

miRNA-27a regulates arthritis via PPAR γ *in vivo* and *in vitro*

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Abstract. The present study investigated the role of microRNA (miR)-27a in the development of arthritis and its mechanism of action. Initially, collagen was used to develop an *in vivo* rat model of arthritis. Changes in the miRs in the rats were analyzed. It was subsequently observed that miR-27a expression was reduced in patients with arthritis, compared with the control group. In the present study an *in vitro* miR-27a overexpression model of arthritis was established and it was observed that miR-27a increased the proliferation of osteoblast-like cells *in vitro*. miR-27a overexpression promoted osteogenic differentiation, increased alkaline phosphatase (ALP) and osteoporosis (OST) content, induced insulin-like growth factor binding protein-5 (IGFBP-5) protein expression, reduced inflammation and suppressed peroxisome proliferator-activated receptor γ (PPAR γ) and matrix metalloproteinase-17 (MMP-17) protein expression in arthritis. However, miR-27a downregulation inhibited osteogenic differentiation, increased inflammation and PPAR γ and MMP-17 protein expression and suppressed ALP and OST content in an *in vitro* model of arthritis. The PPAR γ inhibitor reduced the function of miR-27a downregulation on arthritis. Therefore the results of the present study revealed that miR-27a regulates arthritis via PPAR γ .

Introduction

Osteoarthritis is a common and frequently occurring disease in orthopedics (1). The main pathological manifestations include articular cartilage degeneration, joint edge and subchondral bone hyperosteoecy (2). OA is one of the most serious arthrosis diseases that affect the health and life of human beings (3). It is more common in the middle and old aged people over 50 years old, seriously endanger the physical health of middle-aged and elderly people and affect their life quality. Also, it causes heavy burdens for individuals, families and the society (1).

miRNA is a single stranded non-coding RNA containing 19-25 molecules, commonly existing in plants, animals, and

microorganisms, and involving in regulating a variety of physiological activities in the body (4). In 1993, the first miRNA was found in *Caenorhabditis elegans* (5). miRNA has a wide range of biological functions in the body, and can participate in the regulation of many physiological activities, including cell division, differentiation, apoptosis and organ formation. The research on the function of miRNA has become a hot topic in the field of life science. The expression of miRNA is strictly regulated by the body, and has strict time specificity and tissue specificity (5). Meantime, the biological function of miRNA is also strictly regulated. Studies have shown that many miRNAs play an important role in the regulation of OA (6). Through gene chip and *in situ* hybridization, we found that the expression of nine miRNAs such as miRNA-483, miRNA-22, and miRNA-377 were significantly up-regulated in OA of human knee joint, and the expression of seven miRNAs was obviously downregulated (7).

Matrix metalloproteinases (MMPs) are synthesized by synovial and cartilage cells (8). They play a decisive role in the imbalance between extracellular matrix synthesis and degradation of osteoarthritis (OA) articular cartilage (8). Therefore, MMPs play a key role in OA. Its pathological function is mainly tissue degradation in OA (8). MMP-17 is one of the MMPs gene family members (9). It has strong matrix degradation activity and extensive substrate specificity. These substrates include type IV collagen, laminin, fibronectin, proteoglycans, type I gelatin and soluble elastin (9).

Insulin-like growth factor-I (IGF-I) is a pro-synthetic cytokine (10). It can promote cell proliferation, differentiation and migration, and inhibit apoptosis (10). Thus, it can regulate body metabolism, growth and development, reproduction and immunity, and delay aging (10). Cartilage cell can secrete IGF-I in the manners of paracrine and autocrine. In addition, it can activate the intracellular pathway after binding with its cell surface IGF-I receptor (11). Moreover, it can stimulate division and proliferation, maintain stable phenotype and suppress apoptosis of cartilage cell. Thus, it can promote synthesis of cartilage matrix proteoglycans and collagen (12). Meanwhile, it can actively participate in cartilage matrix repair (11). Consequently, it can delay or prevent articular cartilage degeneration. However, IGF-I is regulated by insulin-like growth factor binding protein (IGFBP) (12). Of them, insulin-like growth factor binding protein-5 (IGFBP-3) can bind with IGF-I (12). In this way, it can inhibit the affinity of IGF-I to IGF-I receptor (IGF-IR) (12,13). Moreover, it will affect the physiological function of IGF-I and inhibit the production of ECM proteoglycans and collagen (13).

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Peroxisome proliferator-activated receptor γ (PPAR γ) can regulate various biological processes, including lipid metabolism, fat formation, cell division and apoptosis (14). In recent years, it has been found that ligand activated PPAR γ has beneficial effects on resisting obesity, hypertension, atherosclerosis, diabetes, cancer and other diseases, which makes the research on the receptor function and ligand screening of PPAR γ become a hot spot in the field of biomedicine and pharmacology (14,15). Here, we investigated the role of miR-27a on arthritis production and its mechanism.

Materials and methods

Experimental animals and collagen-induced arthritis. Female Wistar rats (150–200 g, 6 weeks) was received water and food *ad libitum*, and were kept at room temperature (22–24°C) and 55–60% relative humidity in a standard microbiological regime in a dark-light cycle. All rats were randomly distributed into two groups: Control and arthritis model groups. In arthritis model group, rats were subcutaneously injected with 200 μ g bovine collagen type II from the tail base with 1:1 (v/v) and Incomplete Adjuvant (IFA) (200 μ l/one week; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 3 week.

After collagen-induced arthritis, rat was sacrificed under anesthesia and arthritis were acquired and fixed with 4% paraformaldehyde for 72 h. Then samples were quickly dissected and cut into frozen sections (5 μ m). Samples was stained using H&E sassy for 15 min and observed by microtome (Leica CM1900 UV; Leica, Solms, Germany). Clinical signs was scored as follows: 0 score: no change; 0–5 scores: redness and swelling of the ankle; 6–10 scores: redness and swelling in the paw; 11–20 scores: inflammation of the hind paw multiple joints involved, and/or deformation of joints with function impairment.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from serum of rat with arthritis using a total RNA extraction kit (Omega, Norcross, GA, USA). cDNA was composed using total RNA using a High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). miR-27a expression was amplified using SensiFAST SYBR-Green Master mix (Bioline, London, UK) by an ABI7900HT machine, under the following cycling conditions: polymerase activation for 10 min at 95°C followed by 40 cycles at 95°C for 30 sec and 60°C for 30 sec. miR-27a: primers, 5'-GGCTTAGCTGCTTGTGAGCA-3'; reverse, 5'-GCGGAAGCTTAGCCACTGTGA-3'. The relative expression of the target gene was calculated as $2^{-\Delta\Delta C_q}$.

