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Silencing of circ_0000205 mitigates interleukin-1β-induced apoptosis and extracellular matrix degradation in chondrocytes via targeting miR-766-3p/ADAMTS5 axis

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Abstract

The aim of this study was to explore the role of hsa_circRNA_0000205 (circ_0000205) in chondrocyte injury in osteoarthritis (OA) and the underlying mechanism. Expression of circ_0000205, microRNA (miR)-766-3p and a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS)-5 was detected by quantitative real time (qRT)-polymerase chain reaction (PCR) and Western blot assays. Cell proliferation, apoptosis, and extracellular matrix (ECM) synthesis were examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and 5-ethynyl-2-deoxyuridine assays, flow cytometry, and qRT-PCR and Western blot assays. The target relationship between miR-766-3p and circ_0000205 or ADAMTS5 was confirmed by luciferase reporter assay and RNA immunoprecipitation. IL-1β treatment could attenuate cell viability of primary chondrocytes and proliferating cell nuclear antigen (PCNA) and collagen II type alpha-1 (COL2A1) levels, and elevate apoptosis rate and cleaved caspase-3, ADAMTS5 and matrix metalloproteinase-13 (MMP13) levels, suggesting that IL-1β induced chondrocyte apoptosis and ECM degradation. Expression of circ_0000205 was up-regulated in OA tissues and IL-1β-induced primary chondrocytes, accompanied with miR-766-3p down-regulation and ADAMTS5 up-regulation. Knockdown of circ_0000205 could mitigate IL-1β-induced above effects and improve cell proliferation. Moreover, both depleting miR-766-3p and promoting ADAMTS5 could partially counteract circ_0000205 knockdown roles in IL-1β-cultured primary chondrocytes. Notably, circ_0000205 was verified as a sponge for miR-766-3p via targeting, and ADAMTS5 was a direct target for miR-766-3p. Silencing circ_0000205 could protect chondrocytes from IL-1β-induced proliferation reduction, apoptosis, and ECM degradation by targeting miR-766-3p/ADAMTS5 axis.

Keywords

Osteoarthritis, circ_0000205, miR-766-3p, ADAMTS5

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Introduction

Osteoarthritis (OA) is a degenerative joint disease with the leading cause of disability, and characterized by cartilage damage and inflammatory response.[1] With the aging of the population, this disease is more prevalent, and thus turns into a worldwide concern.[2] The disordered extracellular matrix (ECM) contributes to incapacitated function and articular cartilaginous destruction, eventually leading to OA event.[3] Although much achievements have obtained on the remedy for this illness,[4,5] it is still hard to remove the degradation of ECM or the dysfunction of articular cartilage.[6] Thus, it is of great importance to explore effective treatment to prevent ECM degradation.

Circular RNAs (circRNAs) are a class of long non-coding RNAs that shape a covalently closed continuous loop. Moreover, circRNAs could function as microRNAs (miRNAs) sponges in regulating human diseases including cancer and OA.[7] Very recently, differentially expressed

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circRNAs in intact versus damaged cartilages in OA patients have been screened,[8] and the emerging role of circRNAs in the initiation and progression of OA seems to become a journey to find a treasure.[9–11] Whole-transcriptome sequencing of knee joint cartilage from OA patients showed a top 4 down-regulated circRNAs in intact versus damaged cartilage, including two known circRNAs: hsa_circRNA_circ_0000205 (circ_0000205) and circ_0016733 0000205.[8] Thus, circ_0000205 and circ_0016733 could be selected for further validation and study in OA, from the perspectives of the expression status, functional role and the underlying mechanism. Preliminary experiment showed an up-regulation of circ_0000205 and no alteration of circ_0016733 in this study cohort of OA specimens. Thus, circ_0000205 was ultimately chosen for further research.

MiRNA target prediction and preliminary experiment suggested miRNA (miR)-766-3p as a promising candidate target for circ_0000205. MiR-766-3p is a tumor suppressor in carcinogenesis and osteosarcoma,[12] and a critical regulator in the development and treatment of OA.[13] Proverbially, OA is featured by the inexorable destruction of cartilage,[3] and structural proteoglycan degradation in cartilage mediated by a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS) family is identified as an early step in OA progression.[14] Among ADAMTS family members, ADAMTS5 is the principle "aggrecanase" found in animal and human OA articular cartilage,[15] thus becoming a therapeutic target in OA.[14,16]

IL-1β signaling plays a central role in multiple cell types involved in OA,[17] and the pro-inflammatory cytokine IL-1β-induced chondrocyte has been a popular cell model in OA.[18,19] We intended to delineate the role of circ_0000205 and miR-766-3p in proliferation, apoptosis and ECM degradation of OA-like chondrocytes induced by IL-1β, as well as to determine the potential epigenetically regulatory mechanism involving circ_0000205, miR-766-3p and ADAMTS5.

