European Review for Medical and Pharmacological Sciences 2020; 24: 7927-7936

CircTMBIM6 promotes osteoarthritis-induced chondrocyte extracellular matrix degradation *via* miR-27a/MMP13 axis

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Abstract. – OBJECTIVE: Osteoarthritis is a degenerative disease characterized by degeneration of articular cartilage, but the current mechanism is unclear. Circular RNA (circRNA) plays a significant role in a series of biological processes related to osteoarthritis, but its mechanism remains unclear. The purpose of this study was to investigate the role of the circTMBIM6/miR-27a/MMP13 axis in osteoarthritis.

PATIENTS AND METHODS: The expression levels of circTMBIM6, miR-27a and MMP13 in cartilage of osteoarthritis patients and normal human cartilage were detected by reverse transcription polymerase chain reaction (RT-PCR). Osteoarthritis cell model was induced by IL-1β and TNF-α, and the expression changes of circTMBIM6, miR-27a and MMP13 in the *in vitro* model were detected. In addition, the *in vitro* regulation of circTMBIM6 and miR-27a in osteoarthritis was verified by transfection of circTMBIM6 and miR-27a plasmids, and the regulation of miR-27a on MMP13 was also verified. The dimethylmethylene blue (DMMB) method was used to analyze the secretion and formation of soluble glycosaminoglycan sulfate (sGAG), and the effects of circTMBIM6 and miR-27a chondrocytes were evaluated.

RESULTS: The expression levels of circTMBIM6 and MMP13 in cartilage tissue of patients with osteoarthritis were higher than that of normal group, while the expression level of miR-195 in cartilage tissue of patients with osteoarthritis was lower. After IL-1β and TNF-α treatment, the expression of circTMBIM6 and MMP13 in chondrocytes increased, while the expression of miR-27a decreased. CircTMBIM6 overexpression reduced miR-27a expression but increased MMP13 expression. The circTMBIM6 gene knockout showed the opposite effect.

CONCLUSIONS: CircTMBIM6 promotes osteoarthritis-induced chondrocyte extracellular matrix degradation *via* miR-27a/MMP13 axis.

Key Words:
Osteoarthritis, CircTMBIM6, MiR-27a, MMP13, CeRNA.

Introduction

Osteoarthritis (OA) is a degenerative joint disease, and there are currently no effective treatments that can change its progress[1]. The main pathological features of OA include the progressive loss of articular cartilage, osteophyte formation, and changes in bone around and below the cartilage[2]. Articular cartilage has received great attention in OA research, because the main pathological feature in OA is large articular cartilage injury characterized by progressive cartilage homeostasis, synovial activation, and progressive loss of subchondral bone reconstruction[3].

Circular RNAs (circRNAs) are non-coding RNAs (NNAs) discovered in the 1970s. They are widely found in protozoa, eukaryotes, and a series of animals and plants[4]. Formed by intron cyclization or exon splicing. It has the characteristics of high stability, developmental stage and tissue specificity. CircRNAs have the function of "miRNA molecular sponge", which affects gene transcription regulation and expression; circRNAs also play an important role in the occurrence of bone diseases, osteoblasts and osteoclasts, and interactions with other non-coding RNAs[5].

MiRNA is a type of endogenous non-protein-encoding RNA with a length of 19-25 bases that can induce translational silence, affecting cell proliferation, differentiation, apoptosis, and individual development[6]. MiRNA is highly conserved, so its transcription process is independent of other genes, it plays a regulatory role in a variety of metabolic processes in the body, and does not itself translate proteins. MiRNA plays an important regulatory role in the pathological development of osteoarthritis[7,8].

The process of extracellular matrix degradation is very complicated and involves the interactions between multiple proteases. The role

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of different proteases in the process of matrix degradation is also inconsistent. Type II collagen matrix metalloproteinases (MMPs) and disintegrin are the main components of the extracellular matrix of cartilage. Among them, subtype 13, MMP13 plays an important role in maintaining the stability of the bone environment[9]. However, at present, the specific molecular mechanism of articular cartilage to maintain homeostasis is unclear, which limits the understanding of the pathogenesis of osteoarthritis and restricts the treatment of osteoarthritis.

The purpose of this study was to investigate the regulatory role of circTMBIM6, miR-27a, and MMP13 in the pathogenesis of osteoarthritis. We also elucidated the role of circTMBIM6 in the degradation of osteoarthritis matrix through the circTMBIM6/miR-27a/MMP13 axis.

Patients and Methods

Patient and Sample Collection

Knee cartilage was collected from patients with osteoarthritis and normal people. The normal femoral condyle cartilage of the knee joint was removed due to trauma (10 cases). According to the medical history, X-ray film, intraoperative visual observation and postoperative microscopic pathology, degeneration, tumor, tuberculosis, infection, rheumatoid inflammation, obvious osteoporosis and other systemic diseases such as concurrent immune system diseases and diabetes were excluded. Femoral condyle cartilage (10 cases) was collected from patients diagnosed with osteoarthritis of the knee requiring total knee replacement (TKA), according to the American College of Rheumatology's 1995 diagnostic criteria for osteoarthritis of the knee[10]. According to the medical history, X-ray, intraoperative visual observation and postoperative microscopic pathology, other knee diseases, concurrent immune system diseases, diabetes and other systemic diseases were excluded. OA staging was performed according to the radiological diagnostic criteria of Kellgren Lawrence. General data of patients were collected, including 10 patients in the normal group, aged 49-67 years old, with an average age of 62.2 years old, 3 patients on the left side and 7 patients on the right side. There were 10 patients in OA group, aged 57-85 years, with an average age of 68.1 years, including 6 patients on the left and 4 patients on the right. All patients and healthy volunteers signed the informed consent. This investigation was approved by the Ethics Committee of Second Hospital of Medical University and was in line with the principles of the Helsinki declaration.