Cell culture and cell transfection. A bone marrow-derived human MSC (hMSC) line (BM025SS13) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS (both from Gibco-BRL; Thermo Fisher Scientific) and antibiotics (100 U/ml of penicillin G and 100 μ g/ml of streptomycin) at 37°C in a humidified atmosphere of 5% CO $_2$. miR-27a, anti-miR-27a and negative were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The cells were then transfected with miR-27a mimics, anti-miR-27a mimics and negative mimics using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific). After transfection for

24 h, cell was cultured with 2 ml of fresh DMEM supplemented with TNF- α (20 ng/ml; Invitrogen; Thermo Fisher Scientific).

Enzyme-linked immunosorbent assay (ELISA). Cell was collected at 500 g for 10 min, proteins were extracted using RIPA buffer, and protein concentrations were determined using BCA Protein assay kit (P0009; Beyotime Institute of Biotechnology, Haimen, China). Proteins of 5 μ g were used to analyze IL-1 β (PI303) and IL-6 (PI328) level using ELISA kit (Beyotime Institute of Biotechnology).

MTT assay. After transfection for 24, 48 and 72 h, the MTT solution (20 μ l, 5 mg/ml) was added to each well for 4 h at 37°C. In the last day, old medium was removed and 150 μ l DMSO was added to dissolve purple crystals for 20 min at 37°C. Absorbance was analyzed by Bio-Plex Suspension array (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 492 nm.

Flow cytometry. After induction of arthritis for 48 h, cell were washed twice with PBS and stained with 5 μ l Annexin V-fluorescein isothiocyanate and 5 μ l propidium iodide (BD Biosciences, Franklin Lakes, NJ, USA) for 15 min at darkness. Apoptosis rate was analyzed using flow cytometry (BD C6 flow cytometer; BD Biosciences).

Western blot analysis. Cell was collected at 500 x g for 10 min, proteins were extracted using RIPA buffer, and protein concentrations were determined using BCA Protein assay kit (Beyotime, Beijing, China). Proteins of 50 μ g were loaded onto 8–12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with the primary antibodies overnight at 4°C: IGFBP-5, PPAR γ , MMP-17 and GAPDH (both from Santa Cruz Biotechnology, Dallas, TX, USA) followed by incubation with horseradish peroxidase-conjugated rabbit anti-rabbit IgG (Santa Cruz Biotechnology) at 37°C for 1 h. Protein blank was examined using a chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis. All quantified results are presented as the mean \pm SD of at least three experiments. The differences among multiple groups with normal distribution were evaluated using one-way ANOVA with a Tukey's post-hoc test. Differences were regarded as statistically significant when $P < 0.05$.

Results

miR-27a expression of rate with arthritis. RT-qPCR was used to analyze miR-27a expression in rate with arthritis and to verify the microarray analysis. There was a significant increases of clinical signs in collagen-induced arthritis, compared with control group (Fig. 1A). Fig. 1B showed that there was some cavity in collagen-induced arthritis, compared with control group. As showed in Fig. 1C and D, miR-27a expression of arthritis in rat with arthritis was reduced, compared with control normal group, which showed that miR-27a maybe correlated with arthritis happen.

