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miR-195 contributes to human osteoarthritis via targeting PTHrP

Xiaoming Cao[1] · Zhiqing Duan[2] · Zheyi Yan[3] · Yongping Li[1] · Lu Li[1] · Jian Sun[1] · Pengfei Han[1] · Pengcui Li[1] · Lei Wei[1,4] · Xiaochun Wei[1]

Abstract

The dysregulated expression of the osteoarthritis (OA)-related genes in cartilage, such as matrix metalloproteinase 13 (MMP-13) and type X collagen (Col X), facilitates the onset and progression of OA. Reduced parathyroid hormone-related protein (PTHrP) may also accelerate this progression. Furthermore, miRNAs, endogenous regulators of mRNAs, are thought to play key roles in the pathogenesis of OA. In this study, we found that miR-195 levels were significantly upregulated in OA tissue, while PTHrP mRNA/protein expression was substantially downregulated, and there was a negative correlation between miR-195 and PTHrP. Upregulated miR-195 strongly inhibited Aggrecan, type II collagen (Col II) mRNA/protein expression, while it enhanced the expression of MMP-13 and Col X at mRNA/protein level; conversely, downregulated miR-195 significantly increased Col II mRNA/protein expression, while it decreased the expression of MMP-13 and Col X mRNA/protein. Moreover, our study demonstrated that PTHrP is a novel target of miR-195 using dual luciferase reporter assay. Finally, miR-195-mediated changes of Col II and OA-related genes were substantially attenuated by siRNA$_{PTHrP}$ treatment. These results suggested that miR-195 is involved in the pathogenesis of OA via PTHrP.

Keywords miR-195 · PTHrP · Type II collagen · MMP-13 · Type X collagen

Introduction

Osteoarthritis (OA) is featured by the destruction and loss of articular cartilage [1, 2]. Gradual degeneration of articular cartilage leads to joint pain and dysfunction [3]. The

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✉ Xiaochun Wei
sdeygksys@163.com

1 Department of Orthopedics, The Second Hospital of Shanxi Medical University, Shanxi Key Lab of Bone and Soft Tissue Injury Repair, 382 Wuyi Road, Taiyuan 030001, Shanxi, China

2 Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Shanxi Medical University, 56 South Xinjian Road, Taiyuan 030001, Shanxi, China

3 Department of Ophthalmology, The First Affiliated Hospital of Shanxi Medical University, Taiyuan 030001, Shanxi, China

4 Department of Orthopedics, Warren Alpert Medical School of Brown University/RIH, CORO West, Suite 402H, 1 Hoppin Street, Providence, RI 02903, USA

pathogenesis of OA is highly complex and multifaceted factors like aging, altered mechanical loading, genetic predisposition and epigenetic events are thought to be involved [4, 5]. A disrupted balance between anabolic and catabolic mechanisms maintaining the extracellular matrix (ECM) plays a key role in the pathogenesis of OA [6, 7]. The associated cellular metabolic changes are epitomized by downregulation of anabolic genes, including type II collagen(Col II), Aggrecan, and upregulation of catabolic genes such as matrix metalloproteinases (MMPs) and type X collagen(Col X) [8].

In the progression of OA, collagenase(s) produced by chondrocytes was involved in the cleavage and denaturation of Col II in articular cartilage, and matrix metalloproteinases 13 (MMP-13) may play a significant role in this process [9]. OA chondrocytes synthesized predominantly Col X, while normal chondrocytes synthesized mostly Col II [10]. Col X and MMP-13, standard markers for chondrocyte hypertrophy during development, are not present in healthy articular cartilage, and their concentrations are increased in OA cartilage [11, 12].

Recently, several studies have shown that epigenetic events and changes in gene expression may play a significant

role in OA progression [13]. One of the important epigenetic mechanisms is microRNA (miRNA) regulation, which plays important roles in many physiological and biochemical processes [14, 15]. miRNAs have been implicated in nearly all cellular functions, including lipid metabolism [16], apoptosis [17], differentiation [18], and malignant transformation [19].

According to a recent study by Borgonio et al., who measured expression levels among 380 miRNAs in the plasma of patients with primary knee OA, 12 miRNAs were identified as overexpressed in OA patients compared to expression levels in healthy controls, including miR-16, miR-20b, miR-19c, miR-30b, miR-93, miR-126, miR-146a, miR-184, miR-186, miR-195, miR-345 and miR-885-5p [20]. And we use Affymetrix miRNA 3.0 Gene chip screened and found upregulation of miR-195 in articular cartilage of OA rat models (ID: GLP16384). The function of miR-195 as a tumor suppressor and its possible mechanisms have been widely studied in osteosarcoma [21], lung cancer [22], cervical cancer [23], and breast cancer [24], which suggested that miR-195 could influence the proliferation and anabolic of cells. Nonetheless, the biological function of miR-195 and its underlying molecular mechanisms in OA remain to be fully elucidated.

In this study, we found that miR-195 level was significantly upregulated, while PTHrP level was markedly downregulated in human OA tissues. Moreover, the expression of articular cartilage anabolic genes, including Aggrecan and Col II, catabolic genes, including MMP-13 and Col X, in chondrocytes were profoundly influenced by miR-195. We also found that PTHrP mRNA was a direct target of miR-195. Notably, the miR-195-induced changes of Col II, MMP-13, and Col X at protein levels were reversed with siRNA$_{PTHrP}$ treatment. These findings suggest that miR-195 act as a novel regulator of cartilage homeostasis by suppressing PTHrP expression.

