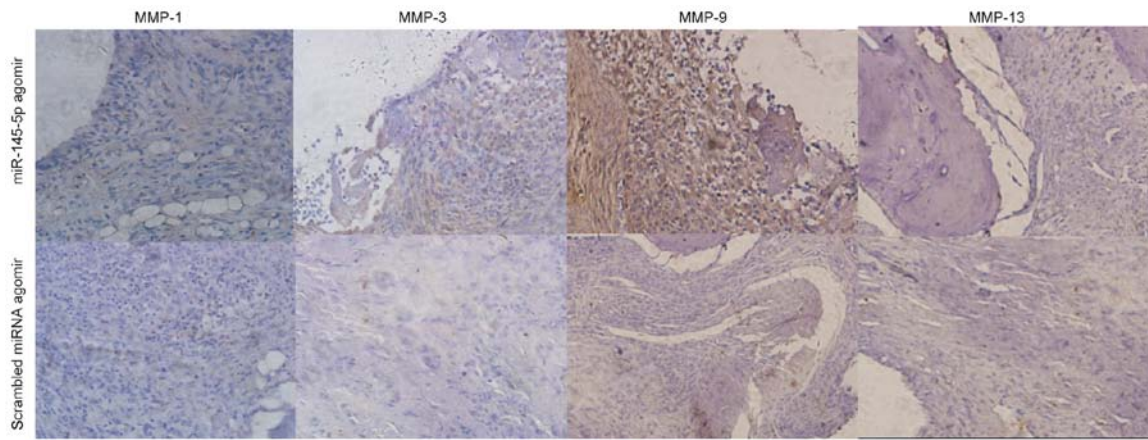


Figure 4. Sections from the hind paws of CIA mice (n=4). IHC and quantification of MMP-1, MMP-3, MMP-9, and MMP-13 in mice with CIA (n=4; magnification, x40). CIA, collagen-induced arthritis; IHC, immunohistochemical; MMP, matrix metalloproteinase.

analysis of variance (ANOVA) followed by Bonferroni's test, for qPCR experiments and western blotting, and differences between the two groups were analyzed using a two-tailed Student's t-test. Differences were considered statistically significant for P-values <0.05 (P<0.05, P<0.01, P<0.001).

## Results

**Overexpression of miR-145-5p enhances MMP-3, MMP-9, and MMP-13 levels in RA-FLS.** FLS samples isolated from synovial tissue derived from patients with RA were used to investigate the effect of miR-145-5p on synoviocytes *in vitro*. FLS were incubated with miR-145-5p mimic, miR-145-5p inhibitor, and their corresponding negative controls for 48 h. *MMP-3*, *MMP-9*, and *MMP-13* RNA levels were significantly increased by miR-145-5p mimic, while they were downregulated by miR-145-5p inhibitor; however, no change in *MMP-1* was detected (Fig. 1A and B). Consistent with the changes in secreted protein levels in cell culture supernatants (Fig. 1C-F), levels of intracellular MMP-3, MMP-9, and MMP-13 proteins were markedly enhanced by treatment with miR-145-5p mimic (Fig. 2A and B), but decreased after treatment with miR-145-5p inhibitor (Fig. 2C and D). These results indicated that miR-145-5p regulates various MMPs in FLS.

**Different mRNA expression levels of MMP-1, MMP-3, MMP-9, and MMP-13 in collagen-induced arthritis mice.** miR-145-5p agomir or scrambled miRNA agomir was injected into CIA mice in the tail vein. It was observed that miR-145-5p agomir significantly upregulated MMP-3, MMP-9, and MMP-13 compared with the mice treated with the scrambled miRNA agomir, however, the level of MMP-1 underwent no apparent alteration (Fig. 3).

**MMP-1, MMP-3, MMP-9, and MMP-13 levels in miR-145-5p-treated mouse joints.** IHC analysis (Fig. 4) revealed increased MMP-3, MMP-9, and MMP-13 levels in the hind ankles of mice treated with miR-145-5p agomir, compared with those in the right hind ankles of control mice.