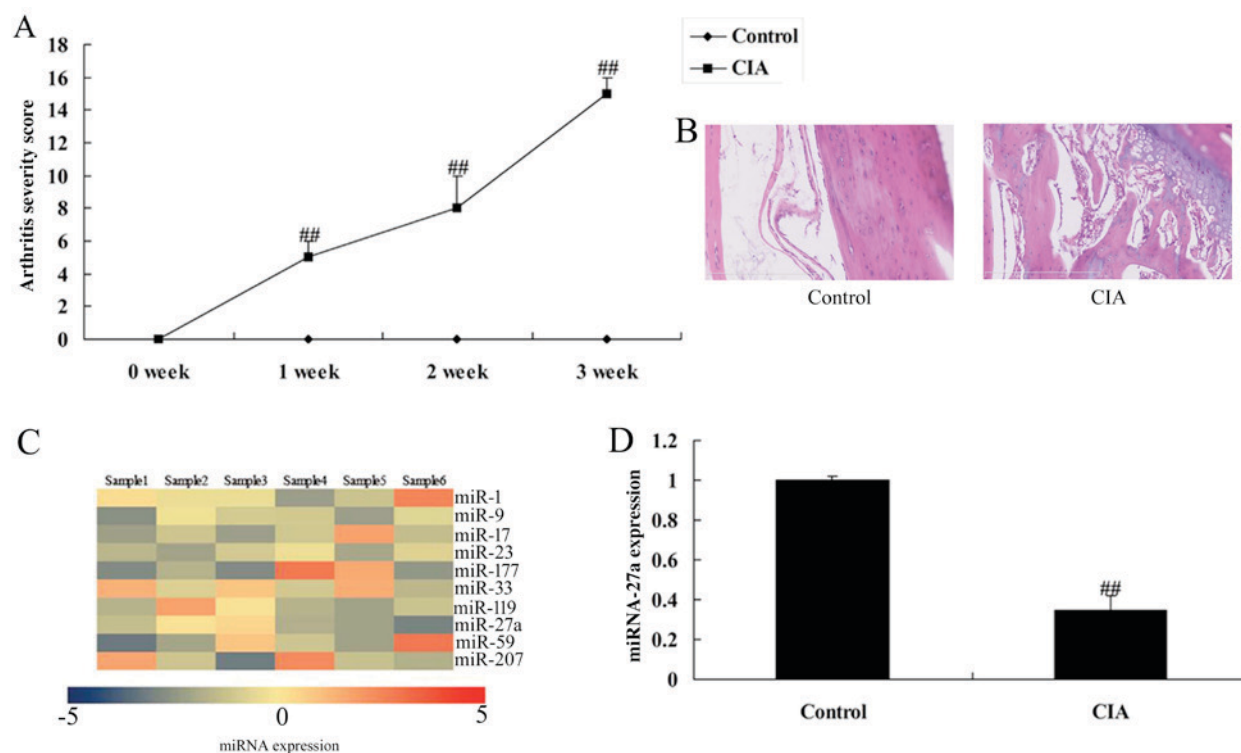


Figure 1. miR-27a expression of rate with arthritis. (A) Clinical signs, (B) H&E staining for joint, (C) microarray analysis for miR-27a expression (blue, -5-fold expression; yellow, 0-fold expression; red, +5-fold expression) and (D) qPCR analysis for miR-27a expression. All quantified results are presented as the mean \pm SD. Control, control group; CIA, collagen-induced arthritis mice group. ##P<0.01 compared with control group.

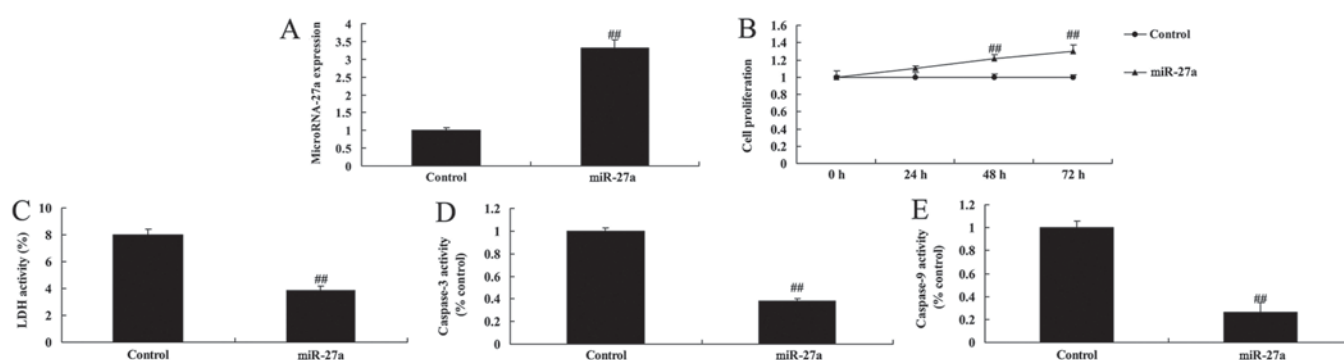


Figure 2. miR-27a overexpression increased cell proliferation of osteoblast-like cell *in vitro* model of arthritis. (A) miR-27a expression, (B) cell proliferation, (C) LDH activity, (D) caspase-3 and (E) caspase-9 activity *in vitro* model of arthritis. All quantified results are presented as the mean \pm SD. LDH, lactate dehydrogenase; control, control negative group; miR-27a, miR-27a overexpression group. ##P<0.01 compared with control group.

miR-27a overexpression increased cell proliferation of osteoblast-like cell in vitro model of arthritis. We transfected human osteoblast-like cell MG-63 cell with miR-27a mimics to detect the interaction between miR-27a and bone cell proliferation of *in vitro* model of arthritis. miR-27a mimics increased significantly increased miR-27a expression, increased cell proliferation, and reduced LDH activity and caspase-3/9 activity of osteoblast-like cell *in vitro* model of arthritis (Fig. 2). So, this study showed that miR-27a promoted bone cell growth and inhibited cell apoptosis in arthritis, and this may be a way for treatment of arthritis.

miR-27a overexpression promoted osteogenic differentiation and inhibited inflammation. Next, we detected osteogenic differentiation and inflammation by miR-27a overexpression.

In addition, significantly promotion of osteogenic differentiation and alkaline phosphatase (ALP) and osteoporosis (OST) content, and inflammation *in vitro* model of arthritis by miR-27a overexpression (Fig. 3). These results showed that miR-27a overexpression promoted osteogenic differentiation, increased cell proliferation and inhibited inflammation.

miR-27a overexpression affects IGFBP-5, PPAR γ and MMP-17 protein expression. We also examined the mechanism of miR-27a on arthritis, IGFBP-5, PPAR γ and MMP-17 protein expression were analyze using western blot analysis. Fig. 4A showed that putative miR-27a binding sites predicted by TargetScan in 3'-UTR of PPAR γ gene. The result of western blotting showed that IGFBP-5 protein expression was induced, and PPAR γ and MMP-17 protein expression