Materials and methods

Patients

The cartilage samples were obtained from OA patients who underwent a total knee replacement surgery ($n = 10$; six women and four men; age range 54–77 years old). OA was diagnosed according to the American College of Rheumatology's criteria. Matched healthy cartilage samples were obtained from traumatic amputees with no known history of OA or rheumatoid arthritis ($n = 10$; seven men and three women; age range 40–59 years old). Informed consent from each patient was obtained, and the protocol of this study was approved by the Ethics Committee of The Second Hospital of ShanXi Medical University (Shanxi, China).

Histology

Articular cartilages dissected from the samples of OA patients and traumatic amputees with no history of OA were fixed in 10% formalin for 3 days and decalcified in ethylenediaminetetraacetic acid (EDTA) solution until the tissues were rubbery soft. Next, the processed tissues were embedded in Paraplast X-tra (Thermo Fisher Scientific, Hampton, NH, USA), and the serial sections of 6 µm thickness were obtained with a rotary microtome (Leica RM2125, Leica Microsystems Ltd, Shanghai, China). Safranin-O staining was performed according to standard procedure, and the damage of articular cartilage was assessed and scored using the modified Mankin grading system [25].

Cell culture and transfection

The cartilage slices dissected from the samples of traumatic amputees with no history of OA were washed in Dulbecco's modified eagle medium (DMEM, Invitrogen, Carlsbad, CA), minced and digested with pronase (2 mg/ml) (Roche, Basel, Switzerland) in Hank's balance salt solution (HBSS, Invitrogen, Carlsbad, CA) for 30 min at 37 °C with shaking, and then were washed with DMEM, and further digested with bacterial collagenase (Type IA, 1 mg/ml) (Sigma-Aldrich, St. Louis, MO) for 6–8 h at 37 °C with shaking. Digestion was stopped by adding DMEM containing 10% FBS (Invitrogen, Carlsbad, CA). The cells were then washed three times with DMEM, and the multicellular aggregates were removed by filtering. The single cells were incubated in DMEM containing 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin) (Sigma-Aldrich, St Louis, MO). Human embryonic kidney (HEK) 293T cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). All cells used in this study were maintained in a humified incubator at 5% CO_2 at 37 °C. Chondrocytes or 293T cells were transfected with 100 nM of miR-195 mimic (designed and synthesized by GenePharma Co.), 100 nM of antisense inhibitor (miR-195 inhibitor, designed and synthesized by GenePharma Co.), or 100 nM scrambled 22-mer oligonucleotides (control, with no homology to mammalian genome). Transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions (transfection efficiency > 75%). 24 or 48 h after the transfection, the cells were harvested for RNA isolation, Western blotting, and an immunofluorescence assay.

RNA extraction and real-time polymerase chain reaction (real-time PCR)

Total RNA was isolated from the human knee joint cartilage or chondrocytes cultured in vitro using the RNeasy isolation kit (Qiagen, Hilden, Germany) [26]. Total RNA (1 μg) was reverse transcribed to complementary DNA (cDNA) using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The cDNA (40 ng) was used to quantify the expression of the target genes by real-time PCR, using the Quanti Tect SYBR Green PCR kit (Qiagen, Hilden, Germany) with the DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Toronto, Canada). GAPDH and U6 were used as internal controls for mRNA and miRNA, respectively. The stem-loop primers for miR-195 were designed and purchased from GenePharma. (Shanghai GenePharma Co., Ltd., Shanghai, China). PTHrP primers were as follows: forward, 5′-CAA CCA GCC CAC CAG AGGA-3′, and reverse, 5′-GGC GGC TGA GAC CCT CCA-3′. SOX9 primers were as follows: forward, 5′-TTC CTC CTC CCG GCA TGA GTG-3′, and reverse, 5′-CAA CTT TGC CAG CTT GCA CG-3′. Col II primers were as follows: forward, 5′-TGA GGG CGC GGT AGA GAC CC-3′, and reverse, 5′-TGC ACA CAG CTG CCA GCC TC-3′. Aggrecan primers were as follows: forward, 5′-CAT TCA CCA GTG AGG ACC TCG T-3′, and reverse, 5′-TCA CAC TGC TCA TAG CCT GCT TC-3′. Runx2 primers were as follows: forward, 5′-GGC AGG CAC AGT CTT CCC-3′, and reverse, 5′-GGC CCA GTT CTG AAG CAC C-3′. Col X primers were as follows: forward, 5′-GCA CGC AGA ATC CAT CTG AGA ATA-3′, and reverse, 5′-GAC CAG GAG TAC CTT GCT CTC-3′. MMP-13 primers were as follows: forward, 5′-TGC TGCATT CTC CTT CAG GA-3′, and reverse, 5′-ATG CAT CCA GGG GTC CTGGC-3′. GAPDH primers were as follows: forward, 5′-TTG TCA AGC TCA TTT CCT GGT AT-3′, and reverse, 5′-TCT CTC TTC CTC TTG TGC TCT TG-3′. FGF-18 primers were as follows: forward: GAT GGG GAC AAG TAT GCC CAG, reverse: GCC TTT GCG GTT CAT GCA C; All experiments were carried out in triplicate.