**miR-145-5p abrogates stimulation of MMP-9 expression by NF- $\kappa$ B inhibitors.** To explore whether there is a direct

functional connection among miR-145-5p, cell signaling pathways, and MMP expression, the effects of NF- $\kappa$ B, p53/MDM2, MAPK and JNK signaling pathways were assessed using specific inhibitors targeting NF- $\kappa$ B (BAY11-7082, 20  $\mu$ M), p53/MDM2 (Nutlin-3, 10  $\mu$ M), MAPK (SB203580, 10  $\mu$ M), and JNK (SP600125, 10  $\mu$ M). In RA-FLS stimulated by miR-145-5p, the NF- $\kappa$ B inhibitor, BAY11-7082, significantly inhibited MMP-9 levels compared to those in the controls (Fig. 5A-C), while inhibitors of the other three cell signaling pathways had no marked effect on the levels of MMP-3, MMP-9, or MMP-13 (Fig. 5D-F).

**miR-145-5p activates the NF- $\kappa$ B signaling pathway in FLS samples from patients with RA.** To explore the mechanism underlying the involvement of miR-145-5p in MMP activation, the effects of the key NF- $\kappa$ B pathway factors were assessed, including p65, p-p65, and I $\kappa$ B-a, in response to miR-145-5p control of MMP-9 synthesis. Levels of p-p65 were significantly increased, while those of I $\kappa$ B-a were markedly decreased in response to miR-145-5p (Fig. 6A and B). Furthermore, reduction of miR-145-5p led to marked downregulation of p-p65 levels, while it increased those of I $\kappa$ B-a (Fig. 6C-D). In conclusion, miR-145-5p can contribute to arthritis progression by activating NF- $\kappa$ B signaling, which controls MMP-9 expression.

**miR-145-5p activates NF- $\kappa$ B by promoting p65 translocation from the cytoplasm to the nucleus.** An immunofluorescence assay was conducted to investigate p65 nuclear translocation in FLS. miR-145-5p induced NF- $\kappa$ B p65 nuclear translocation in FLS; however, this change was reversed by treatment with the NF- $\kappa$ B pathway inhibitor, BAY11-7082 (Fig. 7). These findings demonstrated that BAY11-7082 can attenuate miR-145-5p-induced NF- $\kappa$ B activation.

## Discussion

During normal physiological processes, the synthesis and degradation of joint-cartilage matrix proteins occurs in a state of dynamic equilibrium; however, in patients with RA, this equilibrium becomes disrupted. Matrix protein degradation and excessive bone resorption lead to the loss of cartilage matrix proteins and bone, and consequent gradual loss of joint