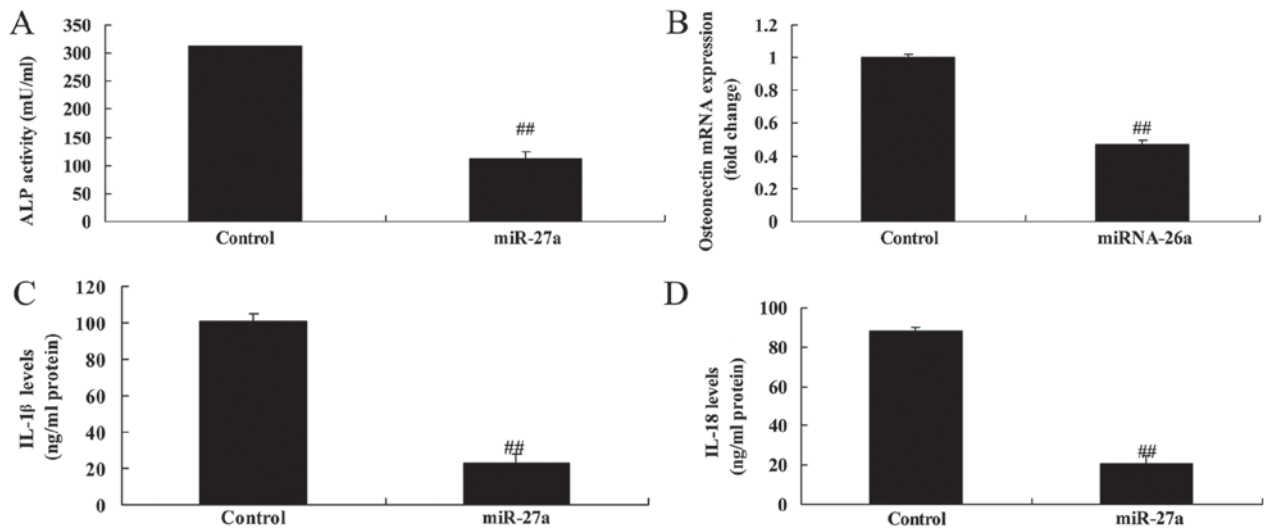


Figure 3. miR-27a overexpression promoted osteogenic differentiation and inhibited inflammation. (A) ALP activity, (B) osteonectin mRNA expression, (C) IL-1 β and (D) IL-18 levels. All quantified results are presented as the mean \pm SD. ALP, alkaline phosphatase; IL-1 β , interleukin-1 β . Control, control negative group; miR-27a, miR-27a overexpression group. ^{##}P<0.01 compared with control group.

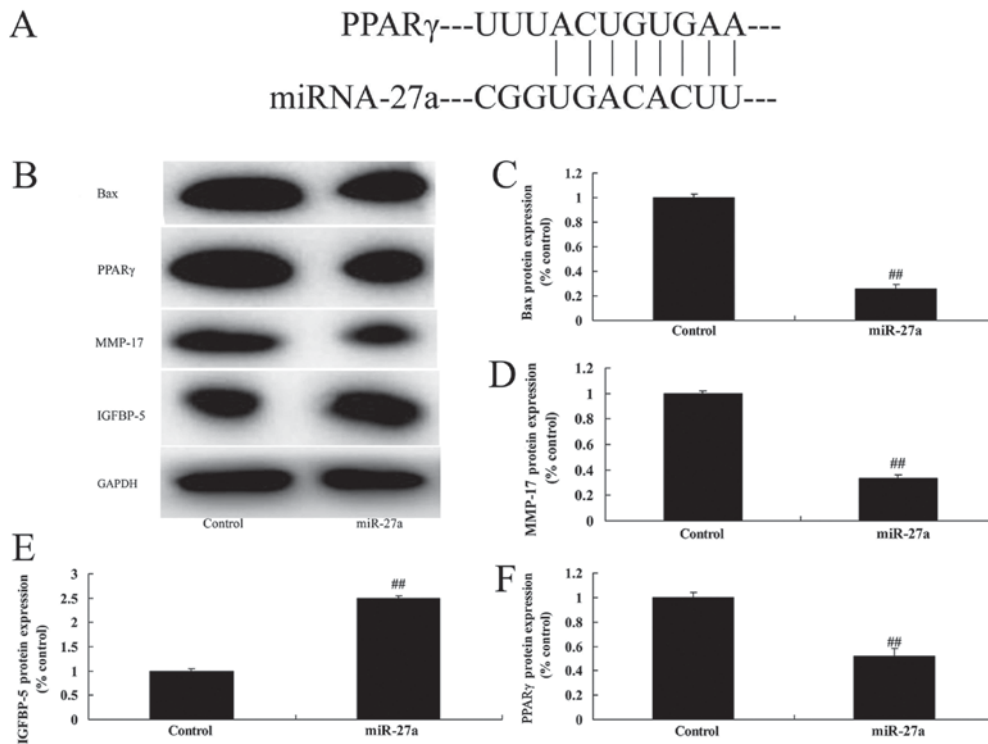


Figure 4. miR-27a overexpression affects IGFBP-5, PPAR γ and MMP-17 protein expression. (A) Putative miR-27a binding sites predicted by TargetScan in 3'-UTR of PPAR γ gene, (B) Bax, PPAR γ , IGFBP-5 and MMP-17 protein expression using western blot analysis. The results of the western blotting were quantified for (C) Bax, (D) MMP-17, (E) IGFBP-5 and (F) PPAR γ . All quantified results are presented as the mean \pm SD. PPAR γ , peroxisome proliferators-activated receptors- γ ; IGFBP-5, insulin like growth factor binding protein-5; MMP-17, matrix metalloproteinase-17; control, control negative group; miR-27a, miR-27a overexpression group. ^{##}P<0.01 compared with control group.

was suppressed *in vitro* model of arthritis by miR-27a overexpression (Fig. 4B-F). miR-27a regulates arthritis through PPAR γ /MMP-17 signaling pathway by IGFBP-5.

miR-27a downregulation decreased cell proliferation of osteoblast-like cell in vitro model of arthritis. Moreover, to further verify the function of miR-27a on arthritis, cell proliferation of osteoblast-like cell by anti-miR-27a was measured by MTT

sassy. As showed in Fig. 5, anti-miR-27a mimics inhibited miR-27a expression *in vitro* model of arthritis, decreased cell proliferation, and increased LDH activity and caspase-3/9 activity of osteoblast-like cell *in vitro* model of arthritis.

miR-27a downregulation suppressed osteogenic differentiation and increased inflammation. Next, *in vitro* model of arthritis by anti-miR-27a mimics, osteogenic differentiation

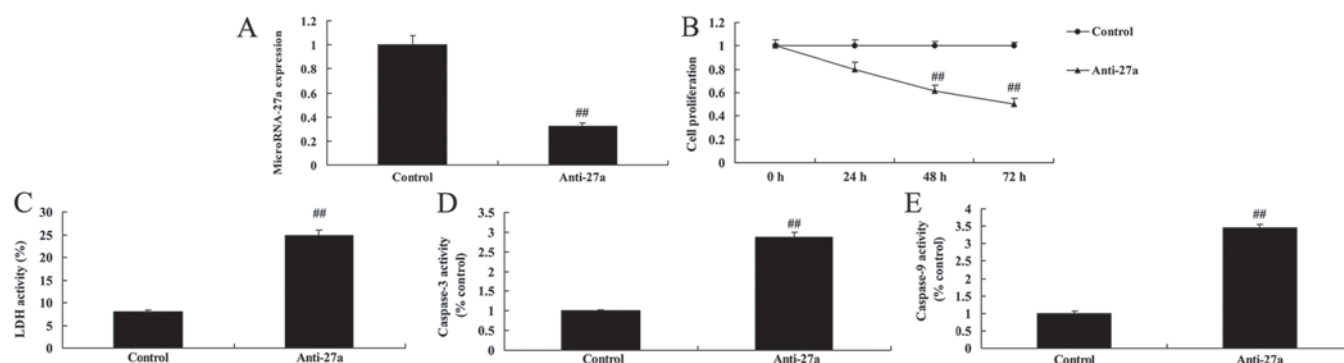


Figure 5. miR-27a downregulation decreased cell proliferation of osteoblast-like cell *in vitro* model of arthritis. (A) miRNA-27a expression, (B) cell proliferation, (C) LDH activity, (D) caspase-3 and (E) caspase-9 activity. All quantified results are presented as the mean \pm SD. LDH, lactate dehydrogenase; control, control negative group; anti-27a, miR-27a downregulation group. ##P<0.01 compared with control group.