Chondrocyte Culture and IL-1β and TNF-α-Induced Cells

Chondrocytes were separated from cartilage tissue, and the separated cartilage tissue was digested with 0.25% trypsin for half an hour, and then incubated with type II collagenase (0.2%) at 37°C for 4 hours. The isolated chondrocytes were in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South Logan, UT, USA) (DMEM; supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin. Chondrocytes were seeded on six-well plates, starved with serum-free medium for 24 hours, and stimulated with IL-1β (10 ng/mL) or TNF-α (10 ng/mL) for 12 hours before use.

PCR Detection Expressions

After the cells were treated in each group, the total RNA of each group of cells was extracted with TRIzol reagent, and the quantitative PCR reaction was performed after reverse transcription according to the instructions. Using the Ct value of U6 (F: 5'-CTCGCTTCGGCAGCACATATACT-3', R: 5'-ACGCTTCACGAATTTGCGTGTC-3') as a reference, $2^{-\Delta\Delta Ct}$ calculates the relative expression level of mRNA. Each experiment was repeated 3 times.

Western Blot Detection of Protein Expression

The cells of each group were collected, washed with pre-chilled phosphate-buffered saline (PBS), and then lysed with radioimmunoprecipitation assay (RIPA). The total protein in the cells was extracted and the protein concentration was determined by the bicinchoninic acid (BCA) method. An equal amount of 50 μg of each group of protein samples was subjected to sodium sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, transferred with polyvinylidene difluoride (PVDF) membrane, diluted with Tris-Buffered Saline and Tween-20 (TBST) antibody diluted solution, diluted primary antibody, incubated at 4°C overnight, added horseradish peroxidase (HRP)-labeled secondary antibody, and incubated at room temperature for 2 h. After washing with PBS, the enhanced che-

miluminescence (ECL) was developed, exposed with a Bio-Rad gel imaging system, and the images were analyzed.

Detection of Soluble Glycosamine Sulfate Additive (sGAG)

Soluble sGAG secretion was measured using dimethylmethylene blue (DMMB) (Sigma-Aldrich, St. Louis, MO, USA). 200 μL DMMB reagents were mixed with 20 μL of cell suspension, and absorbance was measured at 525 nm. The BCA protein assay kit (Pierce, Rockford, IL, USA) was used to normalize total sGAG of total protein for cell division.

Dual-Luciferase Reporter Confirms Targeting Relationship

The target gene and the 3'-untranslated region (3'UTR) wild type (Wt) or mutant (Mut) of MMP13 were transfected into 293T cells at the same time. The predicted mRNA binding sequence was used to clone the predicted gene into GV272 vector. A 3'UTR fragment of MMP13 binding site or a mutated 3'UTR. After verification by DNA sequencing, the target gene plasmid or control plasmid was transfected into 293T cells with Lipofectamine™ 2000 according to the instructions. Cells were collected 48 h after transfection and analyzed using a dual luciferase reporting system.

Statistical Analysis

The experiments were repeated 3 times. Statistical analysis was performed on the experimental data using SPSS 20.0 (IBM, Armonk, NY, USA) software. Comparisons between two groups are performed using the *t*-test. Comparisons between multiple groups are performed using single factor analysis of variance. Co-expression relationships are processed through correlation and linear regression analysis. The difference was statistically significant at $p < 0.05$ or $p < 0.01$.

Results

CircTMBIM6 and MMP13 are Up-Regulated In Osteoarthritis Patients, MiR-27a Is Down-Regulated In Osteoarthritis Patients

To study the role of circTMBIM6 in osteoarthritis, we first explored the expression of circTMBIM6 in cartilage tissue. The experimental results showed that compared with normal control tissues, the expression of circTMBIM6 osteoarthritis patients was up-regulated (Figure 1A). The expression of miR-27a in chondrocytes showed that the expression of miR-27a in normal chondrocytes was higher than that of osteoarthritis chondrocytes (Figure 1B). MMP13 expression in chondrocytes showed that MMP13 expression was higher in osteoarthritis chondrocytes than normal chondrocytes (Figure 1C). Western blot results also showed that MMP13 is highly expressed in osteoarthritis chondrocytes (Figure 1D).