Western blotting

Total proteins were obtained from human samples and chondrocytes cultured in vitro using radioimmunoprecipitation (RIPA) lysis buffer (Beyotime, Beijing, China). The proteins were separated on a 10% SDS-PAGE and then transferred to a nitrocellulose (NC) membrane (Solarbio, Shanghai, China). Non-specific binding was blocked with 5% nonfat milk in TBST (Tris-buffered saline plus 0.1% Tween 20), and then the membranes were incubated with the primary antibodies (Abcam, Cambridge, United Kingdom) against PTHrP (1:1000, ab125700), Col II (1:1000, ab34712), MMP-13 (1:1000, ab39012), and Col X (1:1000, ab58632)

overnight at 4 °C. β-actin (1:1000, ab8227, Abcam, Cambridge, United Kingdom) was used as the loading control. After washing in TBST, the immobilized primary antibodies were detected using a horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit IgG antibody (1:2000, ab205718, Abcam, Cambridge, United Kingdom) and visualized using the ECL Chemiluminescence kit (Thermo, Waltham, USA. Finally, the blots were analyzed quantitatively using BIO-RAD Image lab software. All experiments were performed in triplicates.
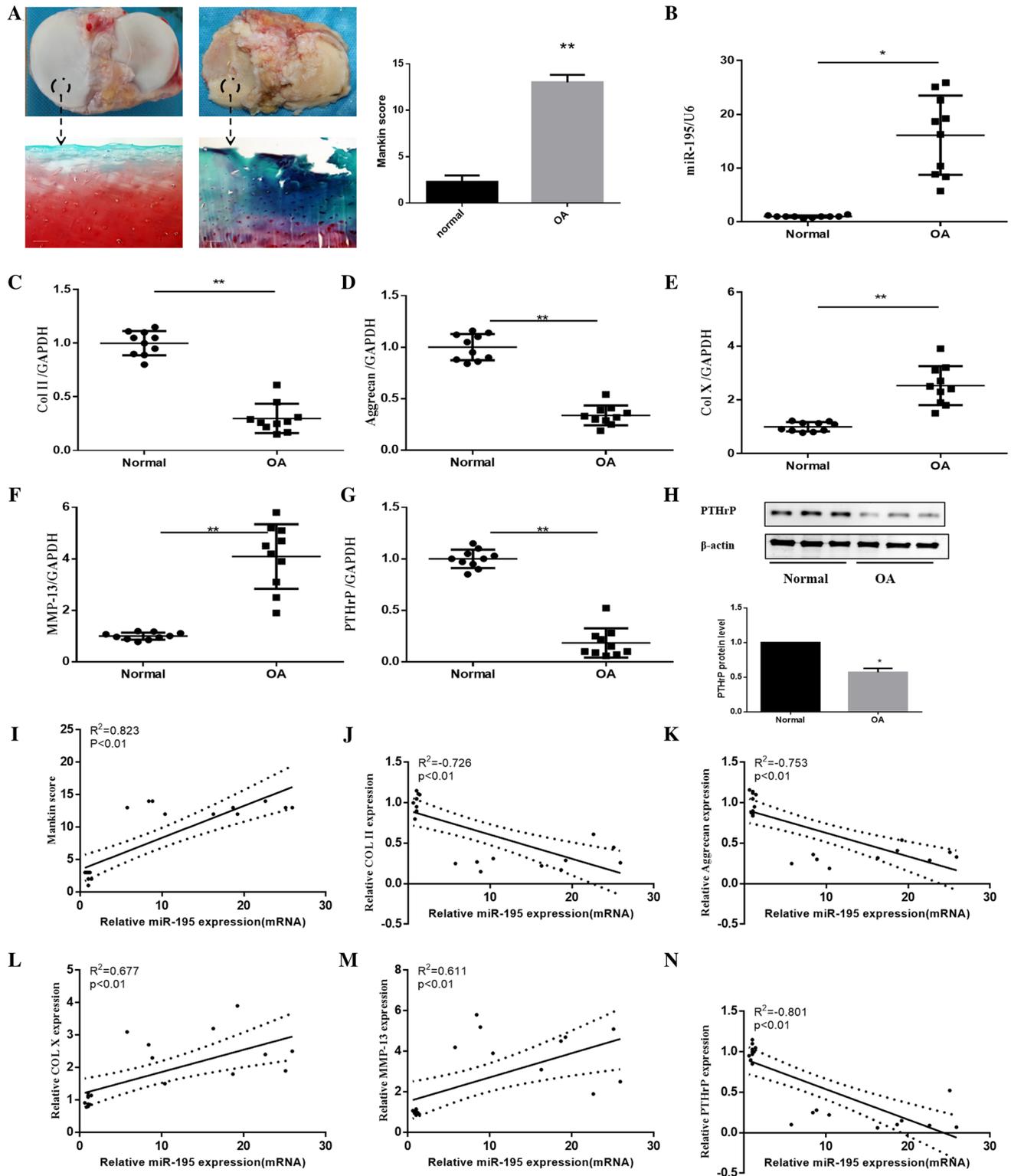
Immunofluorescence assay

Chondrocytes cultured in vitro were rinsed in phosphate-buffered saline (PBS) three times and fixed with 4% formaldehyde in PBS for 15 min at room temperature. Next, goat serum was used to block non-specific binding sites and the cells were then incubated with the Col II antibody (1:200, ab34712, Abcam, Cambridge, United Kingdom) in PBS for 8 h at 4 °C. After three washes with PBS, each for 3 min at room temperature, the cells were incubated for 1 h with a Goat Anti-Rabbit IgG H&L (FITC) (1:1000, ab6717, Abcam, Cambridge, United Kingdom) in PBS. After another three washes, the samples were incubated with 4′,6-diamidino-2-phenylindole (DAPI; Beyotime, Beijing, China) for 5 min. After the final round of three washes, the samples were mounted on slides and examined under a confocal microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA)

The supernatants were collected from the cultured chondrocytes. The human parathyroid hormone-related protein (PTHrP) kit was obtained from Westang Co, China. The kit was used to measure the secreted PTHrP levels according to the manufacturer's instructions. All samples were run in duplicates, and the average value was calculated. The experiment was repeated three times.