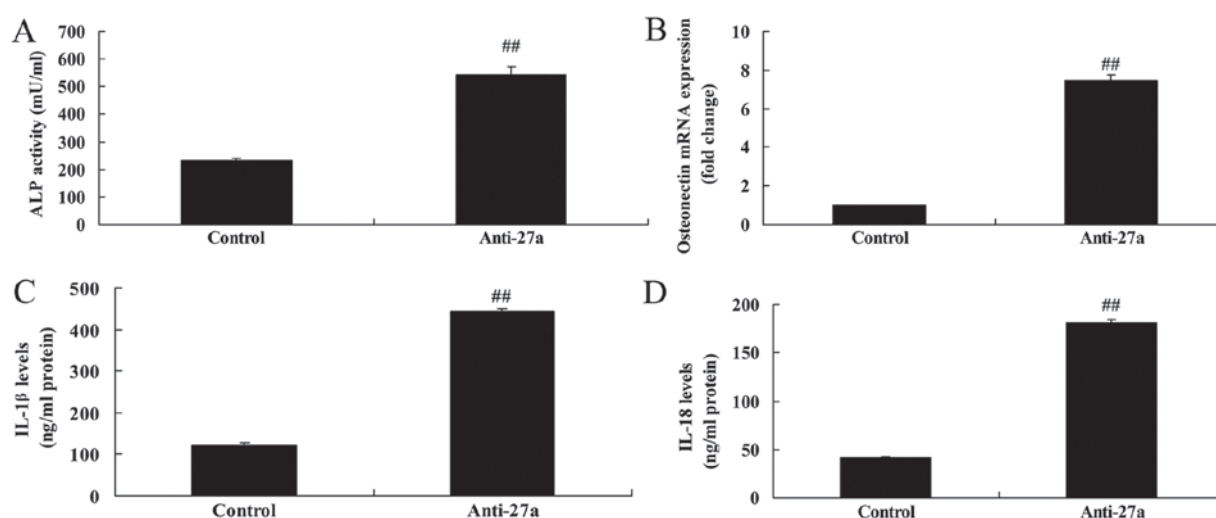


Figure 6. miR-27a downregulation suppressed osteogenic differentiation and increased inflammation. (A) ALP activity, (B) osteonectin mRNA expression, (C) IL-1 β and (D) IL-18 levels. All quantified results are presented as the mean \pm SD. ALP, alkaline phosphatase; IL-1 β , interleukin-1 β ; control, control negative group; anti-27a, miR-27a downregulation group. ##P<0.01 compared with control group.

and ALP and OST content were suppressed, and inflammation also increased (Fig. 6).

miR-27a downregulation affects on IGFBP-5, PPAR γ and MMP-17 protein expression. Lastly, anti-miR-27a mimics suppressed IGFBP-5, and induced PPAR γ and MMP-17 protein expression in osteoblast-like cell. These results suggested that miR-27a affects on PPAR γ expression in arthritis through anti-inflammation and osteogenic differentiation (Fig. 7). These results also showed that downregulation of miR-27a regulate PPAR γ and MMP-17 protein expression by IGFBP-5 to promote arthritis.

PPAR γ inhibitor reduced the function of miR-27a downregulation on arthritis. We next examined whether PPAR γ participated in the unction of miR-27a downregulation on arthritis. As showed in Fig. 8, PPAR γ inhibitor suppressed PPAR γ and MMP-17 protein expression, and induced IGFBP-5 protein expression *in vitro* model of arthritis. The inhibition of PPAR γ inhibited the effects of miR-27a downregulation on cell proliferation, LDH activity and caspase-3/9 activity *in vitro* model of arthritis following miR-27a

downregulation, compared with miR-27a downregulation group (Fig. 9). The PPAR γ inhibition the effects of miR-27a downregulation on osteogenic differentiation and ALP and OST content *in vitro* model of arthritis following miR-27a downregulation, compared with miR-27a downregulation group (Fig. 10).

Discussion

OA is a chronic degenerative osteoarticular disease which seriously endangers human health (16). The degenerative changes in the pathology is mainly expressed as the destruction of articular cartilage degeneration, subchondral bone sclerosis, cystic degeneration joint marginal hyperostosis, synovial hyperplasia, contracture of joint capsule, ligament laxity, muscle weakness and atrophy(2). The incidence of OA is high, commonly occurring in the elderly patients, and female patients are more than male patients (16). The prevalence rate of people under the age of 40 is ~5%, and the prevalence rate of people aged 60-75 years old is up to 50, and 80% among people over 75 years old. There is an increasing trend with age (17). Therefore, we found that miR-27a expression of