In the Chondrocyte Model Induced by IL-1B and TNF-α, the Expressions of CircTMBIM6 and MMP13 are Up-Regulated and MiR-27a Is Down-Regulated

To further study the changes of circTMBIM6, we induced chondrocytes through IL-1B and TNF-α to simulate an *in vitro* model. The experimental results showed that the expression of circTMBIM6 was highly expressed in the IL-1B induced group and the TNF-α induced group (Figure 2A). The expression of miR-27a in chondrocytes showed that the expression of miR-27a was low in the IL-1B induced group and the TNF-α induced group (Figure 2B). MMP13 was highly expressed in the IL-1B induced group and TNF-α induced chondrocytes (Figure 2C, 2D).

CircTMBIM6 Up-Regulates MiR-27a and Up-Regulates MMP13 Expression

Figure 3A shows the binding site information of circTMBIM6 targeting miR-27a. Report carrier experiments further confirmed that circTMBIM6 can bind to miR-27a (Figure 3B). RNA pull down experiments showed that miR-27a probe can enrich circTMBIM6 (Figure 3C). The efficiency of over-expressing circTMBIM6 showed that the over-expression plasmid could effectively up-regulate the expression of circTMBIM6 (Figure 3D). CircTMBIM6 expression trend showed that the circTMBIM6 group was higher than the NC vector group and the control group. The effect of circTMBIM6 overexpression on miR-27a expression showed that overexpression of circTMBIM6 inhibited miR-27a expression (Figure 3E). The effect of circTMBIM6 overexpression on sGAG expression (μg/ml) showed that the content of SGAG in circTMBIM6 group was higher than that in NC vector group and control group (Figure 3F). Co-expression test results show a negative correlation between circTMBIM6 and miR-27a expression (Figure 3G).

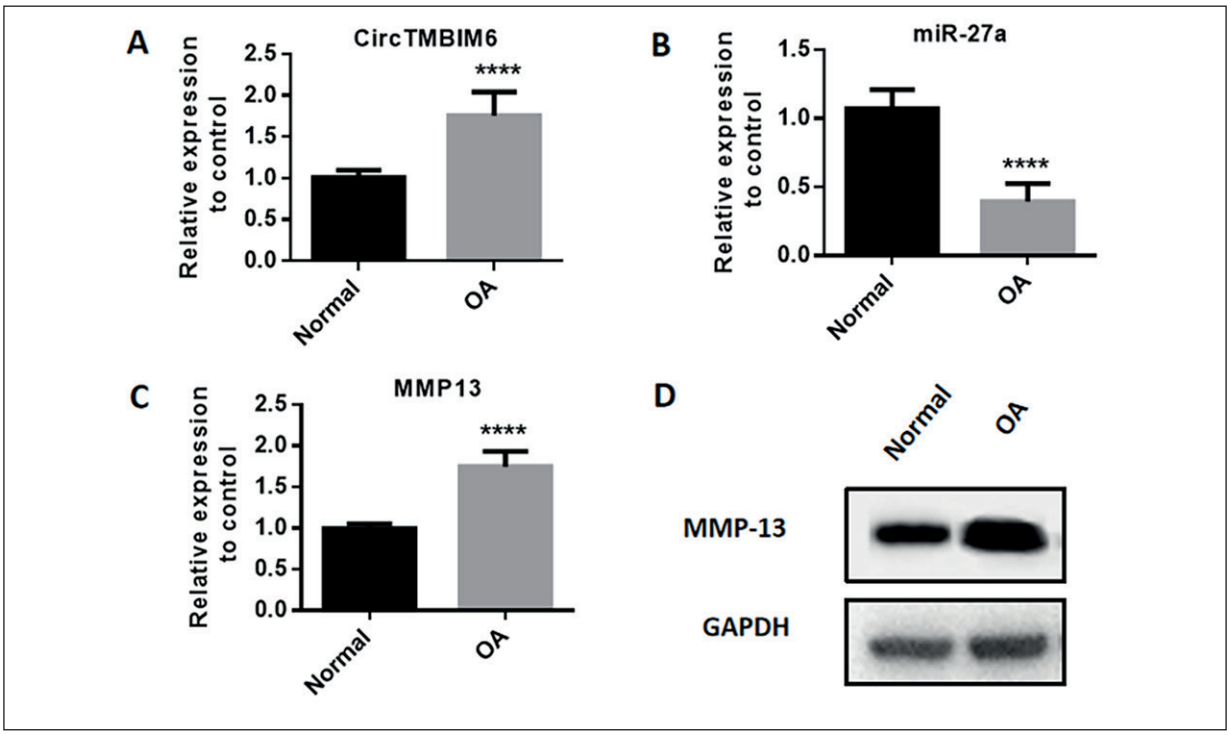

Figure 1. CircTMBIM6 and MMP13 are up-regulated in osteoarthritis patients and miR-27a is down-regulated in osteoarthritis patients. **A**, CircTMBIM6 expression in cartilage tissue. **B**, MiR-27a expression in chondrocytes. **C, D**, MMP13 expression in chondrocytes.