Luciferase reporter assay

For construction of the wild-type PTHrP 3′UTR-Luc reporter plasmid, a fragment of 3′UTR of the PTHrP gene, including the predicted miR-195-binding sites through bioinformatics software (miRanda [http://www.microrna.org], TargetScan [http://www.targetscan.org] and PicTar [http://pictar.mdc-berlin.de/]), was amplified using the following primer set: forward 5′-GUAAAUGUA UCUUGGUGCUGCUG-3′ and reverse 5′-UAGCAGCAC AGAAAUAUUGGC-3′, then cloned into the pmiR-RB-Report vector (Ribo Bio Co., Guangzhou, China) and named as wild-type-PTHrP-3′UTR-Luc reporter plasmid. Site-directed mutagenesis was used to construct

◄**Fig. 1** Expressions of miR-195 and PTHrP in healthy and OA cartilage. **a** Tibial plateaus (upper panel) and Safranin/fast green staining (lower panel) of the cartilage of traumatic amputees and OA patients. Scale bar indicates 400 μm. Modified Mankin scores of control and OA tissues. **b** The expression of miR-195 in OA and normal cartilages ($n = 10$ donors each) was analyzed by real-time PCR. **c–g** The levels of Col II, Aggrecan, Col X, MMP-13 and PTHrP mRNA in tissues from OA and traumatic amputees were analyzed by real-time PCR. **h** The level of the PTHrP protein in tissues from OA patients and from traumatic amputees was analyzed by Western blotting. The relative protein expressions were normalized to β-actin expression. **i** A significant positive correlation was revealed by a two-tailed Pearson's correlation analysis between the expression of miR-195 and Mankin scores. $R^2 = 0.823$. **j** A significant negative correlation between miR-195 and Col II. $R^2 = -0.726$. **k** A significant negative correlation between miR-195 and Aggrecan. $R^2 = -0.753$. **l** A significant positive correlation between miR-195 and Col X. $R^2 = 0.677$. **m** A significant positive correlation between miR-195 and MMP-13. $R^2 = 0.611$. **n** A significant negative correlation between miR-195 and PTHrP, $R^2 = -0.801$. $*P < 0.05$, $**P < 0.01$

Statistics

The results were expressed as the mean ± standard error (SEM). The statistic differences between groups were analyzed by the Student's *t* test when only two groups were compared or by ANOVA when more than two groups were compared with the SPSS software, version 13.0 (SPSS Inc., Chicago, IL). $P < 0.05$ was considered as statistically significant.

Results

Expression of miR-195 and PTHrP in OA and control cartilages

We collected cartilage specimens from the tibial plateaus obtained from OA patients and traumatic amputees (as normal group). Safranin-O staining showed the typical osteoarthritic changes in OA patients, but not in the healthy tissue. Significant differences in the modified Mankin scores were found between the OA and normal groups ($P < 0.01$; Fig. 1a). Real-time PCR data in Fig. 1b demonstrated miR-195 was sharply upregulated ($P < 0.05$) in OA cartilages. Interestingly, OA cartilages also exhibited lower levels of Col II and Aggrecan mRNA (Fig. 1c, d). On the contrary, OA cartilages also exhibited higher levels of Col X and MMP-13 mRNA (Fig. 1e, f). Besides, OA cartilages also exhibited lower levels of PTHrP mRNA (Fig. 1g) and protein (Fig. 1h). Meanwhile, the relationship between miR-195 and Mankin scores, Col II, Aggrecan, Col X, MMP-13 and PTHrP expression was analyzed by a two-tailed Pearson's correlation analysis, as revealed in Fig. 1i–n; a positive correlation between the expression of miR-195 and Mankin scores, Col X and MMP-13 was identified ($P < 0.01$), while

a negative correlation between the expression of miR-195 and Col II, Aggrecan and PTHrP was identified ($P < 0.01$).

miR-195 regulates cartilage-specific genes in human chondrocytes in vitro

Human chondrocytes cells isolated from traumatic amputees were transfected with the miR-195 mimic, the miR-195 inhibitor, or the control vector, respectively. As expected, a significant induction of miR-195 was detected in cells transfected with the miR-195 mimic ($P < 0.01$; Fig. 2a), and a significant suppression of miR-195 was observed in cells transfected with the miR-195 inhibitor, compared to control cells ($P < 0.01$; Fig. 2b).

The expression of the anabolic genes, including SOX9, Aggrecan and Col II, catabolic genes, including Runx2, MMP-13 and Col X, were quantified using real-time PCR after transfection with 24 h. The data demonstrated that the expressions of Aggrecan and Col II were significantly decreased by miR-195 mimic, while miR-195 inhibitor upregulated the expressions of SOX9 and Col II. In contrast, miR-195 mimic significantly upregulated the levels of MMP-13, Col X and Runx2, while it decreased the expressions of MMP-13 and Col X (Fig. 2c). The expressions of the Col II, MMP-13 and Col X were quantified using Western blot analysis after transfection with 48 h. Our results showed that the level of Col II was significantly decreased by miR-195 mimic, while miR-195 inhibitor upregulated the level of Col II. In contrast, miR-195 mimic significantly upregulated the levels of MMP-13 and Col X, while it decreased the expressions of MMP-13 and Col X (Fig. 2d). To further assess the changes in extracellular matrix of the chondrocyte, we estimated the amounts of secreted Col II by immunofluorescence staining. We used the ImageJ to perform semi-quantitative analysis of fluorescence intensity. Similar to the above-mentioned mRNA and protein changes, overexpression of miR-195 decreased Col II level. However, miR-195 inhibitor could increase the level of Col II (Fig. 2e). Collectively, these data suggested that miR-195 inhibited the anabolic genes and upregulated the expression of OA-related genes in human chondrocytes.

miR-195 regulates PTHrP expression in human chondrocytes and PTHrP is a novel direct target of miR-195

