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LncRNA TUG1 promotes osteoarthritis-induced degradation of chondrocyte extracellular matrix via miR-195/MMP-13 axis

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Abstract. – OBJECTIVE: Osteoarthritis is a degenerative disease characterized by articular cartilage degradation. Long non-coding ribonucleic acid (lncRNA) plays important roles in a series of biological processes, but its role in osteoarthritis is still not quite clear. This study aims to investigate the regulatory role of taurine upregulated gene 1 (TUG1) in osteoarthritis.

PATIENTS AND METHODS: The expression level of lncRNA-TUG1 in cartilages of patients with osteoarthritis and those of normal people was compared using Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Primary chondrocytes were induced by interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), followed by expression detection of lncRNA-TUG1, microRNA-195 (miR-195), and matrix metalloproteinase-13 (MMP-13). In addition, in vitro regulatory roles of lncRNA-TUG1 and miR-195 in osteoarthritis were verified by transfection of lncRNA-TUG1 and miR-195 plasmids. The dimethylmethylene blue (DMMB) assay was performed to analyze the secretion and formation of soluble sulfated glycosaminoglycan (sGAG).

RESULTS: The expression levels of lncRNA-TUG1 and MMP-13 in cartilages of patients with osteoarthritis were higher than those in cartilages of normal people, while the level of miR-195 decreased in cartilages of patients with osteoarthritis. After chondrocytes were induced by IL-1β and TNF-α, the expression of lncRNA-TUG1 increased. Overexpression of lncRNA-TUG1 decreased the expressions of miR-195, collagen, and aggrecan, but increased the expression of MMP-13. LncRNA-TUG1 knockdown obtained the opposite results.

CONCLUSIONS: LncRNA-TUG1 regulates the degradation of extracellular matrix in osteoarthritis via lncRNA-TUG1/miR-195/MMP-13 axis.

Key Words:
Osteoarthritis, lncRNA-TUG1, miR-195, MMP-13.

Introduction

Osteoarthritis is a degenerative joint disease, and its major pathological features include articular cartilage degeneration, thickened subchondral bones, and bone spur formation[1]. Osteoarthritis is closely related to joint injury, immune function decline, and inflammatory response[2]. Extracellular matrix degradation is a complex process. As a major component of the chondrocyte extracellular matrix, type II collagen, matrix metalloproteinases (MMPs), and disintegrins play important roles in maintaining bone homeostasis[3]. However, the specific molecular mechanism of maintaining homeostasis by articular cartilages is not quite clear so far, which greatly restricts the treatment for osteoarthritis[4,5].

The human transcriptome includes not only messenger ribonucleic acids (mRNAs) that encode proteins, but also a large number of non-coding transcripts, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). These non-coding RNAs have been reported to play important regulatory roles in various physiological and pathological activities, including growth and development, cell proliferation and apoptosis, stem cell differentiation and tumor formation[6-11]. A study[12] has shown that non-coding genes also play a role in the pathological development of osteoarthritis. LncRNAs are a class of non-coding RNAs longer than 200 nucleotides[13]. A large number of studies[14-16] have revealed that differentially expressed lncRNA lead to abnormal expressions of downstream genes, thereby affecting a variety of diseases. Recently, lncRNA-taurine upregulated gene 1 (TUG1) is considered an oncogenic lncRNA, which is highly expressed in various malignant tumors. The lncRNA-TUG1 expression is negatively correlated to the prognosis. It is re-

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ported[17] that lncRNA-TUG1 promotes cell proliferation, migration, and invasion in patients with osteosarcoma. However, whether lncRNA-TUG1 plays a role in osteoarthritis remains unclear. The expression level of microRNA-195 (miR-195) in patients with osteosarcoma is significantly lower than that in normal people, and its expression level is negatively correlated with the clinical stage[18]. However, the role of miR-195 in osteoarthritis has not been reported yet.