Materials and methods

Clinical specimens

The experiment was authorized by the Ethics Committee of Huizhou Central People's Hospital and executed according to the Declaration of Helsinki Principles. Kellgren-Lawrence grading system for knee of OA patients was used to evaluate OA knee severity,[20] and, according to the performance of knee X-rays, the system was divided from light to heavy as follows: 0 (normal joints), I, II, III, and IV (the most severe degree of knee osteoarthritis).[20] Twenty-five OA patients (III-IV grade) who underwent selective total knee arthroplasty and eighteen trauma control patients (I-II grade) without a history of OA were collected from Huizhou Central People's Hospital. All the participants were graded by two blinded observers. Informed consent was provided for all participants. Secondary OA and other inflammatory joint diseases were ruled out based on clinical data. All cartilage specimens were preserved at −80°C for further analyzes.

Cell culture, transfection and IL-1β stimulation

Chondrocytes were isolated and cultured as previously described.[21,22] For OA model, arthritic chondrocytes at 70% confluence were stimulated with determined concentrations (5, 10 and 15 ng/ml) of human IL-1β recombinant protein for 48 h; cells without IL-1β stimulation served as control.

Short hairpin RNA (shRNA) targeting circ_0000205 (sh-circ_0000205), miR-766-3p mimic (miR-766-3p), miR-766-3p inhibitor (anti-miR-766-3p), and counterpart negative controls (NCs) including sh-NC, miR-NC and anti-miR-NC were all obtained from GenePharm (Shanghai, China). The intact sequence of circ_0000205 and coding domain sequence of ADAMTS5 were separately cloned into pCD5-ciR vector (GENESEED, Guangzhou, China) and pcDNA (zeo+) vector (BioVector Science Lab, Beijing, China) to construct circ_0000205 overexpression plasmid and ADAMTS5 overexpression vector. Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) kit was used to transfect according to the manufacturer's instructions. Transfected cells at 48 h were collected for gene expression analysis or for 10 ng/mL of IL-1β administration for 48 h for functional experiments.

RNA isolation and quantitative real time (qRT)-polymerase chain reaction (PCR)

Total RNA of tissues and cells was extracted by using TRIzol reagent (Thermo Fisher Scientific) and reverse-transcribed using All-in-One™ miRNA Prime Script™RT reagent kit (Takara, Shiga, Osaka, Japan). QRT-PCR was performed using the qRT-PCR Detection Kit (GeneCopoeia, Inc., RockVile, MD, USA) and Synergy brands (SYBR) mix (TaKaRa, Dalian, China) on the 7500 Fast Real-Time PCR system (Thermo Fisher Scientific). U6 small nuclear RNA (U6) or GAPDH was as internal reference gene. Four replicates were set for each reaction. Relative expression of circ_0000205, miR-766-3p, ADAMTS5, matrix metalloproteinase-13 (MMP13), and collagen II type alpha-1 (COL2A1), as well as GAPDH and U6 were calculated by the $2^{-\Delta\Delta Ct}$ method. The sequences of primers for miR-766-3p and U6 were designed and obtained from Sangon Biotech (Shanghai, China), and sequences of primers used in qRT-PCR reactions were listed:

MiR-766-3p forward (5′-ACTCCAGCCCCACAGC-3′),
MiR-766-3p reverse (5′- GAACATGTCTGCGTATCT C −3′);
U6 forward, (5′-CTCGCTTCGGCAGCACA-3′),

U6 reverse, (5′-ACGCTTCACGAATTTGCGT-3′);
Circ_0000205 forward (5′-AGTTGGCTCTCACTGC TTCT-3′),
Circ_0000205 reverse (5′-GGTCACTCCTGCAATA AGACT-3′),
ADAMTS5 forward (5′-TGGCTCACGAAATCGGAC AT-3′),
ADAMTS5 reverse (5′-TTGGACCAGGGCTTAGATG C-3′),
MMP13 forward (5′-ATGACTATGCGTGGCTGGA A-3′),
MMP13 reverse (5′-TGTCCCATTTGTGGTGTGG G-3′).
COL2A1 forward (5′-TGCATGAGGGCGCGGTA-3′),
COL2A1 reverse (5′-GGTCCTGGTTGCCGGAC AT-3′),
GAPDH forward (5′-GACAGTCAGCCGCATCTTC T-3′),
GAPDH reverse (5′-GCGCCCAATACGACCAAAT C-3′).