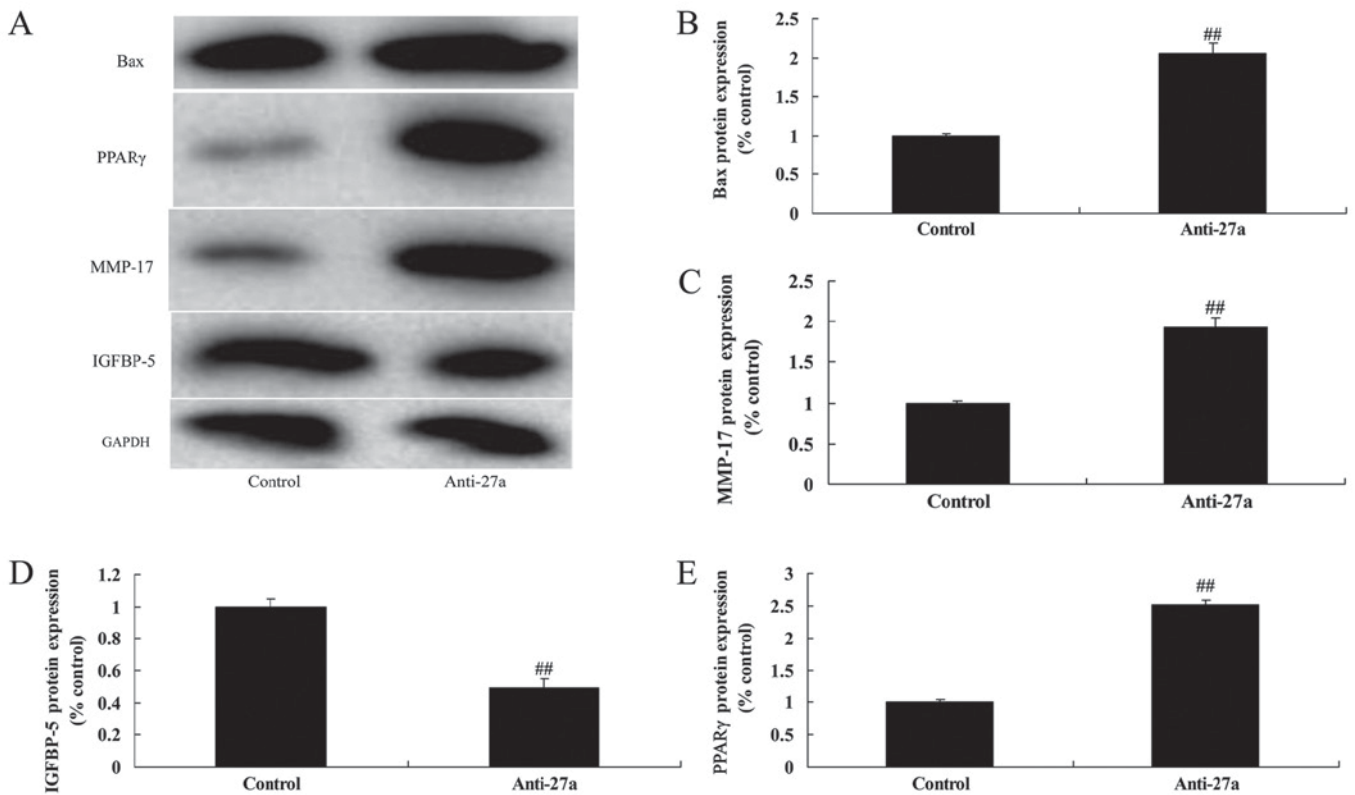


Figure 7. miR-27a downregulation affects on IGFBP-5, PPAR γ and MMP-17 protein expression. (A) Bax, PPAR γ , IGFBP-5 and MMP-17 protein expression using western blot analysis. The results of the western blotting were quantified for (B) Bax, (C) MMP-17, (D) IGFBP-5 and (E) PPAR γ . All quantified results are presented as the mean \pm SD. PPAR γ , peroxisome proliferators-activated receptors- γ ; IGFBP-5, insulin like growth factor binding protein-5; MMP-17, matrix metalloproteinase-17; control, control negative group; anti-27a, miR-27a downregulation group. ^{##}P<0.01 compared with control group.