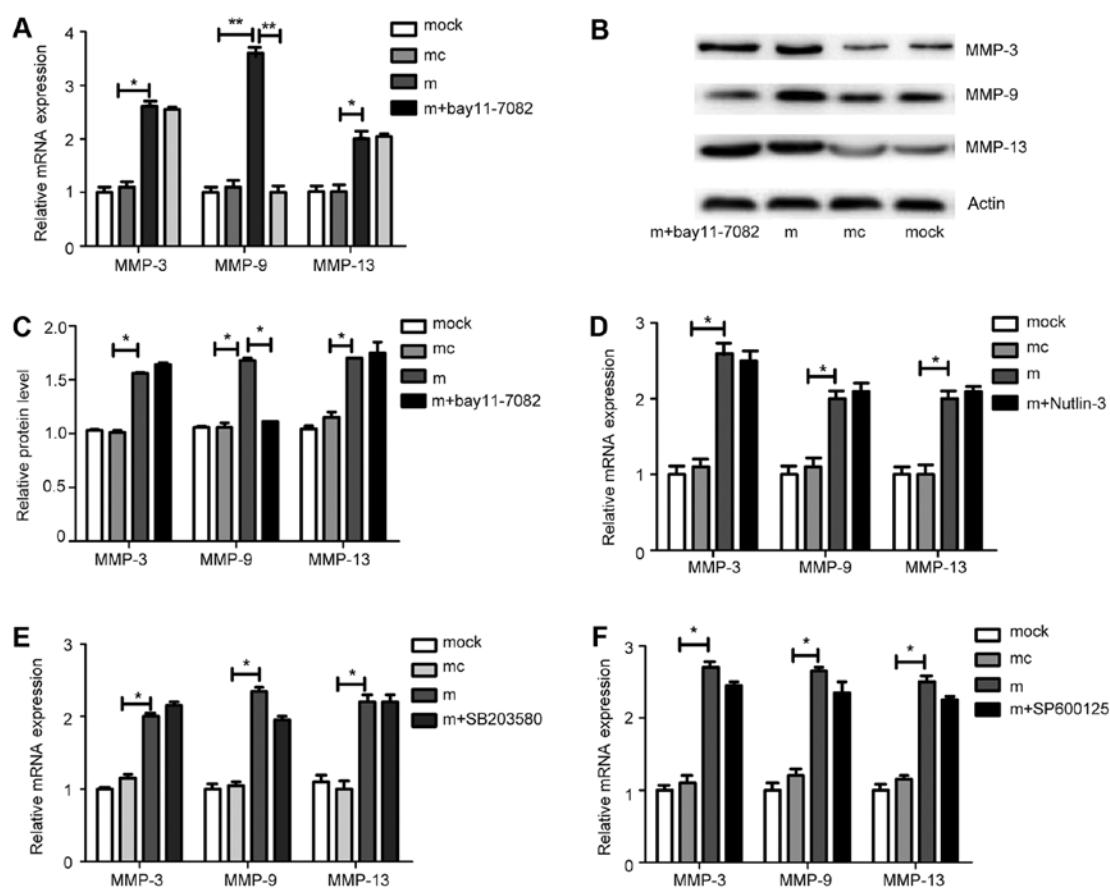


Figure 5. Changes in MMP levels following treatment with JNK, MAPK, NF- $\kappa$ B, and p53/MDM2 pathway inhibitors in FLS overexpressing miR-145-5p. (A and B) An inhibitor of the NF- $\kappa$ B pathway significantly suppressed levels of MMP-9, but not MMP-3 or MMP-13. (C) Quantification data are presented for the corresponding groups. (D-F) Inhibitors of the p53/MDM2, MAPK, and JNK pathways had no effect on MMP-3, MMP-9, or MMP-13 levels. \* $P < 0.05$ , \*\* $P < 0.01$ . Results are presented as the mean  $\pm$  SD of three independent experiments. MMP, matrix metalloproteinase; NF- $\kappa$ B, nuclear factor  $\kappa$ B; FLS, fibroblast-like synoviocytes.

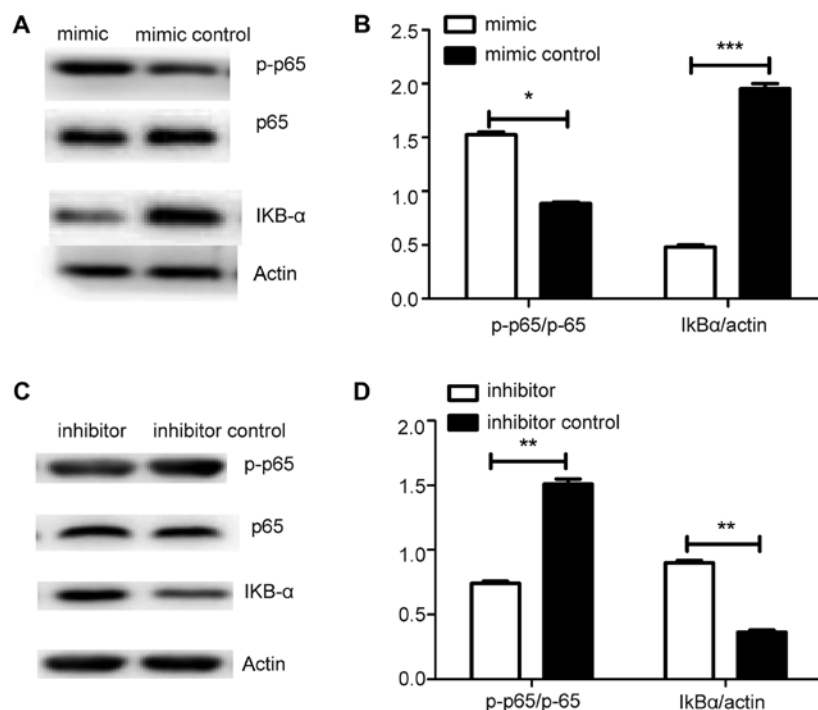


Figure 6. Effect of miR-145-5p on the NF- $\kappa$ B signaling pathway. To explore whether miR-145-5p is involved in the NF- $\kappa$ B signaling pathway, expression of p65, p-p65, and IκB- $\alpha$  were determined by western blotting in cells overexpressing miR-145-5p or with miR-145-5p downregulated. (A and B) Overexpression of miR-145-5p led to a significant increase in p-p65 and a decrease in IκB- $\alpha$  levels. (C and D) Similarly, downregulation of miR-145-5p markedly downregulated the p-p65 levels and increased those of IκB- $\alpha$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Results are presented as the mean  $\pm$  SD of three independent experiments. NF- $\kappa$ B, nuclear factor  $\kappa$ B; p-p65, phosphorylated p65.