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay

Cells (5×10^3/well) were plated in 96-well plate with four replicates for each reaction and maintained for 48 h prior to MTT assay. Simply, MTT reagent (Sigma, St Louis, MO, USA) was added to each well and incubated for further 4 h. After that, cell supernatant was discarded, and 200 µl of dimethylsulfoxide (DMSO) (Solarbio, Beijing, China) was added to dissolve intracellular formazan crystals in each well.[23] Cell proliferation was determined at 490 nm using a microplate reader (Thermo Labsystems, Waltham, MA, USA).

5-Ethynyl-2-deoxyuridine (EDU) assay

BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 594 (Beyotime, Shanghai, China) were utilized to measure cell proliferation. Five thousand cells were transferred in 96-well plate in triplicate, and cells were dyed with 10 µM EdU reagent for 2 h and re-dyed with 4', 6-diamidine-2-phenylindoldihydrochloric acid (DAPI) Staining Solution (Beyotime). Fluorescence detections of Alexa Fluor 594-labelled EdU and DAPI were performed on an inverted fluorescence microscope (Nikon Microsystems, Shanghai, China). EdU positive cell rate was calculated according to three random fields.

Flow cytometry

Cells were collected by digesting with pancreatin and centrifuging. After that, cells were re-suspended with 1× binding buffer after washed with iced phosphate-buffered saline (PBS). Later, these cells were processed following the manufacturer's instructions of Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (BD Pharmingen, San Diego, CA, USA). Apoptotic cells were examined using flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and then apoptosis rate was calculated.

Western blot

Radioimmunoprecipitation assay (RIPA) buffer (Solarbio, Beijing, China) was used to isolate total proteins in cells, and protein concentration was quantified by a NanoDrop 3000 (Thermo Fisher Scientific). sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used to separate proteins, and then proteins were transferred onto polyvinylidene fluoride (PVDF) membranes. After that, membranes were blocked in skim milk for 2 h at 37°C and then incubated with primary antibody (Ab) at 4°C overnight. Following 2-h incubation with secondary Ab marked with horseradish peroxidase (HRP) (1:2000; Cell Signaling Technology, Danvers, MA, USA). The chemiluminescence was developed using an Enhanced chemiluminescence (ECL) detection kit (Beyotime). The primary Abs were as follows: anti-ADAMTS5 (1:1000; Abcam, Cambridge, UK), anti-MMP13 (1:1000; Abcam, Cambridge, UK), anti-COL2A1 (1:1000; Abcam, Cambridge, UK), anti-proliferating cell nuclear antigen (PCNA) (1:1000; Abcam, Cambridge, UK), anti-β-actin (1:4000; Cell Signaling Technology, Danvers, MA, USA), and anti-cleaved caspase-3 (anti-C-caspase3; 1:1000; Cell Signaling Technology).

Luciferase assay and RNA immunoprecipitation (RIP)

The putative binding sites between miR-766-3p and circ_0000205 or ADAMTS5 were predicted by circinteractome and targetScan software online. The full length of circ_0000205 and the fragment of the 3'untranslated region (3'UTR) of ADAMTS5 (ADAMTS5 3'UTR) were separately amplified using PCR method, and the amplified products were inserted into the plasmid of miRNA expression reporter containing firefly luciferase (pMIR-REPORT luciferase vector) (OBio Biology, shanghai, China) to construct wild type (WT) of circ_0000205 (circ_0000205 WT) and ADAMTS5 3'UTR WT luciferase reporters. Besides, the mutant type (MUT) of circ_0000205 (circ_0000205 MUT) and ADAMTS5 3'UTR MUT luciferase reporters were generated via mutating the seed sites based on the corresponding WT vectors. The co-transfection of luciferase reporter and miR-766-3p or miR-NC was performed as prescribed, and each reaction was performed with three replicates. The luciferase activity was tested using Dual-Lucy Assay Kit (Promega, Madison, WI, USA). The primary chondrocytes were lysed, and RIP assay was performed

using Magna RIP kit (Millipore) and Ago2 Ab following the manufacturer's instructions, with lgG as control.

RNA pull-down assay

Cell lysate of primary chondrocytes was isolated in RIPA lysis buffer (Solarbio) and the supernatant was incubated with Streptavidin Dynabeads beads (M-280, Invitrogen) and biotin-labelled circ_0000205 probe or Oligo probe at 4°C for 4 h. After washing with RIPA buffer supplemented with 500 mM NaCl, beads were harvested and the pull-down RNAs were eluted from the beads for further qRT-PCR.