This study aims to investigate the regulatory effects of lncRNA-TUG1, miR-195, and MMP-13 on the pathogenesis of osteoarthritis. We also elucidate whether lncRNA-TUG1 could regulate the degradation of chondrocyte extracellular matrix in patients with osteoarthritis *via* lncRNA-TUG/miR-195/MMP-13 axis.

Patients and Methods

Patients and Samples

Knee articular cartilages were collected from patients with osteoarthritis and normal people. A total of 15 patients with osteoarthritis were enrolled, including 9 females and 6 males at the age of 54-70 years old. Cartilages were taken during the anaplasty for full knee joints. Besides, there were 6 normal people enrolled, including 4 males and 2 females at the age of 27-39 years old, and cartilages were taken from patients with traumas. All tissues were stained using Safranin O, and changes were graded according to a modified Mankin scale[19]. All the recruited patients signed the informed consent. This study was approved by the Ethics Committee of the Second Affiliated Hospital, Zhejiang University School of Medicine.

Cell Culture of Primary Chondrocytes and Induction With Interleukin-1 Beta (IL-1α) and Tumor Necrosis Factor-Alpha (TNF-α)

Isolated cartilage tissues were digested with 0.25% trypsin for half an hour and then incubated with type II collagenase (0.2%) at 37°C for four hours. The isolated chondrocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM; HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 100 U/ml of penicillin. Chondrocytes were inoculated in a six-well plate, starved for 24 h in the serum-free medium, and stimulated with IL-1β (10 ng/mL) or TNF-α (10 ng/mL) for 12 hours.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The total RNA in cartilage tissues was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the concentration and purity of RNA were measured using a spectrophotometer. Reverse transcription kits purchased from TaKaRa (Otsu, Shiga, Japan), were used to reversely transcribe 1,000 ng RNAs per sample. The expression levels of lncRNA-TUG, miR-195, and MMP-13 were detected by the SYBR Premix Ex Taq kit (TaKaRa, Otsu, Shiga, Japan). The $2^{-\Delta\Delta CT}$ method was used for data analyses. The primer sequences were as follows: TUG1: forward: 5'-TAGCAGTTCCCCAATCCTTG-3', reverse: 5'-CACAAATTCCCATCATTCCC-3', miR-195: forward: 5'-GAATTCGCCTCAAGAGAACA-AAGTGGAG-3', reverse: 5'-AGATCTCCCA-TGGGGGCTCAGCCCCT-3', MMP-13: forward: 5'-ACTGAGAGGCTCCGAGAAATG-3', reverse: 5'-GAACCCCGCATCTTGGCTT-3', U6: forward: 5'-TTACATTGCTATCCACAGA-ACGG-3', reverse: 5'CTATGCTGCTGCTTTT-TGCTC-3', and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward: 5'-TGTTCGTCATGGGTGTGAAC-3', reverse: 5'-ATGGCATGGACTGTGGTCAT-3'. U6 and GAPDH were regarded as internal controls. All trials were repeated for three times.

Western Blot

A total of 40 μg protein was loaded into sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Bands were incubated with rabbit anti-MMP-13 antibody (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA, USA) was used to develop the bands. GAPDH was considered as the internal control.

Quantification of Soluble Sulfated Glycosaminoglycan (sGAG)

The cell suspension was collected. Dimethylmethylene blue (DMMB) assay (Sigma-Aldrich, St. Louis, MO, USA) was performed to detect soluble sGAG secretion[20-22]. Briefly speaking, 200 μL of DMMB reagent was mixed with 20 μL of cell suspension, and the absorbance was detected under 525 nm. A standard curve was established

based on shark chondroitin 6-sulfate. Total sGAG was normalized to total protein in cell lysates with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA).

Plasmid and siRNAs Construction and Transfection to Cell

LncRNA-TUG1 overexpression plasmid was constructed (plncRNA-TUG1). SiRNAs against lncRNA-TUG1 (si-TUG1) were produced by Genepharma (Shanghai, China). MiR-195 mimics, inhibitors or negative control (Genepharma, Shanghai, China) was transfected into cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. All measurement results were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used for intergroup comparisons, followed by Post-Hoc Test (Least Significant Difference). The Student's *t*-test was used for comparisons between the two groups. All trials were repeated three times. $p<0.05$ represented that the difference was statistically significant.