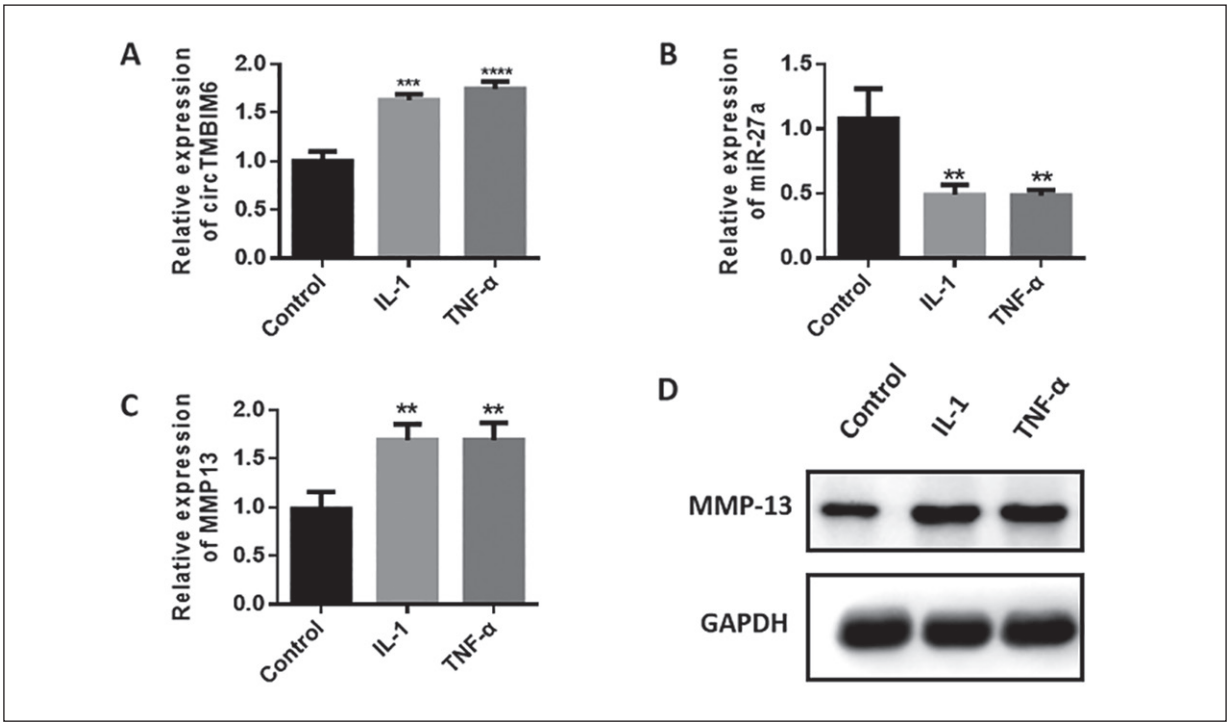

Figure 2. In the chondrocyte model induced by IL-1B and TNF-α, expressions of CircTMBIM6 and MMP13 are up-regulated and miR-27a is down-regulated. **A**, CircTMBIM6 expression in chondrocytes. **B**, MiR-27a expression in chondrocytes. **C**, **D**, MMP13 expression in chondrocytes.