Statistical analysis

The experiments were carried out three times, with at least three replicates of each reaction. All data were expressed as mean ± standard deviation (SD) and analyzed by the SPSS 21.0 software. Comparisons among different groups were analyzed using one-way analysis of variance (ANOVA). A P value less than 0.05 was regarded as statistically significant.

Results

Circ_0000205 and miR-766-3p were dys-regulated in OA patients

Expression of circRNAs (circ_0000205 and circ_0016733) in this group of OA patients was detected, and circ_0016733 level was not significantly altered in these cartilage specimens (data not shown), while circ_0000205 level was dramatically increased in OA cartilages than normal ones (Figure 1a). Besides, miR-766-3p expression was down-regulated in these 25 cases of OA tissues compared to corresponding healthy parts (Figure 1b). Moreover, a negative correlation between circ_0000205 and miR-766-3p expression was observed in OA patients (Figure 1c).

IL-1β administration induced chondrocyte dysfunction in vitro *and altered circ_0000205 and miR-766-3p expression*

We used IL-1β to induce OA-like phenotype of chondrocytes, which was confirmed by measuring proliferation, apoptosis, and extracellular matrix (ECM) degradation. MTT assay showed that 5-15 ng/ml IL-1β treatment caused cell viability inhibition in primary chondrocytes in a concentration-dependent manner (Figure 2a), which was accompanied with decreased proliferation marker PCNA level (Figure 2c). Apoptosis rate was gradually increased in response to IL-1β treatment (Figure 2b), paralleled with promoted apoptosis marker C-caspase3 level (Figure 2c). Meanwhile, expression of matrix-degrading enzymes ADAMTS5 and MMP13 was raised with the treatment of IL-1β, whereas ECM component COL2A1 was suppressed at both messenger RNA (mRNA) level (Figure 2d) and protein level (Figure 2e). These results demonstrated that 5–15 ng/ml IL-1β could induce OA-like phenotypes in these primary chondrocytes, during which circ_0000205 expression was increased and miR-766-3p was descended in concentration-dependent manners (Figure 2f and 2g). These results suggested a potential role of circ_0000205 and miR-766-3p in IL-1β-induced OA model in primary chondrocytes.

Circ_0000205 knockdown promoted cell proliferation of IL-1β-induced chondrocytes and suppressed apoptosis and ECM degradation

To figure out the potential functions of circ_0000205 in osteoarthritic chondrocytes, shRNA was used in a series of loss-of-function experiments. qRT-PCR confirmed that sh-circ_0000205 transfection showed high knockdown

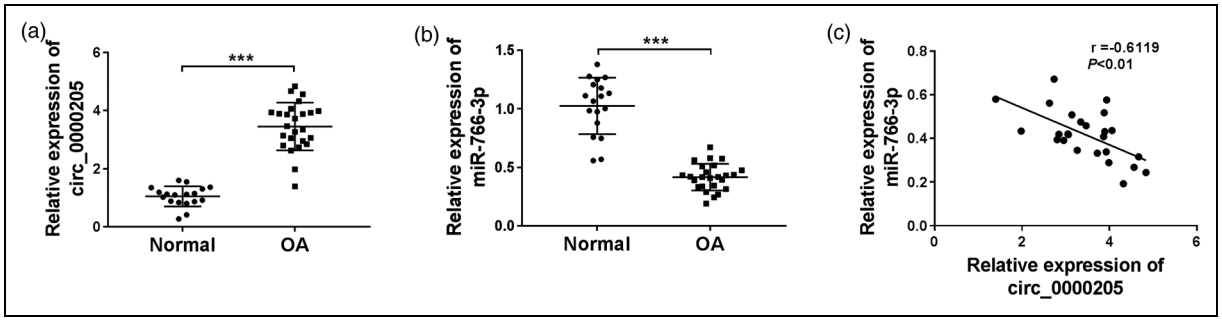

Figure 1. The dys-regulation of circ_0000205 and miR-766-3p in OA tissues. (a) The expression level of circ_0000205 was detected by qRT-PCR assay. (b) The expression level of miR-766-3p was detected by qRT-PCR assay. (c) The potential relationship between circ_0000205 and miR-766-3p. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Experiments were carried out three times, with at least three replicates of each reaction.