Results

Expressions of lncRNA-TUG1, miR-195, and MMP-13 in Cartilages of Patients With Osteoarthritis and Normal People

Compared with cartilages of healthy controls, the expression level of lncRNA-TUG1 in cartilages of patients with osteoarthritis significantly increased (Figure 1A). Bioinformatics analysis was conducted to predict possible target miRNAs of lncRNA-TUG1. It was found that miR-195 could bind to lncRNA-TUG1 and MMP-13. Subsequently, the expression levels of miR-195 and MMP-13 in patients with osteoarthritis and normal controls were examined. The results showed that compared with those in chondrocytes of normal controls, miR-195 was down-regulated, and MMP-13 was significantly up-regulated in chondrocytes of patients with osteoarthritis (Figure 1B-D).

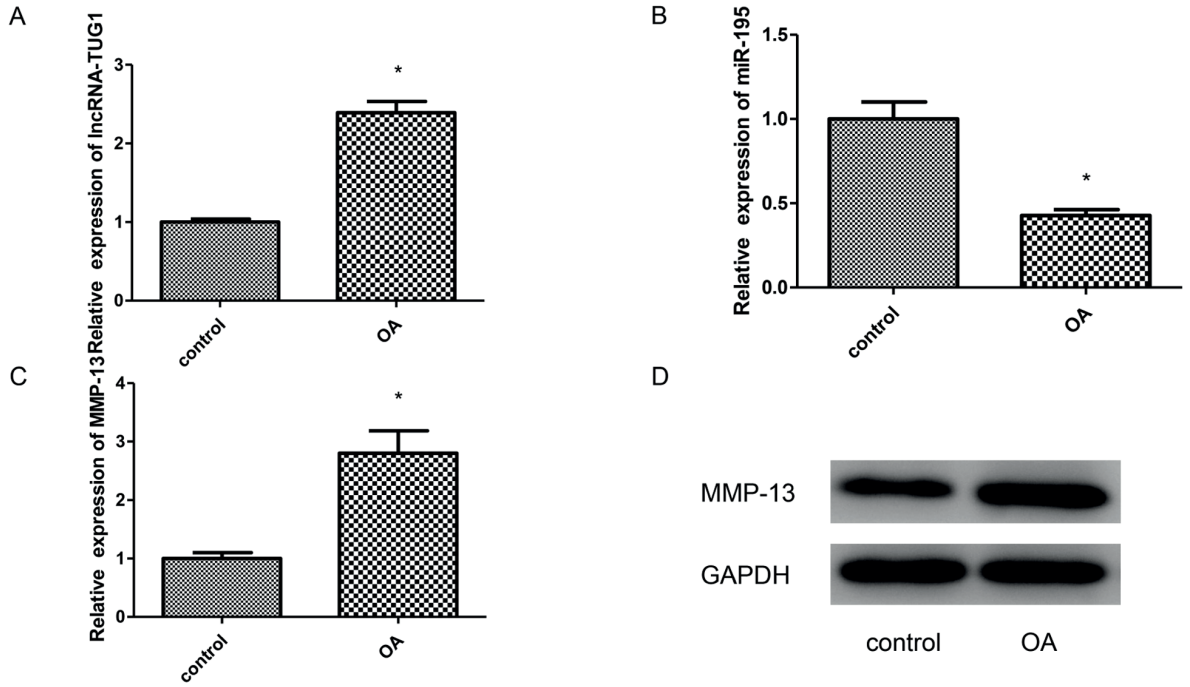

Figure 1. Differentially expressed genes in cartilage of patients with osteoarthritis. ***A,*** The expression level of lncRNA-TUG1. ***B,*** The expression level of miR-195. ***C,*** The mRNA expression level of MMP-13. ***D,*** Protein expression of MMP-13. OA, osteoarthritis. All values were expressed as means ±SD. *, $p<0.05$.