We transfected the human chondrocytes cells with miR-195 mimics, miR-195 inhibitor, or controls, respectively. As expected, we found that overexpression of miR-195 strongly inhibited PTHrP, whereas miR-195 inhibitor could significantly enhance PTHrP mRNA expression in human chondrocytes, based on real-time PCR at 24 h post-treatment ($P < 0.01$; Fig. 3a). In addition, Western blot analysis at
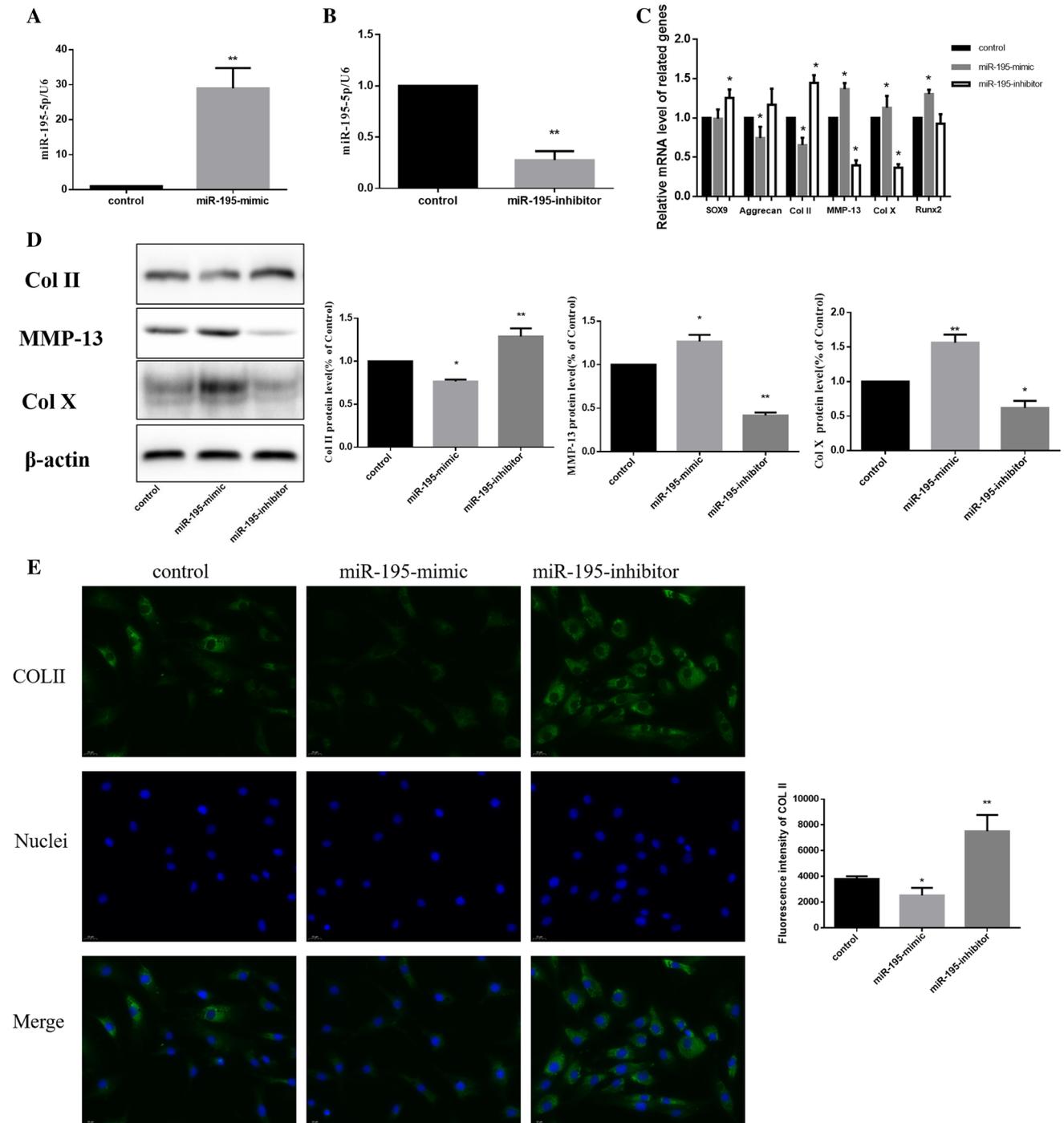
Fig. 2 The expression of cartilage-specific genes in response to treatment of miR-195 mimic or inhibitor. Human chondrocytes cells were transfected with the miR-195 mimic (**a**) and miR-195 inhibitors (**b**). miR-Scrambled 22-mer oligonucleotides was used as a control (*n* = 3), Relative expression levels of miR-195 were analyzed by real-time RT-PCR. **c** The gene expressions of SOX9, Aggrecan, Col II, Runx2, MMP-13 and Col II were analyzed by real-time PCR 24 h after miRNA transfection. **d** The levels of Col II, MMP-13 and Col II were analyzed by Western blotting 48 h after miRNA transfection. The relative protein expressions were normalized to β-actin expression. **e** Col II expression of transfected chondrocytes was assessed using immunofluorescence staining. We used the ImageJ to perform semi-quantitative analysis of fluorescence intensity. Original magnification: ×40. Scale bars are 20 μm. *P < 0.05, **P < 0.01 compared to control group