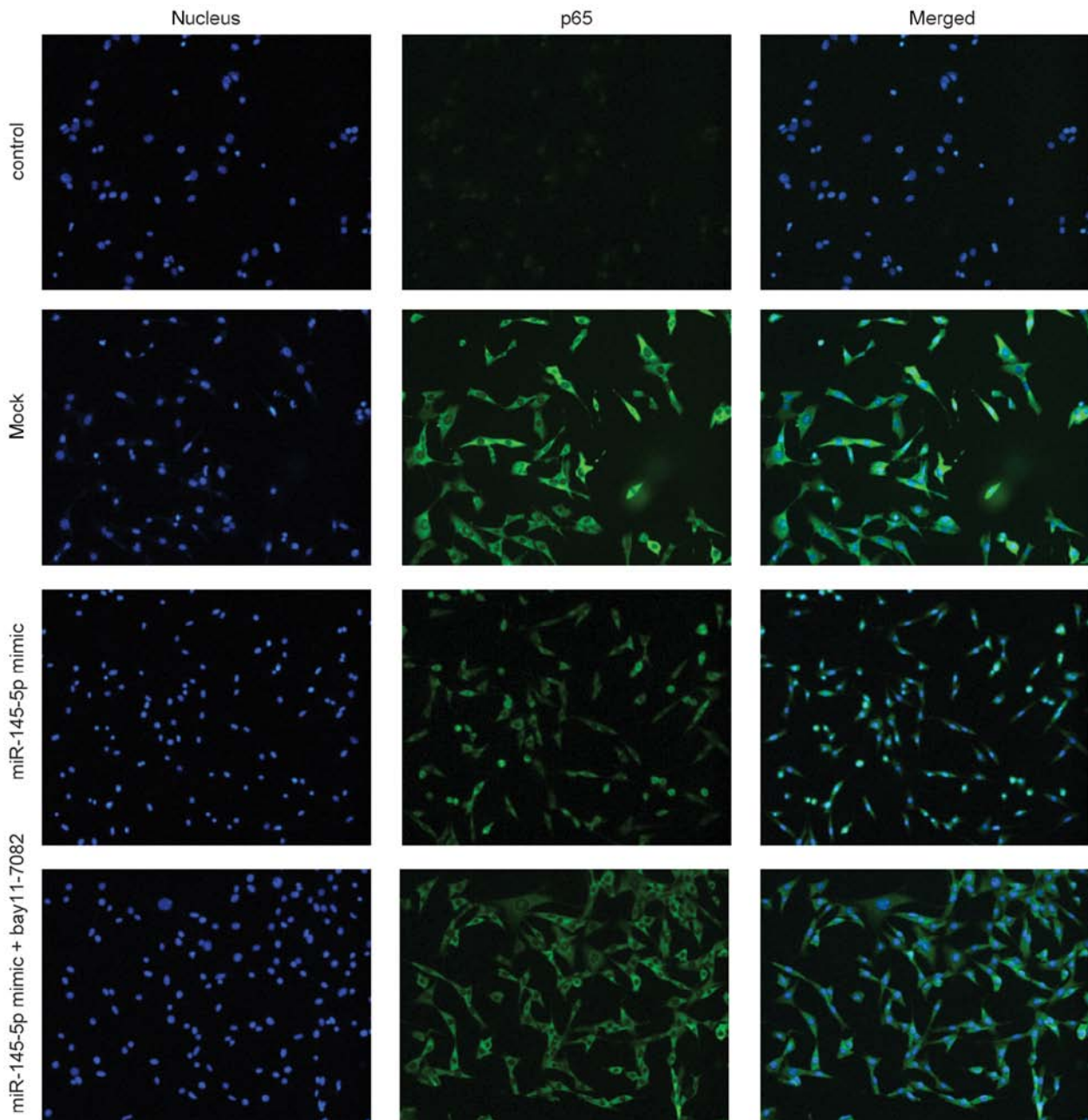


Figure 7. BAY11-7082 inhibits NF- $\kappa$ B signaling in FLS. FLS cells were stimulated as indicated, and subcellular localization of NF- $\kappa$ B p65 was visualized by immunofluorescence assay. p65 was detected by FITC-anti-p65 (green). Nuclei were stained with DAPI (blue). Merged picture reveals p65 translocation into the nucleus (magnification, x80). NF- $\kappa$ B, nuclear factor  $\kappa$ B; FLS, fibroblast-like synoviocytes.

integrity (10). The function of MMPs is mainly the degradation of type II collagen and proteoglycans; however, their aberrant expression can also promote erosion and destruction of articular cartilage in patients with RA (11).