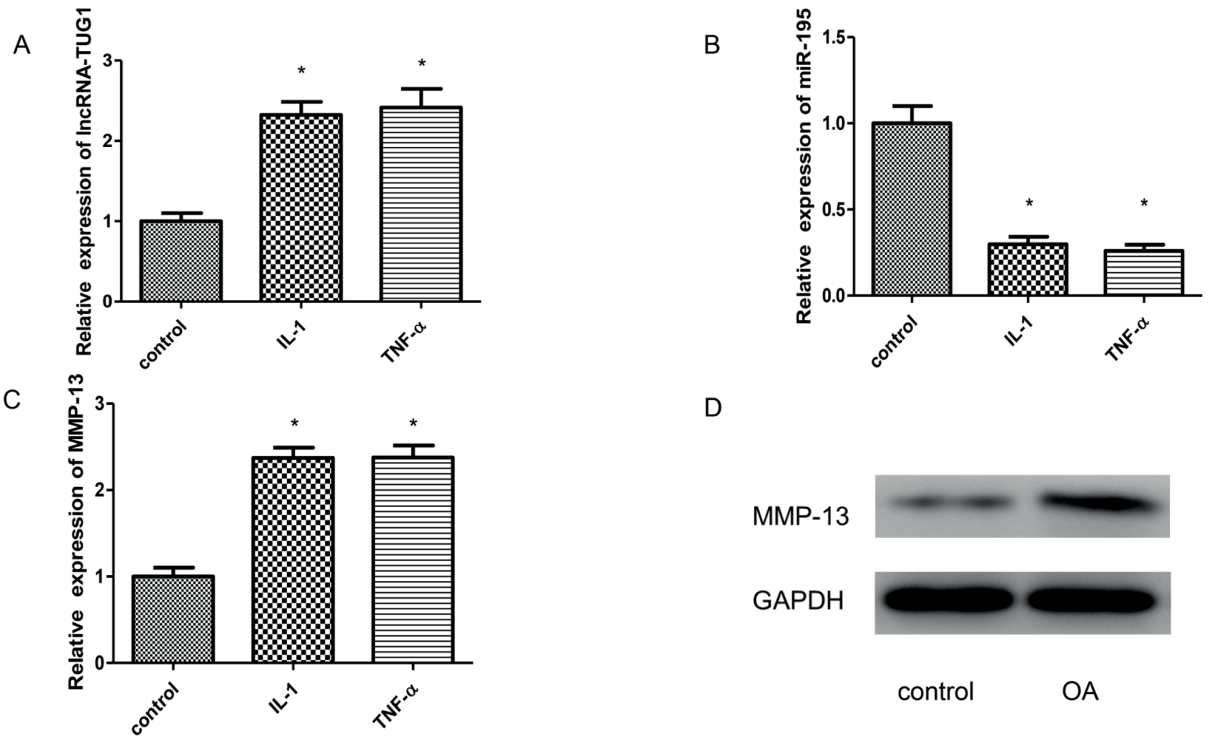

Figure 2. Effects of IL-1 and TNF-α on the expression of genes in cartilage. ***A,*** LncRNA-TUG1 expression. ***B,*** MiR-195 expression. ***C,*** The mRNA level of MMP-13. ***D,*** Protein level of MMP-13. All values were expressed as means ±SD. *, $p<0.05$.

Expressions of lncRNA-TUG1, miR-195, and MMP-13 After Normal Chondrocytes Were Induced With TL-1 and TNF-α

To verify whether inflammation could promote cartilage damage by inducing the degradation of chondrocyte extracellular matrix, osteoarthritis microenvironment was simulated by IL-1 or TNF-α induction in chondrocyte solution. 12 h later, the expressions of lncRNA-TUG1, miR-195, and MMP-13 were detected. It was found that compared with those in the non-stimulation (inflammatory cytokines) group, the mRNA levels of lncRNA-TUG1 and MMP-13 increased, and the mRNA level of miR-195 decreased in the stimulation group (Figure 2).