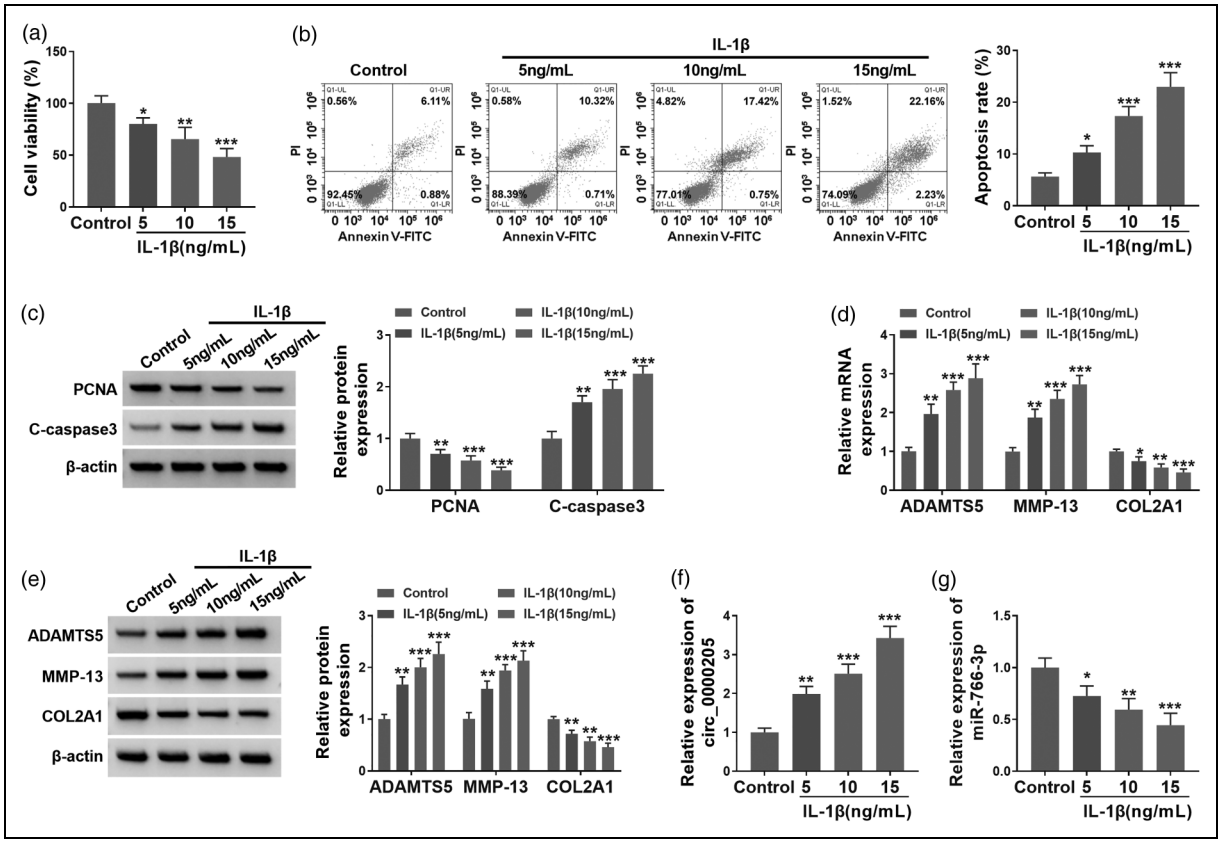

Figure 2. The expression of circ_0000205 and miR-766-3p in IL-1β-administrated human chondrocytes *in vitro*. The isolated human primary chondrocytes were treated with different concentrations (5 ng/ml, 10 ng/ml and 15 ng/ml) of IL-1β for 48 h. (a) Cell viability was analyzed by MTT assay. (b) Apoptosis rate was confirmed by flow cytometry. (c) The protein levels of PCNA and C-caspase3 were explored by Western blot. (d) The mRNA levels of ADAMTS5, MMP13, and COL2A1 were confirmed by qRT-PCR, and (e) their protein levels were tested by Western blot assay. (f, g) The expression of circ_0000205 and miR-766-3p was detected by qRT-PCR. $^{**}P < 0.01$ and $^{***}P < 0.001$. Experiments were carried out three times, with at least three replicates of each reaction.

efficiency in these primary chondrocytes, compared to sh-NC group (Figure 3a). MTT and EdU assays depicted that circ_0000205 knockdown via shRNA improved cell viability and proliferation of IL-1β-insulted primary chondrocytes (Figure 3b and 3c), which was accompanied with increased PCNA level (Figure 3e). The high apoptosis rate under IL-1β treatment was distinctively decreased with circ_0000205 blockage (Figure 3d), paralleled with depressed C-caspase3 level (Figure 3e). Moreover, deletion of circ_0000205 could down-regulate ADAMTS5 and MMP13 expressions and up-regulate COL2A1 expression in primary chondrocytes under IL-1β induction (Figure 3f and 3g). Above data suggested that blocking circ_0000205 could mitigate IL-1β-induced chondrocytes injury by promoting proliferation and restraining apoptosis and ECM degradation.