Fig. 3 miR-195 regulates PTHrP expression in human chondrocytes and PTHrP is a novel direct target of miR-195. Human chondrocytes cells were transfected with the miR-195 mimic, miR-195 inhibitor or control ($n=3$). **a**, **b** PTHrP expression was analyzed by real-time PCR (24 h) and Western blotting (48 h) after miRNA transfection. **c** The concentrations of PTHrP in medium were determined by ELISA 48 h after miRNA transfection. **d** Target Scan 6.2 predicted the target sequence in 3′UTR of human PTHrP mRNA. **e** Cloned target fragments in pmirGV272-PTHrP-WT and pmirGV272-PTHrP-Mut were confirmed by sequencing. Underlined sequence represents mutant sites. **f** Luciferase activity of the PTHrP 3′UTR reporter was analyzed in 293T cells. miR-195 was cotransfected with the wild-type PTHrP 3′UTR or mutant vector. A scrambled oligonucleotide served as a negative control; $n=3$. WT denotes a wild-type PTHrP 3′UTR reporter. Mut refers to mutant-PTHrP-3′UTR reporter. *$P<0.05$, **$P<0.01$ compared to control group
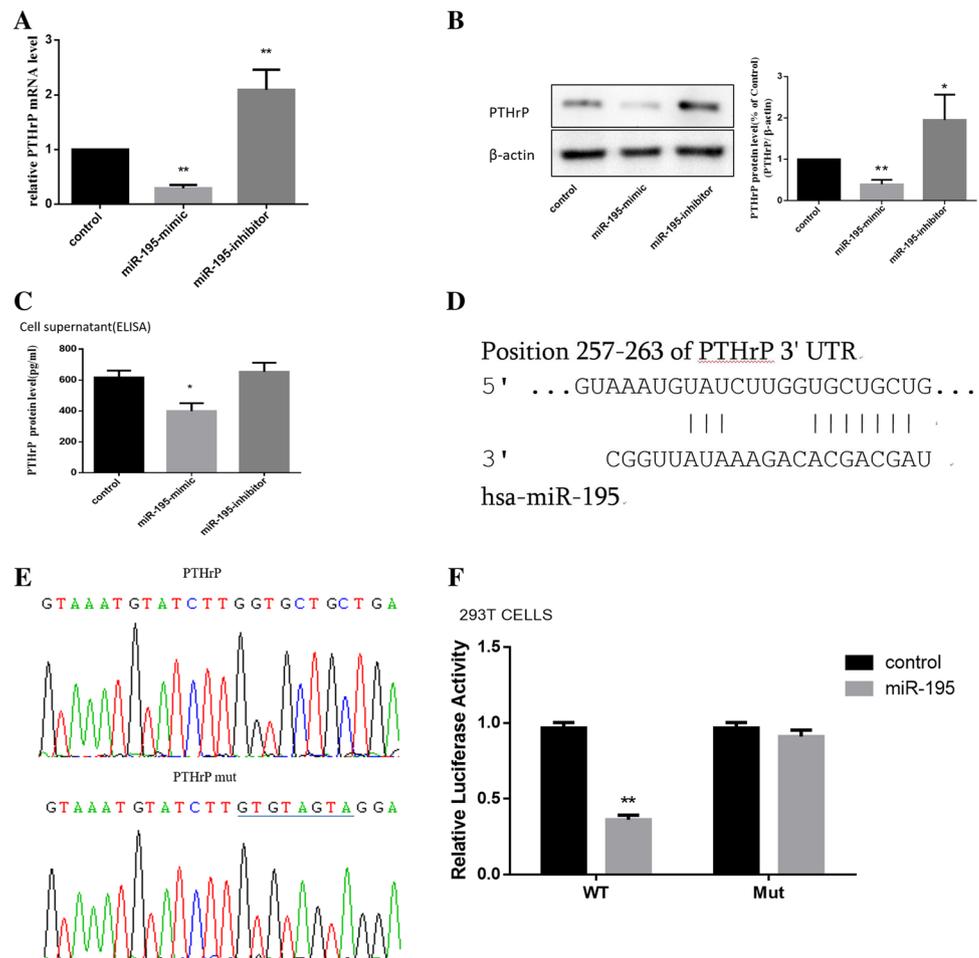
48 h post-treatment demonstrated the same trend ($P<0.05$; Fig. 3b). Furthermore, the PTHrP content in the supernatant was significantly reduced in the miR-195 mimic group after 48 h treatment ($P<0.05$; Fig. 3c).

Target prediction algorithms, including miRanda (http://www.microrna.org), TargetScan (http://www.targetscan.org), and PicTar (http://pictar.mdc-berlin.de/) were used to evaluate the potential relationship between PTHrP and miR-195. We found that 3′UTR of human PTHrP mRNA contained a putative miR-195 binding site (Fig. 3d). This suggested that PTHrP might be a potential target for miR-195. To confirm that miR-195 regulates PTHrP mRNA expression by directly binding to the predicted target sequence, 260 bp (257–263 bp) of 3′UTR of PTHrP mRNA was inserted downstream of the luciferase open reading frame. The recombinant plasmids, pmirGV272-PTHrP wild-type (WT) and pmirGV272-PTHrP-Mutant (Mut), were obtained and confirmed by sequencing (Fig. 3e). 293T cells were used for the co-transfection of sense oligonucleotides of miR-195 and plasmids-pmirGV272-PTHrP-WT/pmirGV272-PTHrP-Mut, respectively. The results demonstrated that luciferase activity was much lower after miR-195 transfection than

after transfection with a scrambled oligonucleotide (as control) (Fig. 3f). Compared with control group, no significant differences of luciferase activity were detected in pmirGV272-PTHrP Mut (Fig. 3f). These results proved that modifications in the target site of PTHrP 3′UTR could block the function of miR-195, suggesting that PTHrP is a direct novel target of miR-195.

