MicroRNA-486 promotes a more catabolic phenotype in chondrocyte-like cells by targeting SIRT6

POSSIBLE INVOLVEMENT IN CARTILAGE DEGRADATION IN OSTEOARTHRITIS

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Aims
Osteoarthritis (OA) is characterized by persistent destruction of articular cartilage. It has been found that microRNAs (miRNAs) are closely related to the occurrence and development of OA. The purpose of the present study was to investigate the mechanism of miR-486 in the development and progression of OA.

Methods
The expression levels of miR-486 in cartilage were determined by quantitative real-time polymerase chain reaction (qRT-PCR). The expression of collagen, type II, alpha 1 (COL2A1), aggrecan (ACAN), matrix metalloproteinase (MMP)-13, and a disintegrin and metalloproteinase with thrombospondin motifs-4 (ADAMTS4) in SW1353 cells at both messenger RNA (mRNA) and protein levels was determined by qRT-PCR, western blot, and enzyme-linked immunosorbent assay (ELISA). Double luciferase reporter gene assay, qRT-PCR, and western blot assay were used to determine whether silencing information regulator 6 (SIRT6) was involved in miR-486 induction of chondrocyte-like cells to a more catabolic phenotype.

Results
Compared with osteonecrosis, the expression of miR-486 was significantly upregulated in cartilage from subjects with severe OA. In addition, overexpressed miR-486 promoted a catabolic phenotype in SW1353 cells by upregulating the expressions of ADAMTS4 and MMP-13 and down-regulating the expressions of COL2A1 and ACAN. Conversely, inhibition of miR-486 had the opposite effect. Furthermore, overexpression of miR-486 significantly inhibited the expression of SIRT6, confirming that SIRT6 is a direct target of miR-486. Moreover, SW1353 cells were transfected with small interfering RNA (si)-SIRT6 and it was found that SIRT6 was involved in and inhibited miR-486-induced changes to SW1353 gene expression.

Conclusion
Our results indicate that miR-486 promotes a catabolic phenotype in SW1353 cells in OA by targeting SIRT6. Our findings might provide a potential therapeutic target and theoretical basis for OA.

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Keywords: Osteoarthritis, miR-486, SIRT6, Chondrocyte catabolic phenotype, SW1353 cell line

Introduction
Osteoarthritis (OA) is a chronic degenerative joint disease that mostly occurs in the middle-aged and elderly population. The main characteristics of OA include progressive wear and tear of articular cartilage, degeneration, joint edge and subchondral bone reactive hyperplasia, joint synovitis, and osteophyte formation. Studies have found that obesity, excessive exercise, ageing, heredity, and trauma are the main pathogenic factors of OA. Articular cartilage is a...
highly differentiated layer of connective tissue that covers the surface of the joint, and acts as a buffer and shock absorber during joint movement.6,7 The main components of articular cartilage include chondrocytes and extracellular matrix (ECM), in which ECM is the extracellular product secreted by cells in living tissues and organs, and the main components are collagen, laminin, elastin, and proteoglycan.8,9 Chondrocytes are the only cellular components of articular cartilage, and their ageing and apoptosis are directly involved in physiological processes such as maintenance and reconstruction of articular cartilage.10,11 Recent studies have found that the main pathogenesis of OA is the apoptosis of articular chondrocytes and the metabolism of ECM caused by inflammatory injury, oxidative injury, immune dysfunction, and other mechanisms.12-14 Therefore, the pathogenesis of OA is a complex pathological process.15,16

In normal cartilage, chondrocytes synthesize a large amount of collagen, type II, alpha 1 (COL2A1) and proteoglycan and secrete them into the ECM to form the extracellular fibrous skeleton, which provides support and protection for the cells.17 Meanwhile, chondrocytes are the main source of cartilage matrix catabolic enzymes. Under normal physiological conditions, a dynamic balance is maintained between the synthesis and metabolism of cartilage components.18 During the pathogenesis of OA, the expression of COL2A1 in chondrocytes is suppressed, and the synthesis and secretion of matrix metalloproteinase (MMP) are increased.19 MMP is a proteolytic enzyme containing zinc, which can degrade COL2A1 in cartilage matrix. MMP-13 has a stronger hydrolysis effect on COL2A1 than other collagenases.20 Recent studies have found that ACANase 4 (ADAMTS4) can significantly degrade the proteoglycan in the ECM of cartilage, and the expression of ADAMTS4 in cartilage from OA patients is significantly upregulated.21 Therefore, this study aimed to investigate the mechanism of metabolism of chondrocyte ECM in OA.