LncRNA-TUG1 Regulates the Expressions of miR-195 and MMP-13 as Well as the Degradation of the Cell Extracellular Matrix

LncRNA-TUG1 overexpression plasmids and small interfering RNAs (siRNAs) were constructed to elucidate the role of lncRNA-TUG1 in osteoarthritis. After transfection with lncRNA-TUG1 overexpression plasmid, the overexpression effect was verified by RT-PCR (Figure 3A). Overexpression of lncRNA-TUG1 decreased miR-195 expression and increased MMP-13 expression (Figure 3B, 3C). RT-PCR confirmed the down-regulation effect of lncRNA-TUG1 after the transfection with lncRNA-TUG1 siRNAs (Figure 3E). After knockdown of lncRNA-TUG1, miR-195 expression increased, and MMP-13 expression decreased (Figure 3F, 3G). In the meantime, sGAG secreted by chondrocytes in the culture medium was measured. The results showed that the content of sGAG decreased after the up-regulation of lncRNA-TUG1 (Figure 3D). On the contrary, sGAG content increased after the down-regulation of lncRNA-TUG1 (Figure 3H).

Roles of miR-195 in the Expressions of lncRNA-TUG1 and MMP-13 as well as the Degradation of the Cell Extracellular Matrix

MiR-195 mimics, miR-195 inhibitor and negative controls were constructed. The regulatory effect of miR-195 on the contents of lncRNA-TUG1, MMP-13, and sGAG were detected. The results revealed that compared with those in the control group, miR-195 expression and sGAG content increased, while the expressions of lncRNA-TUG1

and MMP-13 were reduced after transfection with miR-195 mimics (Figure 4A, 4B, 4C, 4D). In contrast, miR-195 expression and sGAG declined, while the expressions of lncRNA-TUG1 and MMP-13 increased after transfection with miR-195 inhibitor (Figure 4E, 4F, 4G, 4H).

LncRNA-TUG1 Regulated the Expression of MMP-13 As a Competing Endogenous RNA (ceRNA) in Chondrocytes

To validate the interrelations among miR-195, lncRNA-TUG1, and MMP-13, dual-luciferase reporter gene assay was conducted. Chondrocytes were co-transfected with wild-type or mutant lncRNA-TUG1 or MMP-13 and miR-195 mimics. The examination of the relative luciferase activity showed that miR-195 overexpression inhibited the luciferase activity of wild-type MMP-13 or lncRNA-TUG1 compared with those of the negative control and mutant lncRNA-TUG1 or MMP-13 (Figure 5A, 5B). After that, chondrocytes were co-transfected with lncRNA-TUG1 and miR-195 mimics or with lncRNA-TUG1 siRNAs and miR-195 inhibitors. The results showed that lncRNA-TUG1 overexpression alone could promote the expression of MMP-13, while co-transfection with miR-195 mimics decreased the expression of MMP-13 (Figure 5C). On the contrary, MMP-13 expression decreased after transfection with lncRNA-TUG1 siRNAs, while co-transfection with miR-195 inhibitor reversed the decrease (Figure 5D).

Discussion

This study found that endogenous lncRNA-TUG1 regulated MMP-13 expression in osteoarthritis as a sponge of miR-195. In patients with osteoarthritis, the expressions of lncRNA-TUG1 and MMP-13 were significantly higher than those in normal people, while miR-195 was lowly expressed in patients with osteoarthritis. By up-regulating or down-regulating the expression of lncRNA-TUG1 or miR-195 in chondrocytes, it was confirmed that miR-195 might play a role by binding to the three prime untranslated regions (3'-UTRs) of lncRNA-TUG1 and MMP-13. It was concluded that lncRNA-TUG1/miR-195/MMP-13 axis may play a regulatory role in chondrocyte extracellular matrix degradation.