Circ_0000205 was a sponge of miR-766-3p

Circinteractome showed a total of 28 miRNA binding sites in the sequence of circ_0000205, among which there were 7 OA-related miRNAs by retrieving the existing literatures (Figure S1). Later, RNA pull-down assay revealed that, among these 7 miRNAs, miR-776-3p was the most enriched one by circ_0000205 probe in primary chondrocytes (Figure S1), and the presumptive target sites of miR-766-3p in circ_0000205 were intended to be mutated (Figure 4a). Furthermore, luciferase reporter assay was performed and thereby confirmed that the luciferase activity of pMIR-REPOR-circ_0000205-WT was decreased while that of pMIR-REPOR-circ_0000205-MUT was unaltered by miR-766-3p overexpression via mimic transfection (Figure 4b and 4c). RIP assay further confirmed the direct interaction between circ_0000205 and miR-766-3p, since Ago2 mediated a co-enrichment of circ_0000205 and miR-766-3p (Figure 4d). In addition, qRT-PCR analysis was used to observe the potential regulatory effect between miR-766-3p and circ_0000205, and data demonstrated that miR-766-3p level was increased following the knockdown of circ_0000205 via shRNA in primary chondrocytes no matter with or without IL-1β treatment (Figure 4e and 4f).

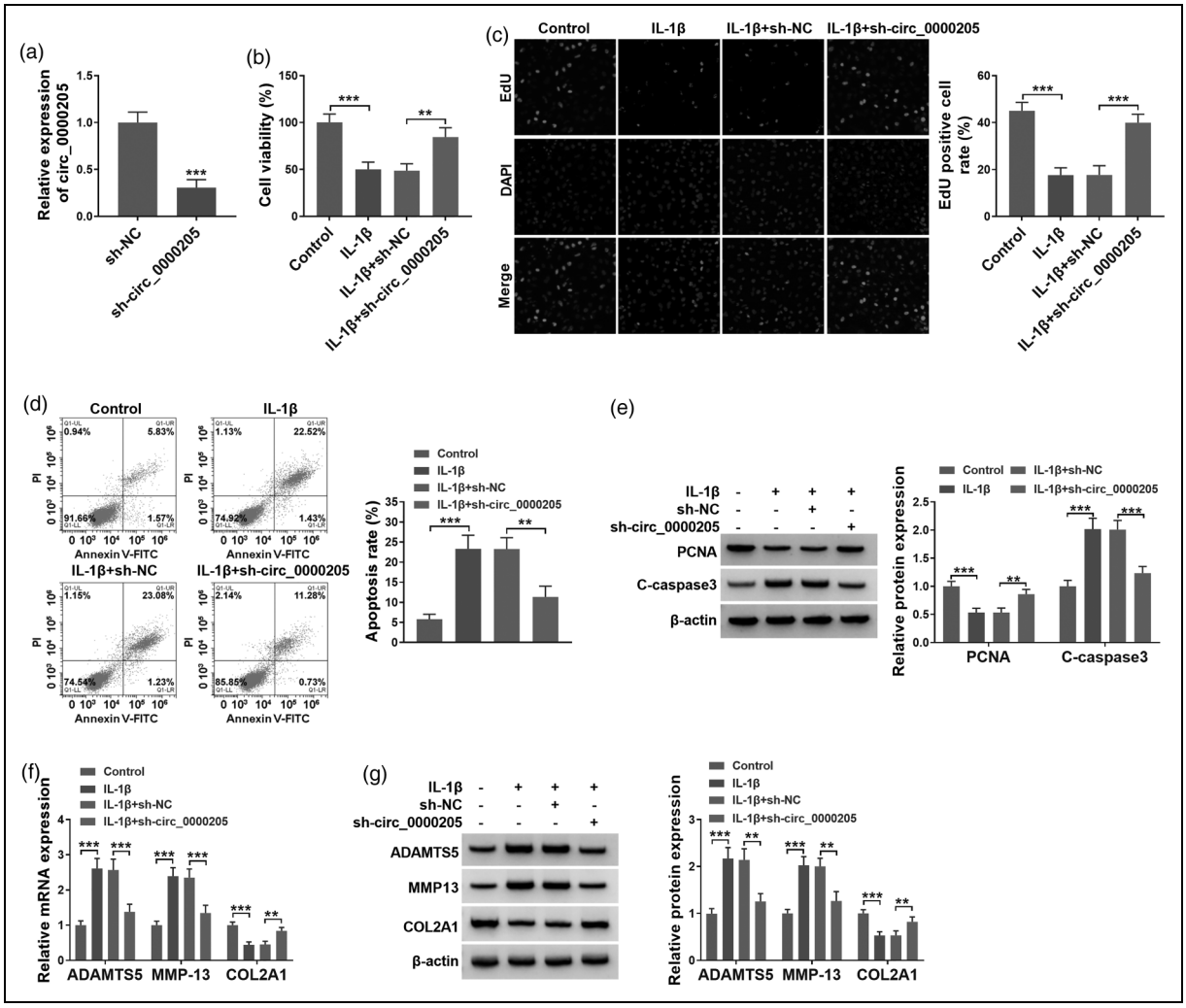

Figure 3. The effect of circ_0000205 knockdown on the proliferation, apoptosis, and ECM degradation of chondrocytes under IL-1β condition. IL-1β-induced human primary chondrocytes were pre-transfected with shRNAs, and this experiment was divided into four different groups: Control, IL-1β, IL-1β + sh-NC, IL-1β + sh-circ_0000205. (a) QRT-PCR was used to confirm the knockdown efficiency