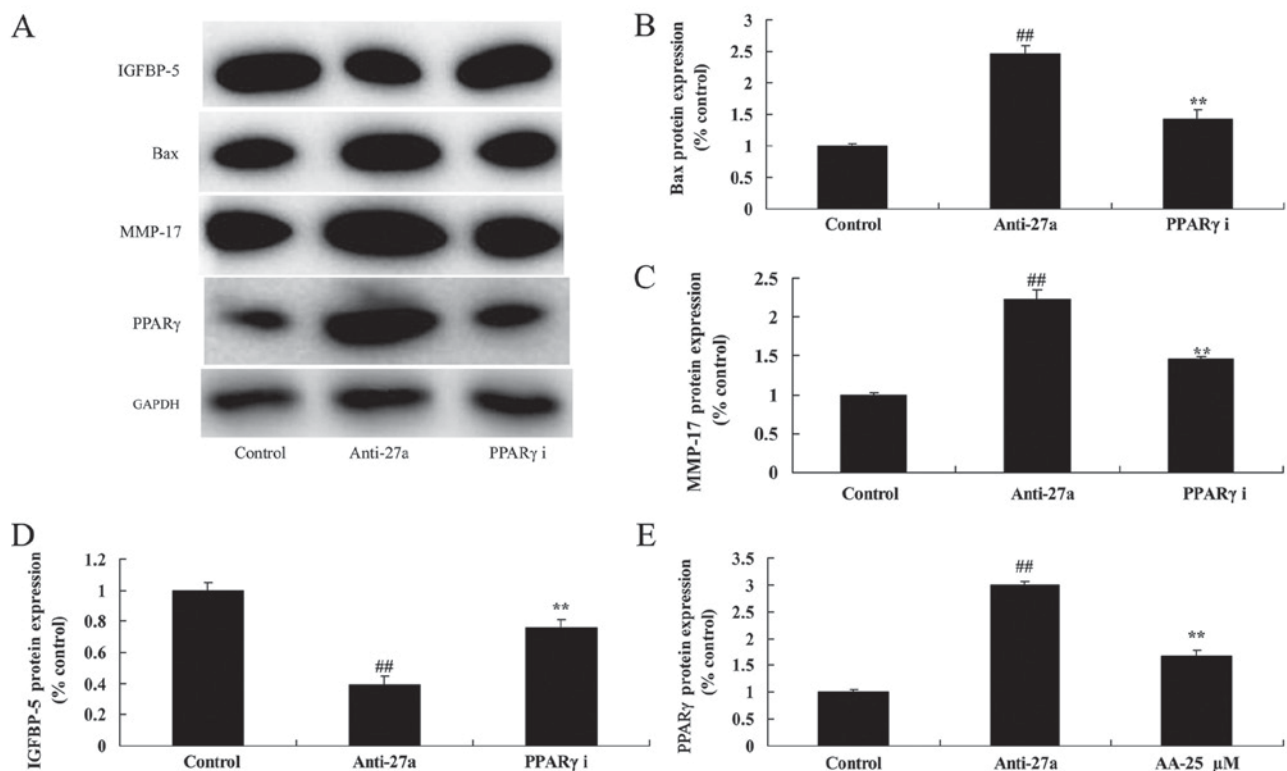


Figure 8. PPAR γ inhibitor reduced the function of miR-27a downregulation on arthritis. (A) Bax, PPAR γ , IGFBP-5 and MMP-17 protein expression using western blot analysis. The results of the western blotting were quantified for (B) Bax, (C) MMP-17, (D) IGFBP-5 and (E) PPAR γ . All quantified results are presented as the mean \pm SD. PPAR γ , peroxisome proliferators-activated receptors- γ ; IGFBP-5, insulin like growth factor binding protein-5; MMP-17, matrix metalloproteinase-17; control, control negative group; anti-27a, miR-27a downregulation group; PPAR γ i, miR-27a downregulation and PPAR γ inhibitor group. ^{##}P<0.01 compared with control group; ^{**}P<0.01 compared with the miR-27a downregulation group.

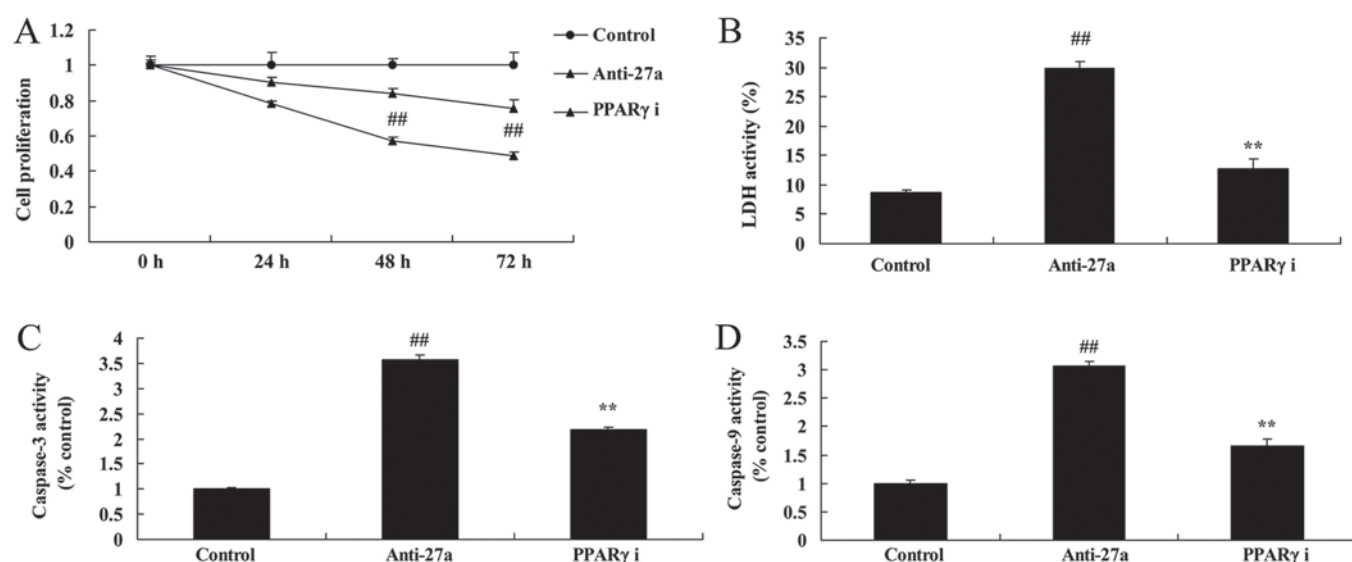


Figure 9. PPAR γ inhibitor reduced the function of miR-27a downregulation on cell proliferation *in vitro* model of arthritis. (A) Cell proliferation, (B) LDH activity, (C) caspase-3 and (D) caspase-9 activity. All quantified results are presented as the mean \pm SD. LDH, lactate dehydrogenase; control, control negative group; anti-27a, miR-27a downregulation group; PPAR γ i, miR-27a downregulation and PPAR γ inhibitor group. ##P<0.01 compared with control group; **P<0.01 compared with miR-27a downregulation group.