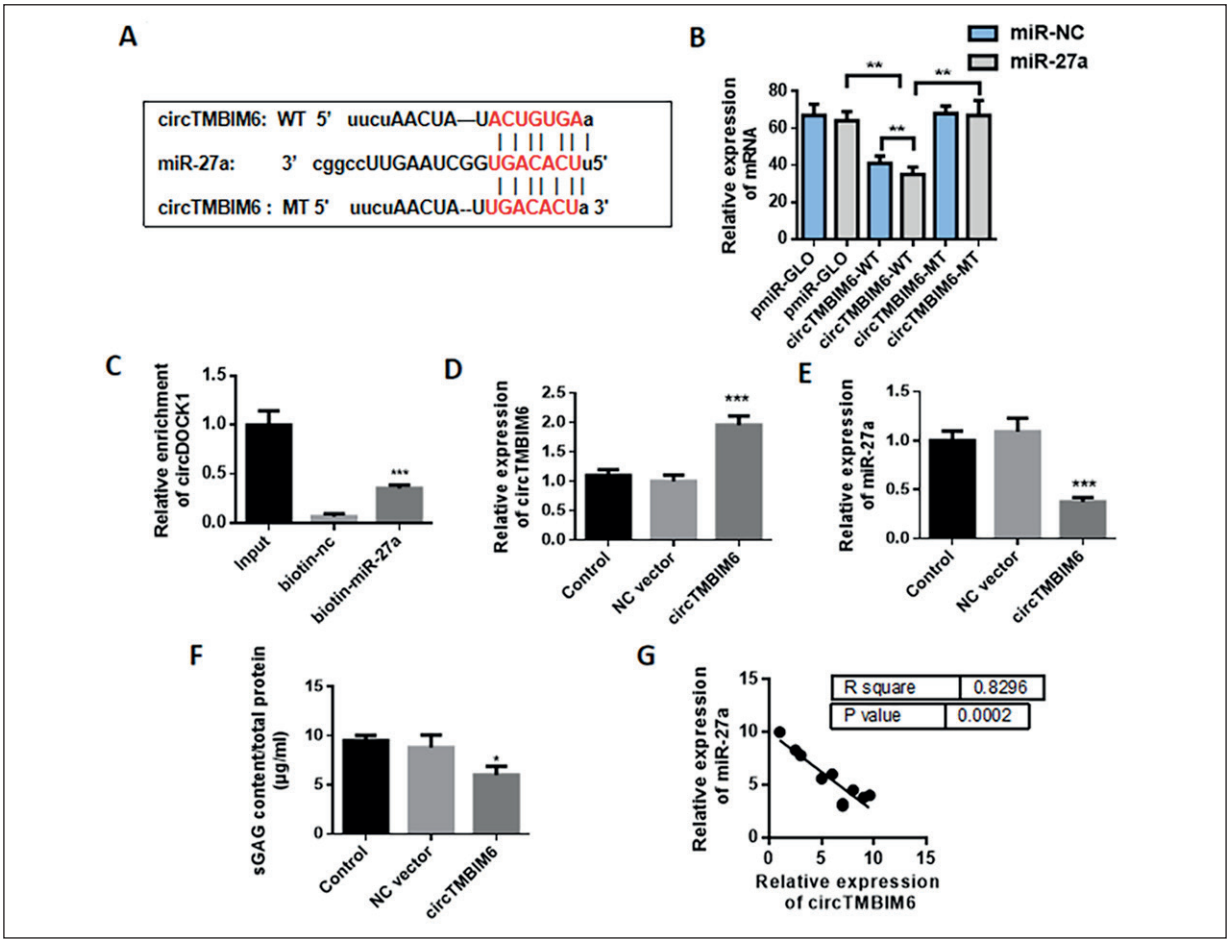

Figure 3. Up-regulation of CircTMBIM6 inhibits miR-27a and up-regulates MMP13 expression. **A**, CircTMBIM6 targets miR-27a binding site information. **B**, Report carrier experiments. **C**, RNA pulled down (miR-27a probe). **D**, Efficient detection of CircTMBIM6 overexpression. **E**, Effect of CircTMBIM6 overexpression on miR-27a expression. **F**, Effect of CircTMBIM6 overexpression on sGAG expression. **G**, Correlation analysis of CircTMBIM6 and miR-27a expression.

Inhibit CircTMBIM6 Up-Regulates the Expression of MiR-27a and Decrease the Expression of MMP13

Results of knockdown of circTMBIM6 showed that knockdown of RNA can effectively reduce the expression of circTMBIM6 (Figure 4A). Further experimental results showed that knockdown of circTMBIM6 up-regulated miR-27a expression (Figure 4B). However, knocking down circTMBIM6 increased the sGAG content. The expression of sGAG was higher in si-circTMBIM6 group than in NC siRNA group and control group.

Up-Regulated MiR-27a Inhibits MMP13 Expression

RNA pulled down experiments show that miR-27a can directly bind to MMP13 (Figure 5A). The overexpression of miR-27a showed that miR-27a mimics significantly increased the expression of miR-27a (Figure 5B). Detection of MMP13 expression showed that miR-27a mimics can inhibit MMP13 expression (Figure 5C). Overexpression of miR-27a can increase sGAG content (Figure 5D). Correlation analysis of miR-27a and MMP13 expression showed that they had a positive correlation (Figure 5E). MiR-27a reverses the effect of circTMBIM6 on the promotion of MMP13 expression (Figure 5F, 5G). The correlation analysis results of circTMBIM6 and MMP13 expression show that circTMBIM6 and MMP13 have a positive co-expression correlation (Figure 5H).

Inhibition of MiR-27a Expression Up-Regulates MMP13 Expression

Results of the knockdown miR-27a efficiency test showed that knockdown RNA can signifi-