Regulating cartilage-specific genes by miR-195 via targeting PTHrP

We showed that miR-195 can repress the expression of PTHrP. To further understand whether PTHrP mediates the downstream effects of miR-195 in human chondrocytes, human chondrocytes were transfected with siRNA against PTHrP (siPTHrP), the miR-195 inhibitor with and without siPTHrP. siPTHrP significantly suppressed the protein expression of PTHrP. As expected, the upregulation of PTHrP by the miR-195 inhibitor was suppressed by the co-transfection with siPTHrP (Fig. 4a). In addition, the data showed that siPTHrP could significantly suppress the expression of COL II, while upregulate the levels of
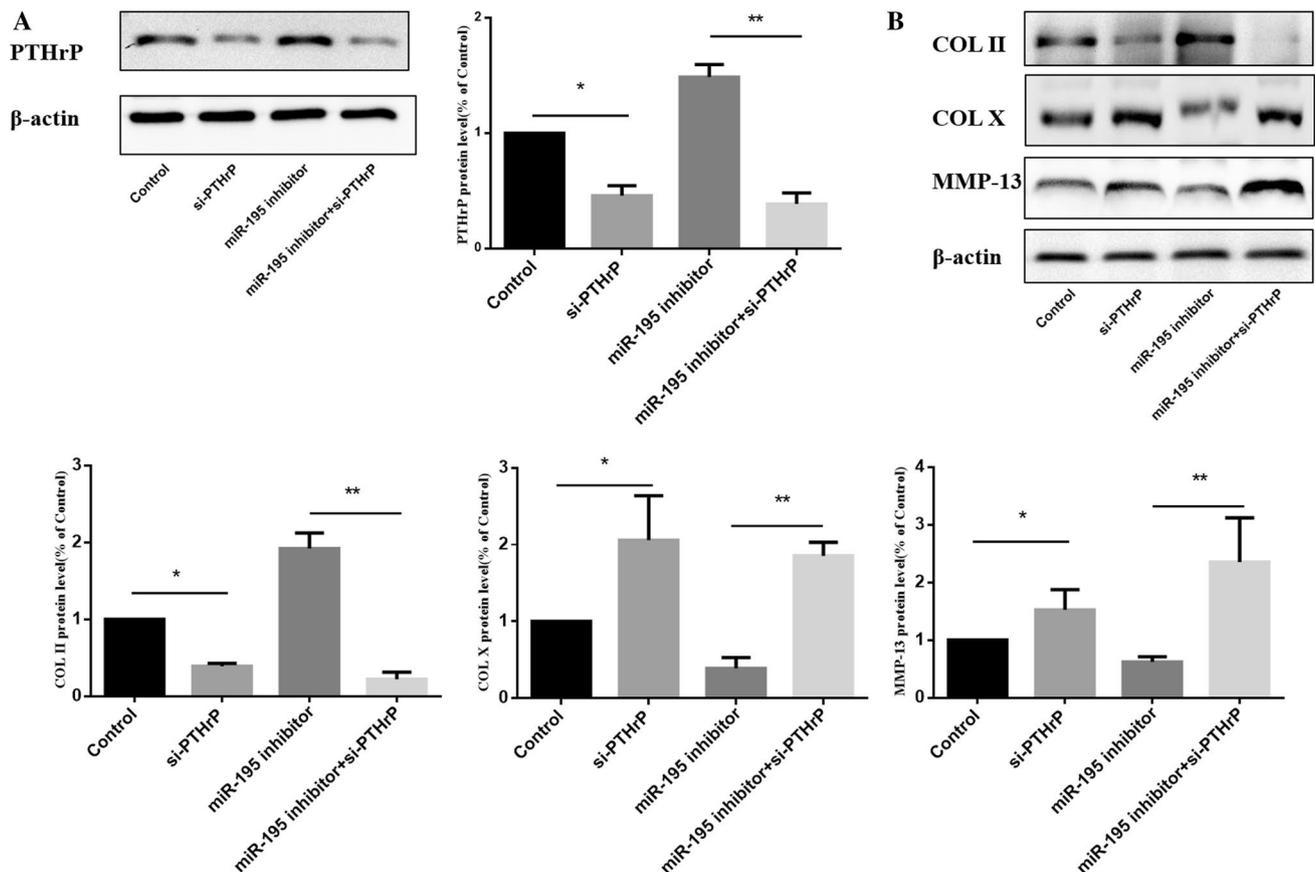
Fig. 4 The effects of miR-195 on cartilage-specific genes with and without PTHrP are evaluated. Human chondrocytes were transfected with a scrambled oligonucleotide miR (control), siRNA against PTHrP (siPTHrP), miR-195 inhibitor, and miR-195 inhibitor with siPTHrP. **a** PTHrP expression was analyzed by Western blotting 48 h after the transfection. Densitometric analysis of PTHrP expressions were normalized to β-actin levels. **b** Expressions of Col II, Col X and MMP-13 were assessed by Western blotting. The lower panel shows densitometric analysis of Col II, Col X and MMP-13 expression, normalized to β-actin levels. Figures are representative images of at least three different samples, *$P < 0.05$, **$P < 0.01$

Col X and MMP-13. Moreover, an upregulated expression of COL II and downregulated expression of COL X and MMP-13 were observed after transfection with the miR-195 inhibitor alone. More importantly, the co-transfection with siPTHrP treatment reversed the changes of Col II, Col X, and MMP-13 caused by the miR-195 inhibitor (Fig. 4b).

Moreover, it has been confirmed that FGF-18, a protective molecule for articular cartilage, is also a target of miR-195 [27]. Therefore, we studied the effects of miR-195 and siPTHrP on FGF-18 expression level. Based on real-time PCR at 24 h post-treatment, we found that overexpression of miR-195 strongly inhibited FGF-18, whereas miR-195 inhibitor or inhibiting miR-195 with siPTHrP could significantly enhance FGF-18 mRNA expression in human chondrocytes, However, we found that FGF-18 expression was not attenuated by siPTHrP (Supplementary Figure 1), which indicated that FGF-18 expression was not positively related to the siPTHrP concentration.