MicroRNAs (miRNAs) are a class of endogenous non-coding single-stranded RNA composed of 20 to 28 nucleotides,22 which can degrade target gene messenger RNA (mRNA) and regulate the expression of target genes at post-transcriptional level.23,24 Micro R-486 is involved in the occurrence and development of various tumours.25-28 Micro R-486 was found to be highly expressed in renal cell carcinoma tissues and pancreatic duct carcinoma, but downregulated in breast cancer, colorectal cancer, non-small cell lung cancer, liver cancer, gastric cancer, prostate cancer, and other malignant tumours.29-31 One study reported that miR-486-5p was highly expressed in OA and inhibited the proliferation and migration of chondrocytes by blocking SMAD2.32 Silencing information regulator 6 (SIRT6) is a nicotinamide adenine dinucleotide (NAD+)-dependent histidine deacetylase that regulates the secretion of inflammatory factors and is involved in the regulation of inflammatory reactions, tumours, anti-ageing, metabolism, and other related diseases.33 SIRT6 was also found to play important regulatory roles in the interactions among OA, ageing, and metabolic syndrome.34 However, there are relatively few studies on the mechanism of SIRT6 and miR-486 in OA.

In this study, the expression levels of miR-486 in cartilage from patients with severe OA were determined by quantitative real-time polymerase chain reaction (qRT-PCR). Meanwhile, the expression of COL2A1, MMP-13, aggrecan (ACAN), and ADAMTS4 in SW1353 cells was determined by qRT-PCR, western blot, and enzyme-linked immunosorbent assay (ELISA). In addition, double luciferase reporter gene assay, qRT-PCR, and western blot assay were also used to explore whether SIRT6 was involved in miR-486-induced SW1353 cell gene expression. Our finding will provide the theoretical basis and search for possible targets for the treatment of OA.

Methods

Materials. In this study, healthy human articular cartilage from both femoral condyles and tibial plateaus were obtained from victims of road traffic accidents during surgery who had no known history of OA or rheumatoid arthritis (13 males and seven females, mean age 69 years (standard deviation (SD) 5)). Articular cartilage tissues from 20 OA patients undergoing total knee arthroplasty surgery (16 males and four females, mean age 64 years (SD) 6) were collected and used as the experimental group. Since OA is characterized by cartilage degeneration and/or osteophyte formation, necrotic bone tissues that cannot be observed with cartilage degeneration or osteophyte formation were used as negative control samples. All sample tissues were stored at -80°C. All studies were approved by the institutional ethics review committee of Honghui Hospital. All participants provided written informed consent.

Cells. Human osteochondroma cell line SW1353,35,36 originally obtained from a primary grade II osteosarcoma of the right humerus of a 72-year-old white Caucasian woman,34,35 was obtained from Honghui Hospital. The SW1353 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Hyclone, USA) medium containing 10% fetal bovine serum (FBS) at 37°C for 24 hours. Cells were then seeded into 96-well plates (9 × 10³ cells/well).