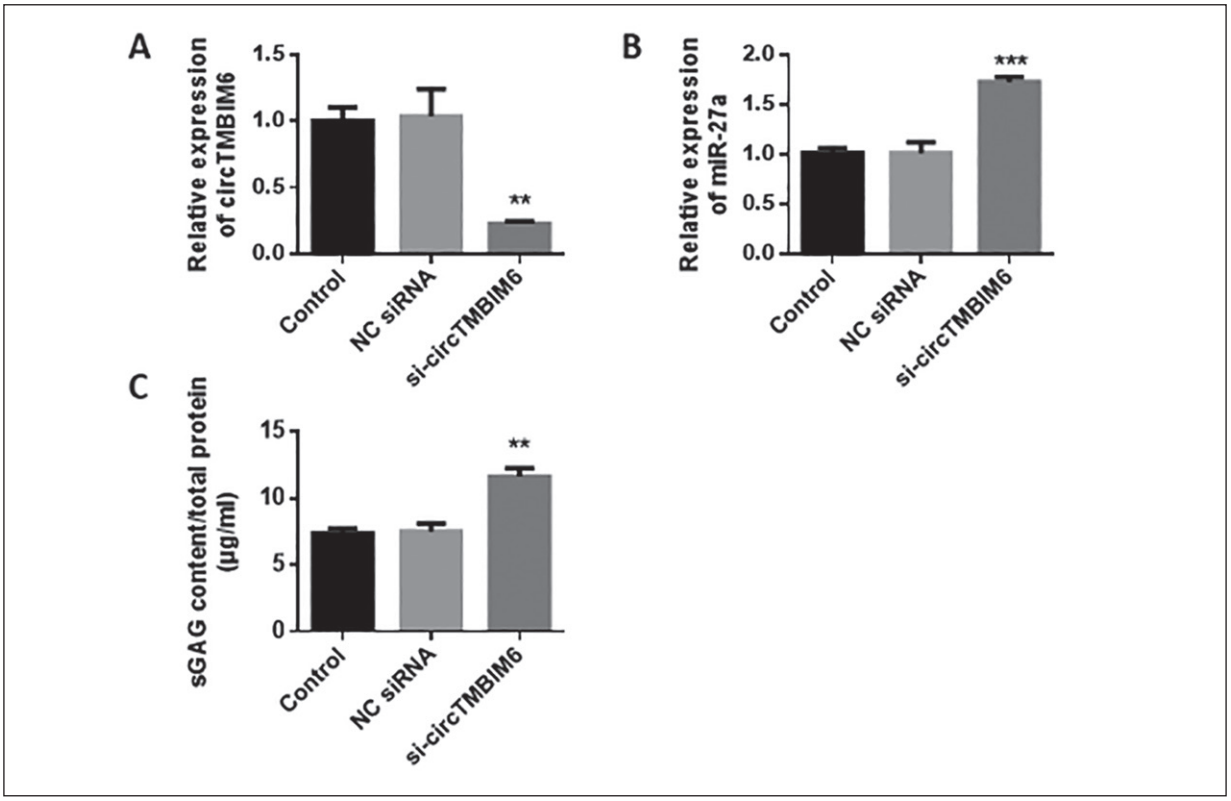

Figure 4. Inhibition of CircTMBIM6 up-regulates miR-27a expression and decreases MMP13 expression. **A**, Knockdown CircTMBIM6 efficiency test. **B**, Effect of CircTMBIM6 knockdown on miR-27a expression. **C**, Effect of CircTMBIM6 knockdown on sGAG expression.

cantly reduce the expression of circTMBIM6 (Figure 6A). Detection of MMP13 expression showed that inhibitor miR-27a was able to up-regulate MMP13 expression (Figure 6B). Inhibition of miR-27a down-regulated sGAG content in chondrocytes (Figure 6C).

Discussion

Osteoarthritis (OA) is a degenerative joint disease caused by cartilage degradation, bone thickening, and bone spur formation[11]. The main clinical manifestations are chronic pain and joint mobility disorders, which seriously affect patients' quality of life. The disease is more common in middle-aged and elderly people, and osteoarthritis occurs in about 80% of people aged ≥60 years[12]. Osteoarthritis is a complex multifactorial disease caused by mechanical, genetic, and environmental factors. It is also the most common disease of the joints. Many environmental factors such as hormones, diet, infection, trauma, alcohol intake, and exposure to tobacco smoke can increase bone risk of arthritis. Chondrocytes are the only cells in articular cartilage. Degradation of extracellular matrix, chondrocyte apoptosis and production of cytokines are essential for the pathological progress of osteoarthritis[13,14]. Therefore, inhibition of extracellular matrix degradation, reduction of chondrocyte apoptosis and inflammatory response can delay the pathology of osteoarthritis.

Competitive endogenous RNA (ceRNAs) reveals a new mechanism by which RNA regulates each other at the post-transcriptional level. MRNA, lncRNA, circRNA and pseudogene, as ceRNA molecules, regulate the expression level of target genes by competing for miRNA response components (MREs). CeRNA is widely involved in important life processes such as cell differentiation, inflammation and apoptosis *in vivo*. Reports have shown that the expression level of non-coding RNA GAS5 is significantly increased in osteoarthritis chondrocytes. Moreover, miR-21 was competitively bound as ceRNA, thereby