Osteoarthritis seriously affects life quality of affected patients[23]. Although the incidence rate of osteoarthritis is high, and the disease exerts huge impacts on public health, the etiology of osteoarthritis remains unclear. Evidence has shown the degradation of the chondrocyte extracellular matrix is one of the important causes of osteo-

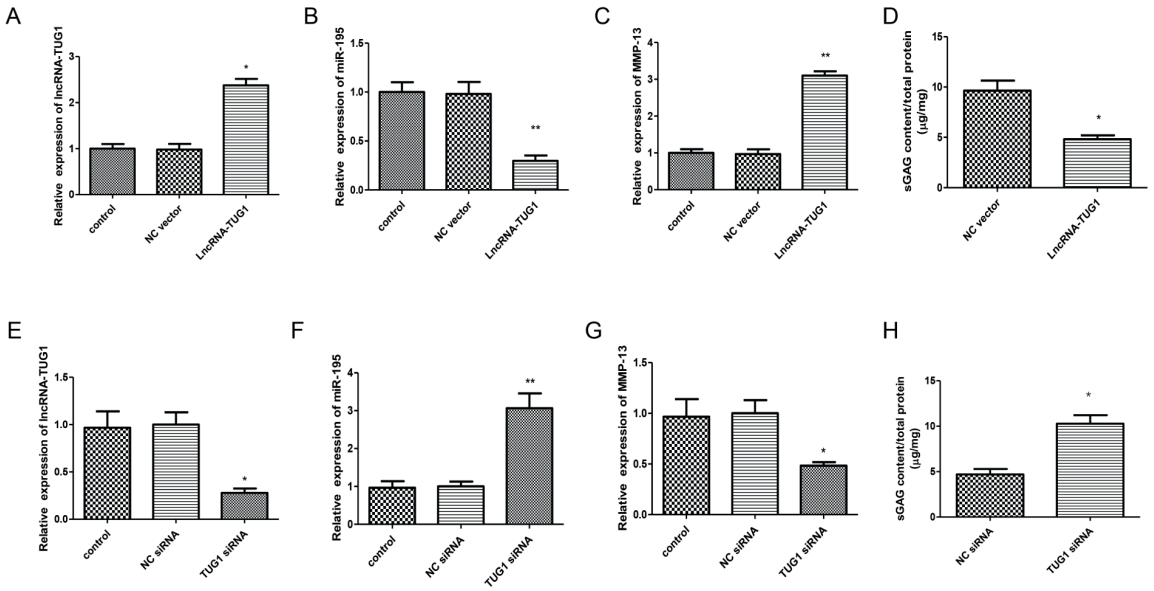

Figure 3. The effects of lncRNA-TUG1 on the expression of genes and the degradation of the ECM in chondrocytes. ***A,*** and ***E,*** LncRNA-TUG1 expression. ***B,*** and ***F,*** MiR-195 expression. ***C,*** and ***G,*** The mRNA level of MMP-13. ***D,*** and ***H,*** The content of sGAG in culture. NC siRNA: negative control siRNA; TUG1 siRNA: lncRNA-TUG1 siRNA; NC vector: negative control vector; lncRNA-TUG1: lncRNA-TUG1 overexpression plasmid. All values were expressed as means ±SD. *, $p<0.05$. **, $p<0.01$