Cell transfection. SW1353 cells were inoculated into a six-well plate at 2.5 × 10⁴ cells per well. After 24 hours of incubation, cells were transfected for six hours in serum- and antibiotic-free DMEM using Lipofectamine 2000 (Thermo Fisher Scientific, USA). Cells were then transfected with the miR-486 mimic (uuccuuuguaaucuuggcc; Thermo Fisher Scientific) or antisense inhibitor anti-miR-486 (aggcaaugagacuagggga; Thermo Fisher Scientific) at a concentration of 50 nM using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific). Cells transfected with Lipofectamine RNAiMAX (Thermo Fisher Scientific) with a scrambled control miRNA were used as the control cells. Cells were harvested after 48 hours of transfection.
Table I. Sequences of primers used for quantitative real-time polymerase chain reaction.

| Genes   | Forward (5′–3′) | Reverse (5′–3′) |
|---------|----------------|----------------|
| SIRT6   | CACCCAGGATGTCGACCAT | CTCCTCGCAGTTTGTCCCTG |
| COL2A1  | AGAATGGATGGAGGCAAAGA | ACCGGCCGGTAAAGGCTC |
| ACAN    | TGAGCAGGAGCAGCAGTTCGAG | TGAATACGAGGAGGAGTCCGAG |
| MMP-13  | ACTCGAGGACCTGCGAATTG | GACCCGGCATTGCGTT |
| ADAMTS4 | GAGGAGAGACTGTCCTTCTCA | CCCGCTCTACTGACGCTC |
| U6      | CTGGCTTCCGGGAGCACA | AAGCGTTCCAGATTGCGT |
| GAPDH   | GGACCGAGATCCCTCCCAATT | GGCCGTCTGTACCTCTCATGG |

ACAN, aggrecan; ADAMTS4, ACANase 4; COL2A1, collagen, type II, alpha 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP, matrix metalloproteinase; SIRT6, silencing information regulator 6.

Quantitative real-time polymerase chain reaction. Total RNAs in cartilage tissues and cells were extracted using Trizol reagent (Thermo Fisher Scientific). Total RNAs were reverse transcribed into complementary DNA (cDNA) using the cDNA Synthesis kit (TakaRa, Japan). PCR amplifications were performed using a 7500 real-time PCR system (Applied Biosystems, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references for the expression of mRNA and miRNA, respectively. The relative expression levels of genes were determined by the 2-ΔΔCt method. The sequences of all primers are shown in Table I.

Western blotting. Total proteins of the cells were extracted with RIPA (Biocolors Biotechnology, China) lysis buffer, and protein concentrations were determined using a Pierce BCA Protein assay kit (Pierce Biotechnology, USA). Protein samples (30 µg/lane) were separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA). After transfer, the membrane was blocked with 5% non-fat milk for one hour at room temperature, and incubated with the following primary antibodies (BioTeke, China) at 4°C overnight: SIRT6 (1:1000), COL2A1 (1:1000), and GAPDH (1:1000; all Abcam, UK). Then, the membranes were incubated with goat anti-rabbit IgG secondary antibody, and signals were detected by chemiluminescence. GAPDH was used as a reference. All bands were detected using ECL Western Blot Kit (Amersham Biosciences, UK) following the manufacturer’s protocol.

ELISA assay. The secretion of COL2A1, MMP-13, aggrecan, and ADAMTS4 in SW1353 cells was determined using an ELISA kit (Elabscience Biotechnology, USA). The ELISA kits were: Human Aggrecan ELISA Kit (catalogue number E-EL-H0294c), sensitivity of 100 pg/ml; Human MMP-13 ELISA Kit (catalogue number E-EL-H0134c), sensitivity of 190 pg/ml; Human ADAMTS4 ELISA Kit (catalogue number E-EL-H0266c), sensitivity of 37.50 pg/ml; and Human Collagen II ELISA Kit (catalogue number E-EL-H0777c), sensitivity of 380 pg/ml (all kits produced by Elabscience Biotechnology Co.).