of circ_0000205. (b) Cell viability was analyzed by MTT assay. (c) EdU positive cell rate was examined by EdU assay. (d) Apoptosis rate was confirmed by flow cytometry. (e, g) The protein levels of PCNA, C-caspase3, ADAMTS5, MMP13, and COL2A1 were explored by Western blot. (f) The mRNA levels of ADAMTS5, MMP13, and COL2A1 were confirmed by qRT-PCR. $^{**}P < 0.01$ and $^{***}P < 0.001$. Experiments were carried out three times, with at least three replicates of each reaction.

MiR-766-3p knockdown abrogated the role of circ_0000205 knockdown in IL-1β-induced chondrocytes

To explore the relationship between miR-766-3p and circ_0000205 in functional effects in IL-1β-induced primary chondrocytes, anti-miR-766-3p was used to be co-transfected with sh-circ_0000205, and the silencing efficiency of this anti-miRNA was determined by qRT-PCR (Figure 5a). Compared with sh-circ_0000205 and anti-miR-NC co-transfection group, sh-circ_0000205 and anti-miR-766-3p co-transfection descended cell viability (Figure 5b), lessened EdU positive cell rate (Figure 5c), and improved apoptosis rate (Figure 5d), as well as depressed PCNA expression and promoted C-caspase3 level (Figure 5e). Besides, circ_0000205 interference-mediated protection of ECM synthesis under IL-1β condition could also be diminished with anti-miR-766-3p co-transfection, as described by the rescued mRNA and protein expression levels of ADAMTS5 and MMP13 and the abated COL2A1 levels (Figure 5f and 5g). These outcomes demonstrated that exhausting miR-766-3p could abate circ_0000205 knockdown roles in IL-1β-induced chondrocytes, suggesting a counteractive effect between circ_0000205 and miR-766-3p.