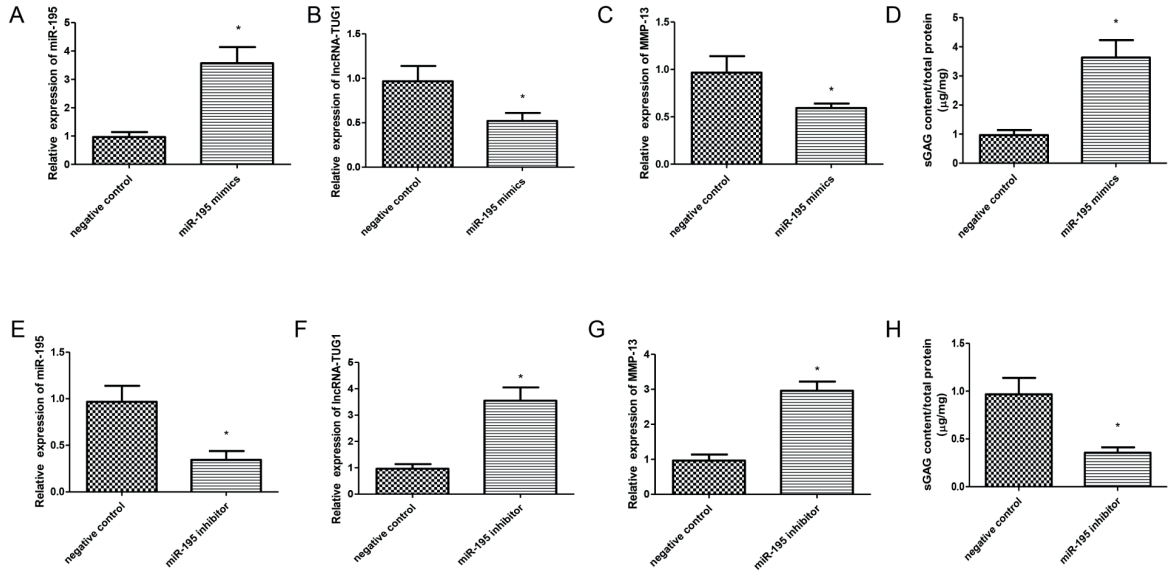

Figure 4. The effects of miR-195 on the expression of genes and the degradation of the ECM in chondrocytes. ***A,*** and ***E,*** MiR-195 expression. ***B,*** and ***F,*** LncRNA-TUG1 expression. ***C,*** and ***G,*** The mRNA level of MMP-13. ***D,*** and ***H,*** The content of sGA in culture. All values were expressed as means ±SD. *, $p<0.05$.