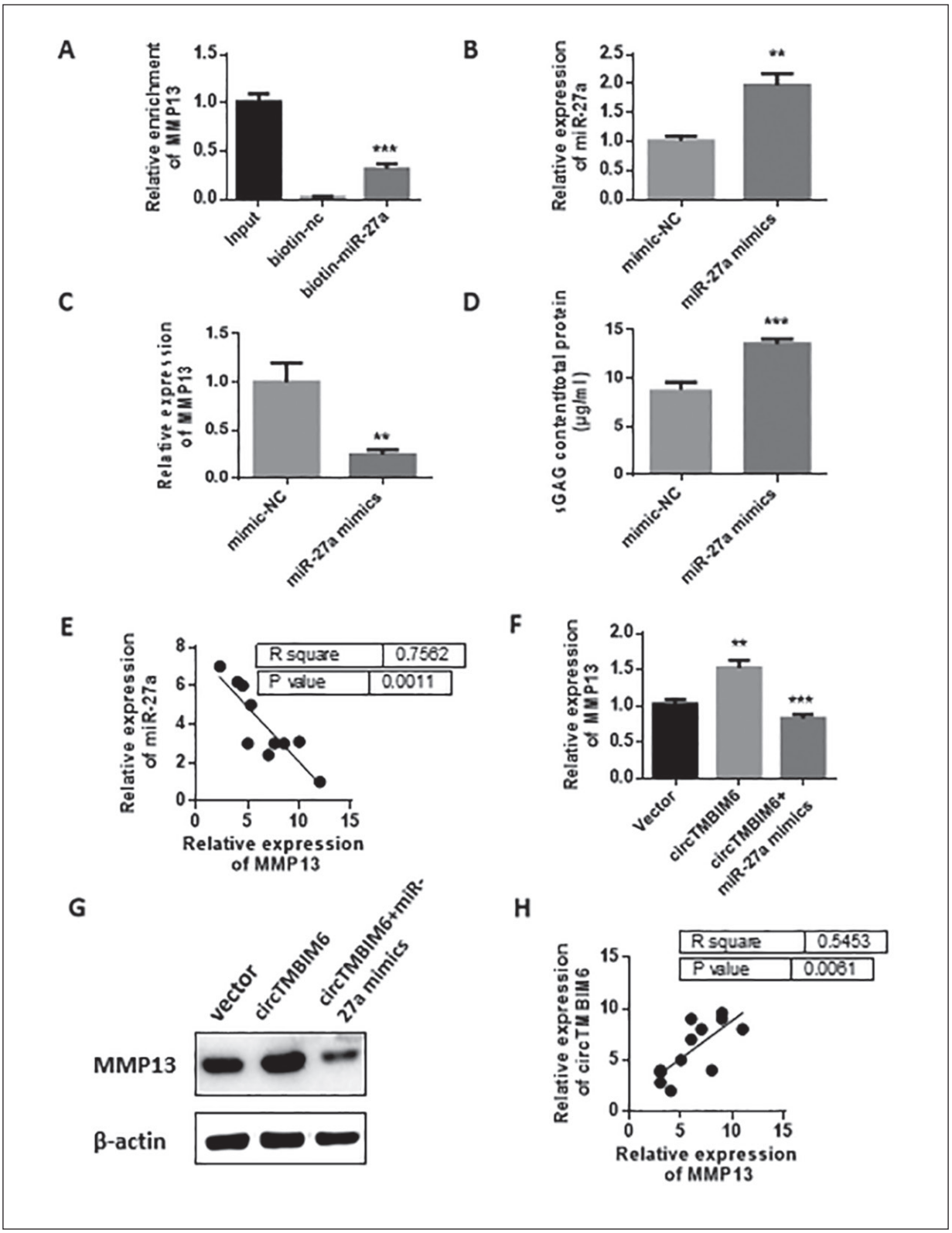

Figure 5. Up-regulation of miR-27a inhibits MMP13 expression. **A**, RNA is pulled down to detect whether miR-27a bind to MMP13. **B**, Overexpression of miR-27a efficiency test. **C**, Detection of MMP13 expression. **D**, Effect of miR-27a on sGAG expression. **E**, Correlation analysis of miR-27a and MMP13 expression. **F, G,** MiR-27a reversed the effect of circTMBIM6 on the promotion of MMP13 expression. **H**, Correlation analysis of CircTMBIM6 and MMP13 expression.

promoting the expression of MMP13 and exacerbating the degeneration of bone and joints. In addition, the upregulation of MMP13 in patients with osteoarthritis can also be achieved by binding miR-136 competitively to circRNA-CER, promoting extracellular matrix degradation. It has been reported that circRNA-CER can be up-regulated by the expression of interleukin-1 (IL-1β) and tumor necrosis factor (TNF-α). It regulates MMP13 expression through endogenous competition of miR-136 and participates in the extracellular matrix damage of chondrocytes process[15]. In this study, IL-1β and TNF-α were used to induce chondrocytes to simulate an *in vitro* osteoarthritis cell model. TNF-α can promote the expression of miR-193b, miR-293b can inhibit early cartilage formation, and its target gene IGF1R that regulates early cartilage formation can be promoted by circRNA_0045714. Due to the important role of the gene IGF1R in early cartilage formation, circRNA_0045714. It may also play an important role in arthritic diseases. In addition, some researchers have used circRNAs chips to screen differential expression of circRNAs and co-expression analysis of ceRNAs network in OA patients and normal people, and identified 16 circRNA expressions are up-regulated and 55 expressions are down-regulated. They involve in the development of cartilage damage and arthritis. CircRNA is stable in structure, complex in function, diversity in the source, and widely distributed. In the future, its protein coding function and the function of "miRNA molecular sponge" need to be further studied. At present, there are few research results on circRNA and bone-related diseases, but miRNAs and bone diseases. There are a number of proven functions, such as miR-222-3p and miR-7067-5p associated with osteoblasts are regulated by circRNA5846 and circRNA19142. In view of the rich possible interactions between circRNAs and miRNAs, circRNAs are likely to affect hereditary bone disease through interaction with miRNAs or their own coding ability. Collagenase is a group of matrix metalloproteinases (MMPs) that function at neutral pH (1-4). It plays an important role in bone development and bone reconstruction[16]. It has several common