These results suggested that PTHrP plays a key role in the downstream effects of miR-195 on the cartilage-specific genes to some degree

Discussion

OA is an age-related disease, and its incidence increases with age [28, 29]. Significant changes in extracellular matrix were observed after OA, such as downregulation of Col II, Aggrecan, and upregulation of MMPs and Col X [8]. In addition, previous study also found that epigenetic regulation is also related to the development of OA [20]. Currently, the available treatments are limited to pain management for most OA patients due to limited understanding of the molecular mechanisms of OA. Therefore, study of the mechanisms of OA is urgently needed.

miRNA plays important roles in the processes of OA [13]. Our lab has previously screened 16 age-related miRNAs (mi-195, -497, -16, -15b, etc.), and their levels increased with age in rat models. However, the effects of miR-195 on OA are unclear in humans due to potential species differences. In this study, we found that miR-195 was notably upregulated in OA cartilage compared to control cartilage, which was consistent with the results that miR-195 is higher in the plasma of OA patients [20]. We used human chondrocytes to study the relationship between miR-195 and OA, based on the assumption that cartilage degeneration in OA may be similar to the cartilage degradation in the growth plate, in which Col X and MMPs are essential [30]. In fact, it was previously reported that MMP-13 plays a key function in the pathogenesis of OA [31]. Our data showed that MMP-13 and Col X were significantly increased by the miR-195 mimic or decreased by the miR-195 inhibitor.

Meanwhile, we also found that overexpression of miR-195 could downregulate Col II, and inhibition of miR-195 could reverse the effect; therefore, it is reasonable to argue that miR-195 has a pro-OA capacity. The question is: how does this promotion take place?

PTHrP is known to maintain the function of proliferating chondrocytes and inhibits chondrocyte differentiation toward hypertrophy in the growth plate [32]. In this regard, it is interesting to note that it has been reported that the transition from the proliferative to hypertrophic phase may be accelerated in PTHrP null mice [33, 34], and these data seem to suggest that downregulation of PTHrP contributes to an OA phenotype in the OA experimental models [35], while overexpression of PTHrP enhances the growth and differentiation of chondrocyte during chondrogenesis [36]. Chang et al. indicated that PTH(1-34) inhibits the terminal differentiation of human articular chondrocytes in vitro and inhibits progression of OA in rats in vivo, and may be used to treat OA [37], and Kozhemyakina et al. found that PTHrP represses chondrocyte hypertrophy through a protein phosphatase 2A/histone deacetylase 4/MEF2 pathway [38]. PTHrP could increase COL2 expression, which could potentially be used to prevent undesirable hypertrophic chondrocyte differentiation during cartilage repair or regeneration [39]. Indeed, PTHrP was closely associated with OA in our study. We found that PTHrP was significantly decreased in human OA tissue, which was consistent with the idea that a reduction of PTHrP leads to OA phenotype [35], which is also supported by the report indicating that PTHrP is a protective factor in OA [40].

Abnormal expression of miR-195 has been widely reported in diverse biological systems [41, 42]. miR-195 has been widely characterized as a tumor suppressor gene in various cancers, including breast cancer, hepatocellular carcinoma and osteosarcoma [43–45]. Gu et al. reported that miR-195 could inhibit the chondrocytes' proliferation by targeting G-protein coupled receptor kinase-interacting protein-1 (GIT1) [46]. Bai et al. also found that miR-195 could target hypoxia-inducible factor 1 α (HIF-1α) and promote apoptosis in hypoxic chondrocytes [47]. In fact, some miRNA may target hundreds of different mRNAs [48].

Could there be other targets that mediate the effects of miR-195 in OA? It is well-known that miRNAs could regulate their downstream genes via binding to the 3′UTR of the targeted genes [49]. Through an online bioinformatics analysis and the dual luciferase assay, we verified that miR-195 could target and bind to a PTHrP 3′UTR and regulate PTHrP mRNA and protein expression. We found that PTHrP interacts directly with miR-195. These results indicate that PTHrP is a potential new target of miR-195 in vitro. Moreover, miR-195 does indeed downregulate the expression of PTHrP at both mRNA and protein levels in chondrocytes, strongly suggesting that PTHrP may be involved in the process of OA formation and progression.

Subsequent studies with Western blotting confirmed our findings, i.e., miR-195 inhibitor could upregulate PTHrP, promote the expression of Col II, and inhibit hypertrophy-associated genes (Col X, MMP-13). By dual inhibition of miR-195 and PTHrP (siRNA$_{PTHrP}$), the downregulated effect of miR-195 can be reversed, confirming that PTHrP is downstream of miR-195 action. These results further indicated that PTHrP is involved in miR-195 induced OA.

However, it is worthy to mention that most miRNAs have multiple target genes. For example, it has been confirmed that FGF-18 is also a target of miR-195 [27]. Thus, we can not ignore the effect of FGF-18 or exclude the possibility that other targets of miR-195 might be involved in regulating the key molecules of OA. However, we found that FGF-18 expression was not attenuated by siRNA$_{PTHrP}$, which indicated that FGF-18 expression was not positively related to the PTHrP concentration. Indeed, it is difficult to evaluate the specific relationship of FGF18 and PTHrP, due to the complex feedback regulation. In the present study, our data could only suggest that PTHrP is involved in the regulation of key molecules of OA induced by miR-195.

In conclusion, our study identifies that miR-195 is upregulated in OA, and PTHrP is a novel target of miR-195. Furthermore, PTHrP is involved in the progress of miR-195 which mediated the form of OA. Therefore, the downregulation of miR-195 or prevention of its upregulation could be a novel approach for the potential treatment of OA.

Compliance with ethical standards

Conflict of interest All authors have no conflicts of interest.

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