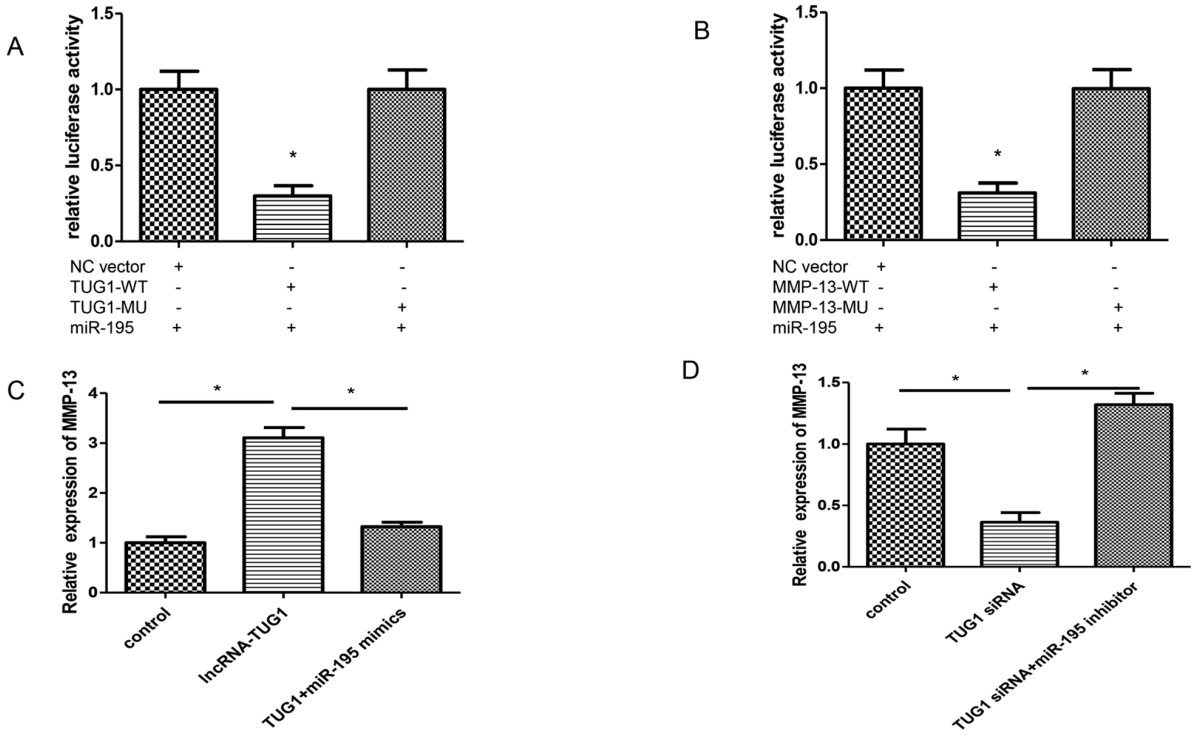

Figure 5. The effects of lncRNA-TUG1 on MMP13 expression as a ceRNA in chondrocytes. ***A,*** and ***B,*** Relative luciferase activity. ***C,*** and ***D,*** The mRNA level of MMP-13. NC vector: negative control vector; TUG1-WT: report gene containing wild-type lncRNA-TUG1 3'UTP; TUG1-MU: report gene containing mutated lncRNA-TUG1 3'UTP; miR-195: miR-195 mimics; MMP-13-WT: report gene containing wild-type MMP-13 3'UTP; MMP-13-MU: report gene containing mutated MMP-13 3'UTP; NC miRNA: negative control miRNA; TUG1-siRNA: specific siRNA of lncRNA-TUG1; lncRNA-TUG1: lncRNA-TUG1 overexpression plasmid. All values were expressed as means ±SD. *, $p<0.05$.

arthritis. MMP-13 is an extracellular matrix degradation-related enzyme produced at the site of bone and joint. Under physiological conditions, MMP-13 is an important regulatory factor for tissue plasticity and repair. However, under pathological conditions, such as inflammation, MMP-13 overexpression is an important factor leading to the degradation of extracellular matrix[24,25]. This study also revealed that MMP-13 was indeed highly expressed in patients with osteoarthritis. However, the specific molecular mechanism of extracellular matrix degradation remains unclear.

In recent years, a large number of studies have focused on the epigenetic aspects of the pathogenesis of osteoarthritis and sought to find molecular targets for the treatment of osteoarthritis, including miRNAs and lncRNAs. LncRNAs have been proven to be involved in the regulation of various life events, such as cell cycle regulation and immune surveillance[26]. LncRNAs have been reported to be involved in the regulation of osteoarthritis. Cartilage injury-related lncRNA (lncRNA-CIR), maternally expressed gene 3 (MEG3), and prostate cancer gene expression marker 1 (PCGEM1) can relieve cartilage injury and degradation[27]. In addition, lncRNA-UFC1 can promote the proliferation of chondrocytes by interacting with miR-34a in osteoarthritis[28]. However, the role of lncRNA-TUG1 in osteoarthritis has not been reported yet. Scholars[8,29] have revealed that many differentially expressed microRNAs in osteoarthritis, such as miR-27 and miR-558. Besides, miR-9 and miR-98 are highly expressed in osteoarthritis and involved in the inflammatory response by inducing the releases of MMP-13 and inflammatory cytokines[8]. MiR-195 plays important regulatory roles in thyroid cancer, non-small cell lung cancer, and osteosarcoma[30], but its role in osteoarthritis has not been reported. This study found that miR-195 was lowly expressed in osteoarthritis and negatively correlated with the expression of MMP-13. Further research showed that lncRNA-TUG1 could regulate extracellular matrix degradation in osteoarthritis through the interaction with miR-195.

Conclusions

We showed that lncRNA-TUG1 plays an important role in the regulation of gene expressions in osteoarthritis. The lncRNA-TUG1/miR-195/MMP-13 axis played an important role in regulating the degradation of chondrocyte extracellular matrix in osteoarthritis. An in-depth study on the molecular mechanism of lncRNA-TUG1 in the regulation of bone and joint is of great importance for comprehending the pathophysiology of osteoarthritis. Our results may provide a novel suggestion for the targeted therapy of osteoarthritis.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgements

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