Luciferase reporter assay. Online tools TargetScan, miRanda, and RNAhybrid were used to predict the three prime untranslated region (3′-UTR) of SIRT6 containing a binding site for miR-486. The pGL3 vector (RiboBio, China) was inserted with the wild type (WT) or mutant (MUT) SIRT6 3′-UTR segment containing the miR-486 binding site. SW1353 cells were co-transfected with WT-SIRT6 or MUT-SIRT6 and miR-486 mimic or mock control for 48 hours. The luciferase reporter assay was used to detect the luciferase activity of transfected cells. Two pairs of small interfering RNA (si)-SIRT6 were designed and their knockdown efficiency was verified (si-SIRT6-1: 5′-CGAGGAUGUGCUGAUAUUA-3′; si-SIRT6-2: 5′-TCATGCCCGGCTCTGAA-3′). To verify whether SIRT6 was involved in miR-486-induced promotion of a catabolic phenotype in SW1353 cells, luciferase reporter gene vectors containing si-SIRT6 or si-control (si-con) were transfected into SW1353 cells, and co-transfected with miR-486. For gene knockdown, SW1353 cells were seeded for 24 hours, and 50 nM si-SIRT6 or si-con was transiently transfected into the cells by 1.5 µl/well using Lipofectamine 2000 (Thermo Fisher Scientific). Knockdown efficiency was determined by western blotting after transfection for 48 hours. Cell transfection was performed following the manufacturer’s instructions. For gene knockdown, SW1353 cells were seeded for 24 hours, and 50 nM si-SIRT6 or si-con was transiently transfected into the cells by 1.5 µl/well using Lipofectamine 2000. Knockdown efficiency was determined by western blotting after transfection for 48 hours.

Statistical analysis. Data were analyzed using SPSS v.18.0 software (SPSS, USA). Data were expressed as means and SDs. Significance analysis was performed by independent-samples t-test or analysis of variance (ANOVA). The post-hoc test following ANOVA was used to compare differences among multiple groups. The correlation between the expression of miR-486 and SIRT6 was assessed using the Pearson correlation coefficient. Each experiment included three replicates, and one-way ANOVA with Tukey’s post-hoc test was used for multiple comparisons. A p-value < 0.05 indicated statistically significant differences.

Results

Expression levels of miR-486 in osteonecrosis tissues and cartilage from subjects with severe OA. The expression of miR-486 in osteonecrosis tissue and cartilage from
Fig. 1

Analysis of the expression levels of microRNA 486 (miR-486) in osteonecrosis tissue and cartilage from subjects with severe osteoarthritis (OA) by quantitative real-time polymerase chain reaction (qRT-PCR). ***p < 0.001, independent-samples t-test.

Fig. 2

Relationship between microRNA 486 (miR-486) and extracellular matrix (ECM) metabolism of SW1353 cells. a) and b) Detection of the messenger RNA (mRNA) and protein expressions of miR-486, collagen, type II, alpha 1 (COL2A1), aggrecan (ACAN), matrix metalloproteinase (MMP)-13, and ACANase 4 (ADAMTS4) by quantitative real-time polymerase chain reaction (qRT-PCR) (a) and Western blotting (b). c) Detection of the secretions of COL2A1, aggrecan, MMP-13, and ADAMTS4 by enzyme-linked immunosorbent assay (ELISA). Compared with the control group (con), **p < 0.01, independent-samples t-test.

The online tools TargetScan, miRanda, and RNAhybrid were used and the 3'-UTR of SIRT6 containing a binding site for miR-486 was predicted (Figure 3a). SW1353 cells were co-transfected with WT-SIRT6 or MUT-SIRT6 and miR-486 mimic or mock control. Luciferase reporter assay results showed that the luciferase activity in the WT-SIRT6 group was significantly lower than that in the MUT-SIRT6 group after co-transfection with the miR-486 mimic (Figure 3b, t = 15.46, p < 0.001, independent-samples t-test). In addition, the miR-486 inhibitor, miR-486 mimic, or control was transfected into SW1353 cells to determine the expression of SIRT6. The results showed that the expression levels of SIRT6 in the miR-486 mimic
Silencing information regulator 6 (SIRT6) as a microRNA 486 (miR-486) target gene binding site. a) Schematic representation of miR-486 predicted binding sites in the three prime untranslated region (3'-UTR) of SIRT6. b) Determination of the luciferase activity of SW1353 cells by luciferase assay. c) and d) Detection of the messenger RNA (mRNA) and protein expression levels of SIRT6 after transfection of SW1353 cells with miR-486 mimic, miR-486 inhibitor, or its negative control by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot. e) Determination of the mRNA expression of SIRT6 in cartilage from subjects with severe OA and osteonecrosis tissue by qRT-PCR. f) Correlation analysis of SIRT6 and miR-486 expression in human osteonecrosis tissue and cartilage from subjects with severe OA. Compared with the control group, **p < 0.05, ***p < 0.001, independent-samples t-test. con, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mut, mutant; wt, wild type. hsa-miR-486, homo sapiens miR-486.