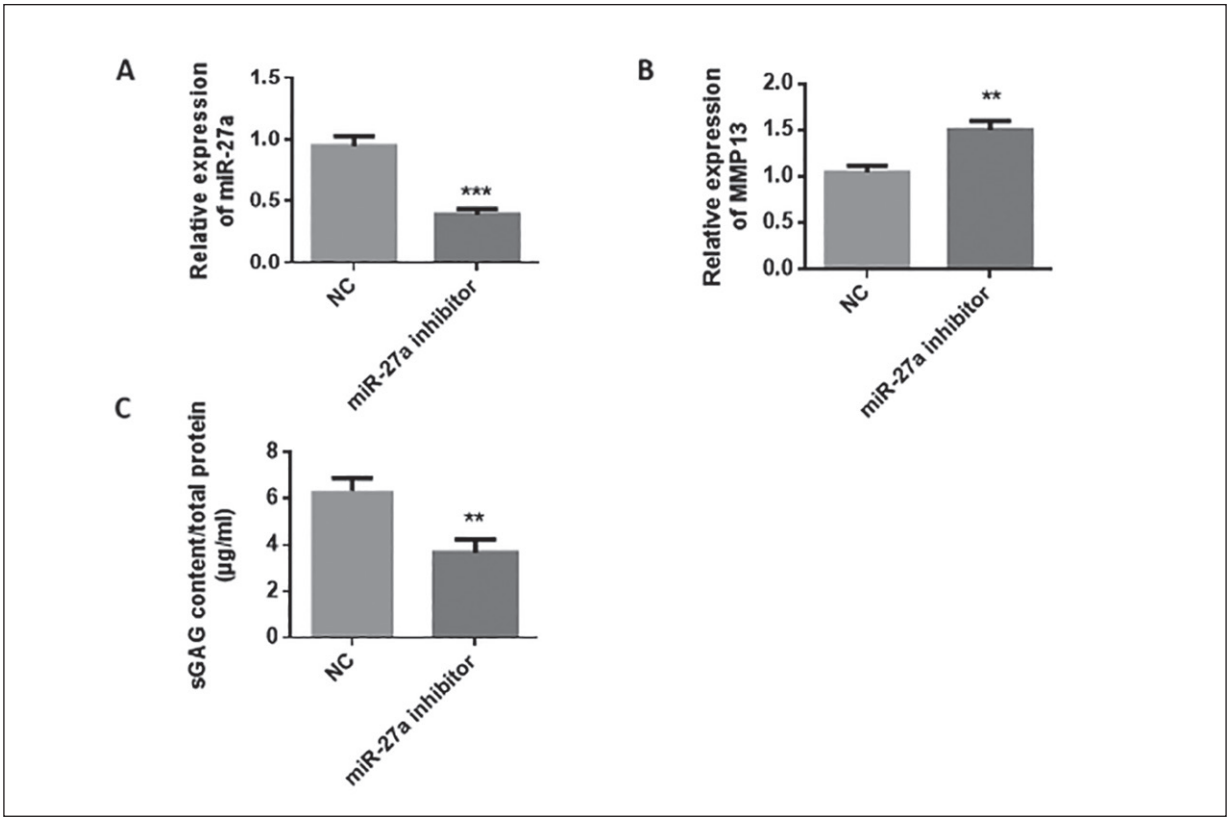

Figure 6. Inhibition of miR-27a expression up-regulates MMP13 expression. **A**, Detection of knockdown miR-27a efficiency. **B**, Detection of MMP13 expression. **C**, Effects of knockdown of miR-27a on sGAG expression.

structural characteristics, including there is conserved zinc-binding catalytic domains[17-20]. However, only the products of specific MMP genes such as MMP1, 2, 8, 13, and 14 have the ability to cut natural, undenatured interstitial collagen at specific helix loci[21,22]. MMP13 (matrix metalloproteinase 13) is a major enzyme that targets cartilage degradation. Compared with other MMPs, MMP13 expression is more restricted to connective tissue[23]. It can degrade not only type II collagen in cartilage, but also proteoglycan, type IV and IX collagen, osteoadhesin and basement membrane proteoglycan in cartilage[24]. Therefore, MMP13 plays an important role in OA. Under normal physiological conditions, MMP13 is an important regulator of tissue plasticity and tissue repair. However, under pathological conditions such as osteoarthritis and inflammation, MMP13 overexpression is an important factor leading to the degradation of extracellular matrix. The experimental results in this paper show that MMP13 is highly expressed in cartilage tissue of patients with osteoarthritis. However, the specific molecular mechanisms that regulate extracellular matrix degradation are unclear.

Our study produced several new discoveries. First, the expression of circTMBIM6 was negatively correlated with that of miR-27a, with the former up-regulated and the latter down-regulated in OA. Second, overexpression of circTMBIM6 significantly promoted the progression of OA disease. Third, as the target gene of miR-27a, MMP13 may mediate the role of circTMBIM6/miR-27a axis in promoting OA. Fourth, circRNA acts as ceRNA through sequence complementation, limiting the functional availability of miR-27a. Therefore, we revealed new aspects of the cellular function and pathophysiological roles of circTMBIM6 and miR-27a, both of which can be considered as potential molecular targets for the treatment of OA.

Conclusions

We found that circTMBIM6 plays an important role in regulating gene expression in osteoarthritis. CircTMBIM6 plays an important role in regulating the degradation of the chondroextra cellular matrix in osteoarthritis through the miR-27a/MMP13 axis. Further study of the molecular mechanism of CircTMBIM6 in bone and joint regulation is of great significance for understanding the pathophysiology of osteoarthritis. Our results provide a new idea for the targeted therapy of osteoarthritis and a potential drug therapeutic target for the treatment of osteoarthritis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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