Patients with RA have increased levels of various MMPs in their synovial fluid and peripheral blood, with these levels closely related to disease activity and bone and joint destruction (12). Erosion of the articular cartilage, the ECM of which is comprised of proteoglycans and collagen (mainly type II collagen), is the most prominent manifestation of RA, and loss of structural integrity directly affects the mechanics of the joint (13).

Aggrecanase, which is secreted by synoviocytes, can degrade the proteoglycan matrix, while MMP-9 can degrade

type II collagen. Giannelli *et al* (14) previously revealed that, in an arthritic mouse model, MMP-2 and MMP-9 have roles in arthritis pathogenesis. Various signaling pathways can promote MMP activity, including MAPK, signal transduction and transcriptional activator (STAT), and SMAD (15) pathways.

miR-146b was revealed to reduce glioma cell invasion by targeting MMP-16 gene expression in human patients with glioma (16). Stanczyk *et al* (7) and Wu *et al* (17) revealed that miR-155 regulated MMP-1 and MMP-3 levels by acting on the target molecule, SOCS1, to activate the APK/JNK signaling pathway, thereby affecting osteoblast and osteoclast metabolism. Furthermore, Akhtar *et al* (18) revealed that negative regulation of miR-27b expression by activation of MAPK and

NF- $\kappa$ B signaling may be necessary for IL-1 $\beta$ -mediated stimulation of MMP-13 production in OA chondrocytes.

In the present study, overexpression of miR-145-5p in RA-FLS significantly increased MMP-3, MMP-9, and MMP-13 levels. Analysis of MMP levels after the addition of JNK, MAPK, NF- $\kappa$ B, or p53-MDM2 pathway inhibitors demonstrated that only MMP-9 levels were significantly decreased by the NF- $\kappa$ B inhibitor, BAY11-7082.

NF- $\kappa$ B is a transcription factor that regulates the expression of multiple genes and a key signaling factor in the control of inflammation, synovial hyperplasia, and matrix degeneration. This transcription factor also regulates the expression of many pro-inflammatory genes (19,20). An NF- $\kappa$ B binding site was first identified in the MMP-9 promoter and associated with TNF- $\alpha$  gene induction, and subsequently reported to mediate the synergistic effects of growth factors and cytokines on MMP-9 (21). p65 is a key factor in the NF- $\kappa$ B pathway, and p-p65 can serve as a marker for NF- $\kappa$ B activation (22), while I $\kappa$ B- $\alpha$  is an inhibitor of NF- $\kappa$ B, and decreased I $\kappa$ B- $\alpha$  levels are also indicative of NF- $\kappa$ B pathway activation (23).

Herein, the regulatory effects of miR-145-5p on MMPs were assessed in bone-induced rheumatoid arthritis. The results revealed that miR-145-5p overexpression significantly increased MMP-9 levels and enhanced p65 nuclear translocation, while increasing the levels of p-p65 and decreasing those of I $\kappa$ B- $\alpha$ ; these effects were reversed by the NF- $\kappa$ B inhibitor, BAY11-7082. In conclusion, these studies suggest that miR-145 promotes increased MMP-9 levels in patients with RA by activating the NF- $\kappa$ B pathway, which accelerates the process of bone erosion, increasing disease severity of RA patients; however, predictions using two informatics software tools, TargetScan and miRDB, failed to identify a direct target of miR-145-5p in the NF- $\kappa$ B pathway (data not shown), suggesting that miR-145-5p may not affect MMP levels by directly regulating this pathway, and that other cytokines may also be involved in the regulatory process. For example, it has been reported that tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 and IL-6 can play an important role in cartilage catabolism as major pro-inflammatory cytokines (24). These cytokines may activate more signaling pathways to form a complex regulatory network. Therefore, further studies should be performed to confirm the results.

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## Availability of data and materials

All datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

YHY conceived and designed the experiments. XXW, KT, YYW, YQC, and MCY performed the cell culture, RT-qPCR, and IHC experiments, and interpreted the data. XXW, KT, YW, JW, and CGG performed the ELISA and western blotting experiments and interpreted the data. XXW wrote the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

## Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Tianjin Medical University General Hospital (no. IRB2015-YX-081) and the Animal Ethical and Welfare Committee (AEWC; no. TMUaMEC 2018035). Informed consent was obtained from all participants.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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