The expression levels of SIRT6 in cartilage from subjects with severe OA were significantly lower than those in the control group at both mRNA and protein levels, while the expression levels of SIRT6 in the miR-486 inhibitor were significantly higher than those in the control group (Figures 3c and 3d). Furthermore, the expression levels of SIRT6 in bone necrosis and cartilage from subjects with severe OA were measured to explore the correlation between the expression of SIRT6 and miR-486 in vivo. The expression levels of SIRT6 in cartilage from subjects with severe OA were significantly lower than those in osteonecrosis (Figure 3e, t = 14.37, p < 0.001, independent-samples t-test), and there was a significant negative correlation between the expression of SIRT6 and miR-486 (Figure 3f, r = -0.483, p = 0.042, Pearson correlation coefficient). These results indicate that overexpression of miR-486 significantly inhibited the expression of SIRT6, confirming that SIRT6 was a direct target of miR-486.

**SIRT6 as a target for miR-486-induced change to SW1353 gene expression.** The qRT-PCR results showed that transfection of si-SIRT6 significantly inhibited the expression of SIRT6 in SW1353 cells, indicating successful transfection (Figure 4a, si-SIRT6-1 vs si-con, t = 8.296, p < 0.001; si-SIRT6-2 vs si-con, t = 11.01, p < 0.001, both independent-samples t-test). The silence efficiency of si-SIRT6-2 is better than that of si-SIRT6-1 (Figure 4a, t = 2.632, p = 0.0251). As shown in Figure 4a, the knockdown efficiency of the two pairs of designed si-SIRT6 was verified, and the later experiments were conducted with si-SIRT6-2 that showed relatively high knockdown efficiency. In addition, the expression of COL2A1, MMP-13, ACAN, and ADAMTS4 was measured after miR-486 inhibitor or si-SIRT6 was transfected into SW1353 cells. The results showed that compared with the control group, the expression of COL2A1 and ACAN were significantly downregulated, while the expression of MMP-13 and ADAMTS4 were significantly upregulated in the si-SIRT6 group at both mRNA and protein levels, indicating that interference of the expression of SIRT6 could significantly inhibit the chondrocyte-like cell catabolism (Figures 4b and 4c, p < 0.001, independent-samples t-test). Furthermore, the expression of COL2A1 (t = 8.899, p < 0.001) and ACAN (t = 10.91, p < 0.001, both independent-samples t-test) was significantly downregulated in miR-486 inhibitor and si-SIRT6 co-transfected cells, compared with that in the miR-486 inhibitor group, while the expression of MMP-13 and ADAMTS4 was significantly upregulated at both mRNA (t = 3.390, p = 0.007) and protein levels (t = 9.92, p < 0.001, both independent-samples t-test), indicating that suppressed expression of SIRT6 significantly attenuated the inhibition of chondrocyte-like cell by miR-486 inhibitors. These results indicate that SIRT6 inhibited miR-486-induced change to a more catabolic cell phenotype.
Silencing information regulator 6 (SIRT6) as a target for microRNA 486 (miR-486)-induced chondrocyte extracellular matrix (ECM) metabolism. a) Detection of the messenger RNA (mRNA) expression level of SIRT6 in small interfering RNA-control (si-con) or si-SIRT6 transfected SW1353 cells by quantitative real-time polymerase chain reaction (qRT-PCR). b) Detection of the mRNA expression levels of collagen, type II, alpha 1 (COL2A1), aggrecan (ACAN), matrix metalloprotease (MMP)-13, and ADAMTS4 in miR-486 inhibitor or si-SIRT6 co-transfected SW1353 cells by qRT-PCR. c) Detection of the protein expression levels of COL2A1, aggrecan, MMP-13, and ADAMTS4 in miR-486 inhibitor or si-SIRT6 co-transfected SW1353 cells by western blot. Compared to the control group, **p < 0.001; compared to the miR-486 inhibitor group, ##p < 0.001. All statistical analyses were conducted with independent-samples t-test.