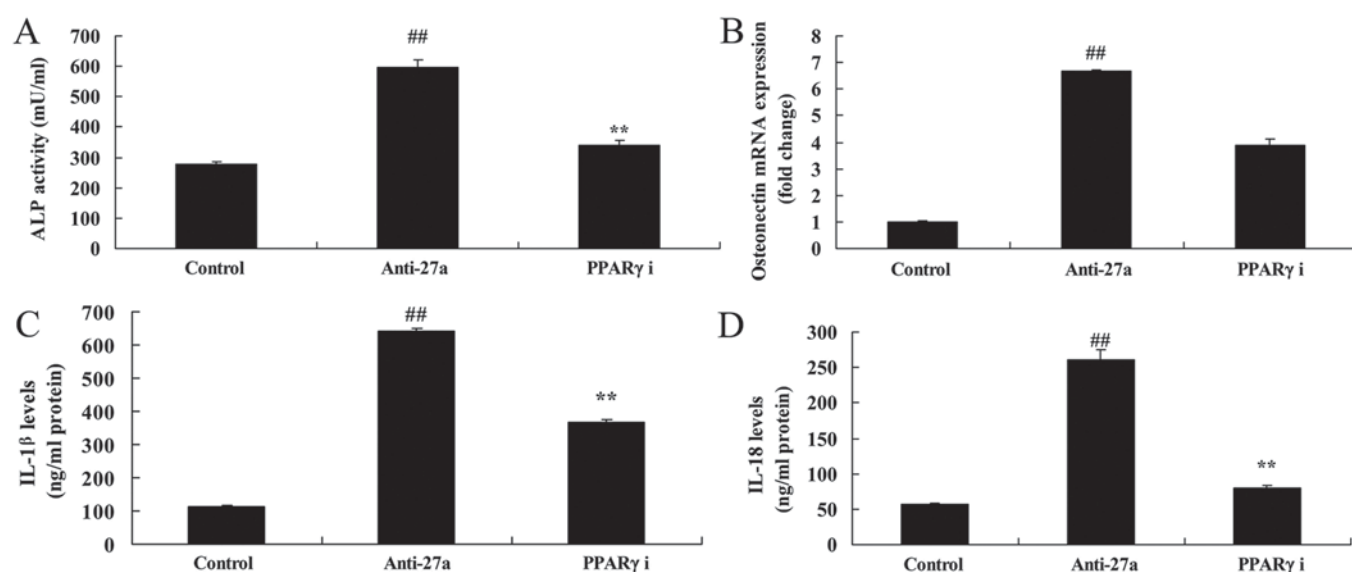


Figure 10. PPAR γ inhibitor reduced the function of miR-27a downregulation on osteogenic differentiation and inflammation *in vitro* model of arthritis. (A) ALP activity, (B) osteonectin mRNA expression, (C) IL-1 β and (D) IL-18 levels. All quantified results are presented as the mean \pm SD. ALP alkaline phosphatase; IL-1 β , interleukin-1 β ; control, control negative group; anti-27a, miR-27a downregulation group; PPAR γ i, miR-27a downregulation and PPAR γ inhibitor group. ##P<0.01 compared with control group; **P<0.01 compared with miR-27a downregulation group.

arthritis in rat with arthritis was reduced, compared with control normal group.

Studies have shown that miRNA is associated with disease progression and pathogenesis (5). For example, miRNA plays an important role in the pathogenesis of cancer and cardiovascular disease (18). Research on tissue specificity miRNA in mice also showed that miRNA could have both pathogenic and tissue protective functions (19). Along with the gradually deepened research on miRNA in terms of the regulation of cell function in science, and the discovery of new miRNA targets, increasing research has shown that miRNA can regulate the expression of OA-related genes in multiple aspects (18). Our

study showed that miRNA-27a overexpression increased cell proliferation and inhibited bone cell apoptosis *in vitro* model of arthritis.

In the process of OA development, matrix degradation and severe damage is often accompanied by excessive apoptosis of cartilage cells (20). Cell apoptosis, also known as programmed death, refers to the physiological process that nucleated cells trigger programmed cell death under the regulation of gene, which causes the natural death of cells and automatic removal of the non-functional, damaged and senescent cells (21). There is a small amount of apoptosis in the normal cartilage tissue, which is usually confined to the surface of cartilage tissue (22).

It is an essential physiological process to ensure the normal growth and development of cartilage, regulate function and maintain homeostasis (22). The excessive apoptosis in cartilage cells indicates pathological changes of cartilage tissue, which is considered to be the key factor leading to the onset of OA (20). In this study, we found that miR-27a overexpression promoted osteogenic differentiation *in vitro* model of arthritis.

Cartilage abnormality is a key link in the pathogenesis of OA, and closely related the mechanical properties, cartilage cell proliferation and apoptosis, inflammatory factor and the secretion of MMPs (23). It has been reported that IL-1 β can stimulate the release of NO from the cartilage cells so as to promote apoptosis. Inflammatory factor and its harm are important mechanisms of OA (23). In the process of OA, the pathological changes of cartilage tissue can lead to the increase of inflammatory factor expression, while the increased inflammatory factors in turn intensify the cartilage tissue lesions by mediating signal activities, forming a vicious circle (24). Inflammatory factors can affect the expression of ECM catabolic proteins, such as MMPs and integrins, so that the contents will be significantly increased, which leads to ECM metabolism disorder and promotes the development of OA (25). We found that miR-27a overexpression inhibited inflammation *in vitro* model of arthritis. Wang *et al* suggested that microRNA-27a mediate physcion 8-O- β -glucopyranoside-induced apoptosis through MMPs expression in osteosarcoma cells (26). These results showed that microRNA-27a negatively modulates inflammatory response in lipopolysaccharide-stimulated microglia.

Insulin-like growth factor-1 (IGF-1) is one of the major mediators in the synthesis and metabolism of articular cartilage (27). OA model showed that IGF-1 can reduce the destruction of articular cartilage (28). Insulin-like growth factor binding proteins (IGFBPs) are an important factor in regulating and maintaining the activity of anabolic factors IGF-1 (29). The increase of IGFBP5 concentration in joints will enhance IGF-1 (28). Also, it has been found that the expression level of IGFBP5 in OA cartilage was significantly lower than that in normal subjects (29). This study showed that miR-27a overexpression induced IGFBP-5 protein expression, and suppressed PPAR γ and MMP-17 protein expression *in vitro* model of arthritis. Tardif *et al* reported that miRNA-27 regulated the IGFBP-5 and MMP-13 genes in human osteoarthritic chondrocytes (30).

The inhibitory effect of PPAR γ on OA is mainly expressed as inhibition of inflammatory reaction and regulation of cell proliferation and migration (31). PPAR γ ligand or agonist can reduce the expression of inflammatory factor IL-1 β , IL-6, tumor necrosis factor- α (TNF- α), inducible nitric oxide synthase (iNOS) and matrix metalloproteinase-9 (MMP-9) (32). Also, it can inhibit the activity of monocyte/macrophage transcription factor AP-1, NF- κ B and transcription activator (Stat) (33). The activated PPAR γ may regulate the inflammatory response in OA (32). In this study, we found that PPAR γ inhibitor reduced the function of miR-27a downregulation on arthritis. Xie *et al* demonstrated that miR-27a mediates endothelin-1-induced PPAR γ reduction and proliferation of pulmonary artery smooth muscle cells (34).

In conclusion, we found that miRNA-27 play essential roles in regulating osteoblast differentiation and bone

apoptosis by arthritis through PPAR γ expression *in vivo* and *in vitro* model. miRNA-27 has been proposed as a candidate therapeutic modality to treat for arthritis, and provides novel insights into the pathogenesis of AI and potential preventative or therapeutic interventions.

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