**Discussion**

Studies have demonstrated that miRNAs are involved in critical biological processes such as cell proliferation and differentiation, micro-angiogenesis, and tumorigenesis. Micro R-486 is associated with the occurrence and development of various tumours, such as pancreatic duct carcinoma, renal cell carcinoma, breast cancer, and non-small cell lung cancer. In addition, miR-486 is also reported to be associated with the proliferation and migration of OA chondrocytes, but the underlying mechanism remains unclear. In this study, qRT-PCR results showed that the expression of miR-486 in cartilage from subjects with severe OA was significantly upregulated compared with that in osteonecrosis tissues. These results suggest that miR-486 might be involved in the regulation of the occurrence and development of OA, which is consistent with the results reported in previously published literature.

Cartilage destruction and chondrocyte ECM metabolism are clinical-pathological features of OA. Cartilage matrix is mainly composed of aggrecan and COL2A1, accounting for about 90% of the normal articular cartilage without water weight. It has been confirmed that MMPs and proteoglycan enzyme are involved in the metabolism of chondrocyte ECM. COL2A1 can be degraded by MMPs, of which MMP-13 is the most efficient. Moreover, aggrecan can be degraded by proteoglycan enzyme, with ADAMTS4 and ADAMTS5 having the highest decomposition efficiency. One previous study found that the expression levels of collagen II and aggrecan in the ECM of OA chondrocytes were significantly lower than normal values, indicating that the levels of COL2A1 and aggrecan in the ECM of chondrocytes could reflect the degree of cartilage tissue destruction. In this study, when miR-486 was overexpressed, the expression of ADAMTS4 and MMP-13 was upregulated, and the expression of aggrecan and COL2A1 was downregulated. This suggests that ECM homeostasis was destroyed by miR-486 in OA. Overexpressed miR-486 accelerated the reduction of aggrecan and COL2A1 expression by upregulating the expression of ADAMTS4 and MMP-13, producing a more catabolic phenotype in chondrocyte SW1353 cells. This indicates that miR-486 may promote a more catabolic phenotype in chondrocytes.

SIRT6 has physiological functions such as inhibiting inflammation, delaying ageing, and stabilizing the genome. One recent study has found that SIRT6 is also related to the occurrence and development of OA, but its specific mechanism of action is still unclear. In this study, bioinformatics analyses were used to predict the binding site of miR-486 in the 3′-UTR of SIRT6. The results suggest that SIRT6 was a direct target gene of miR-486. Expression of SIRT6 in cartilage from subjects with severe OA was significantly lower than that in cartilage from subjects with osteonecrosis. In addition, when miR-486 inhibitor and si-SIRT6 were co-transfected, the expression of COL2A1 and ACAN in cells was significantly downregulated, while the expression of MMP-13 and ADAMTS4 was significantly upregulated, indicating that SIRT6 inhibited the catabolic phenotype of SW1353 cells induced by...
miR-486. As far as we know, this study is the first to report the targeting effect between miR-486 and SIRT6 in cartilage from subjects with severe OA.

In our work, we also face some drawbacks regarding the limitations of our project. The effect of osteonecrosis on cell metabolism due to altered mechanical load is unknown and potentially different from normal cartilage. SW1353 cells are tumour cells and therefore could behave differently to normal chondrocytes. The results indicate that miR-486 promotes a more catabolic phenotype in chondrocyte-like cells by targeting SIRT6, thus providing a possible target and theoretical basis for the treatment of human OA.

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