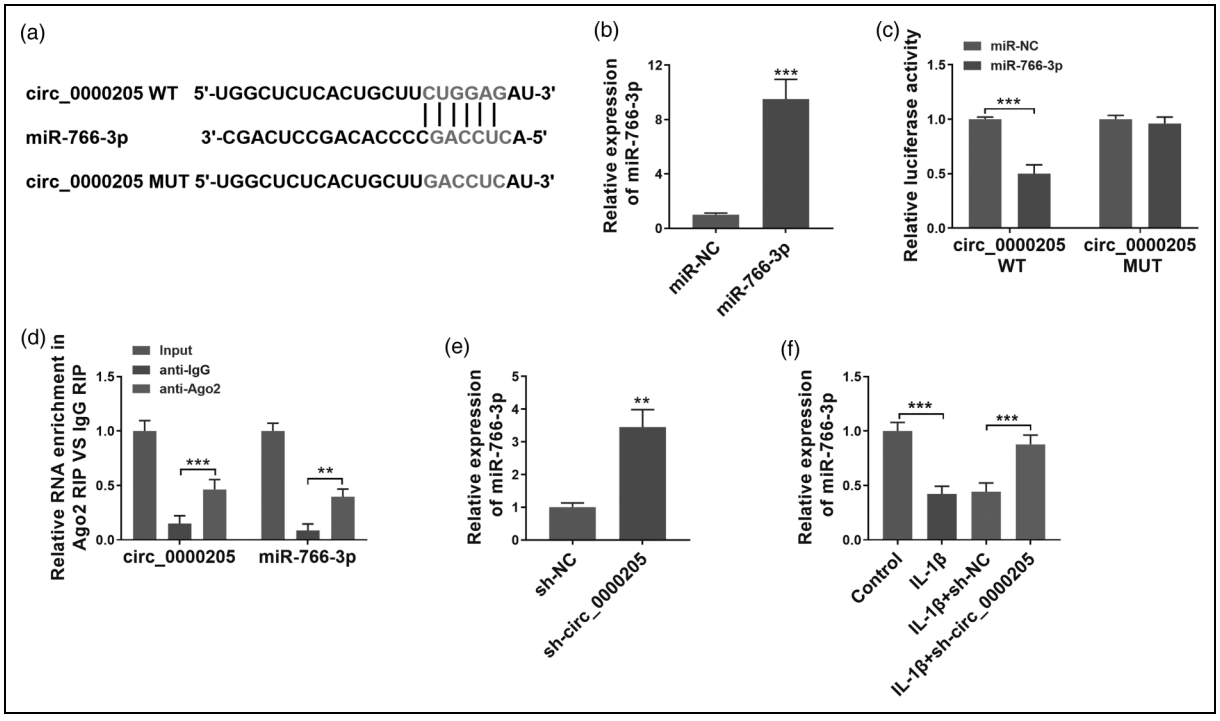

Figure 4. The identification of circ_0000205 as a sponge for miR-766-3p. (a) The putative binding sites of miR-766-3p and circ_0000205 are showed. (b) QRT-PCR was used to detect miR-766-3p level in miR-NC- or miR-766-3p-transfected primary chondrocytes. (c) The prediction sites were identified by dual-luciferase reporter assay by co-transfecting circ_0000205 WT/MUT vectors and miRNAs. (d) The RIP assay-measured expression of miR-766-3p and circ_0000205 in the samples bound to anti-Ago2 Ab or anti-IgG in primary chondrocytes. (e) QRT-PCR was used to detect miR-766-3p level in sh-NC or sh-circ_0000205-transfected primary chondrocytes. (f) QRT-PCR was used to detect miR-766-3p level in IL-1β-induced human primary chondrocytes transfected with shRNAs. $^{**}P < 0.01$ and $^{***}P < 0.001$. Experiments were carried out three times, with at least three replicates of each reaction.

MiR-766-3p was a direct regulator for ADAMTS5 via target binding

TargetScan also identified the potential target of miR-766-3p in functional genes. TargetScan showed a total of 6609 potential mRNA targets for miR-766-3p, and among them ADAMTS5 was selected for further validation due to its crucial role in OA progression and treatment. To further validate this predicted target relationship, the putative miR-766-3p binding sites in ADAMTS5 3'UTR were mutated (Figure 6a), and luciferase reporter assay depicted that the activity of pMIR-REPOR-ADAMTS5 3'UTR MUT vector was not like that of WT vectors to be reduced with miR-766-3p mimic (Figure 6b). What's more, we examined the regulatory effect of miR-766-3p on ADAMTS5 using loss-of-function and gain-of-function experiments, and data showed that miR-766-3p overexpression deleted ADAMTS5 mRNA and protein levels in these primary chondrocytes, and its deletion could elevate that (Figure 6c and 6d); besides, circ_0000205 knockdown also resulted in the down-regulation of ADAMTS5 in IL-1β-cultured primary chondrocytes, and silencing miR-766-3p via inhibitor transfection could salvage that (Figure 6e and 6f). These data implied a target relationship between miR-766-3p and ADAMTS5 in human chondrocytes.

Circ_0000205 knockdown ameliorated IL-1β-induced chondrocyte dysfunction through down-regulation of ADAMTS5

The interaction between circ_0000205 and ADAMTS5 in regulating OA phenotypes was further deciphered. Western blot data showed that ADAMTS5 overexpression vector resulted in an up-regulation of ADAMTS5 (Figure 7a), and this up-regulation could weaken the promoting role of circ_0000205 deficiency in cell proliferation of IL-1β-induced primary chondrocytes, as evidenced by the diminishment of cell viability (Figure 7b), EdU positive cell rate (Figure 7c) and PCNA expression (Figure 7e). Contrarily, apoptosis rate and C-caspase3 expression in IL-1β-induced primary chondrocytes were inhibited by depleting circ_0000205, whereas this inhibition was eliminated by restoring ADAMTS5 (Figure 7d and 7e). Consistent with ADAMTS5 expression, MMP13 expression in IL-1β-induced primary chondrocytes was attenuated in the presence of ADAMTS5 vector (Figure 7f and 7g);

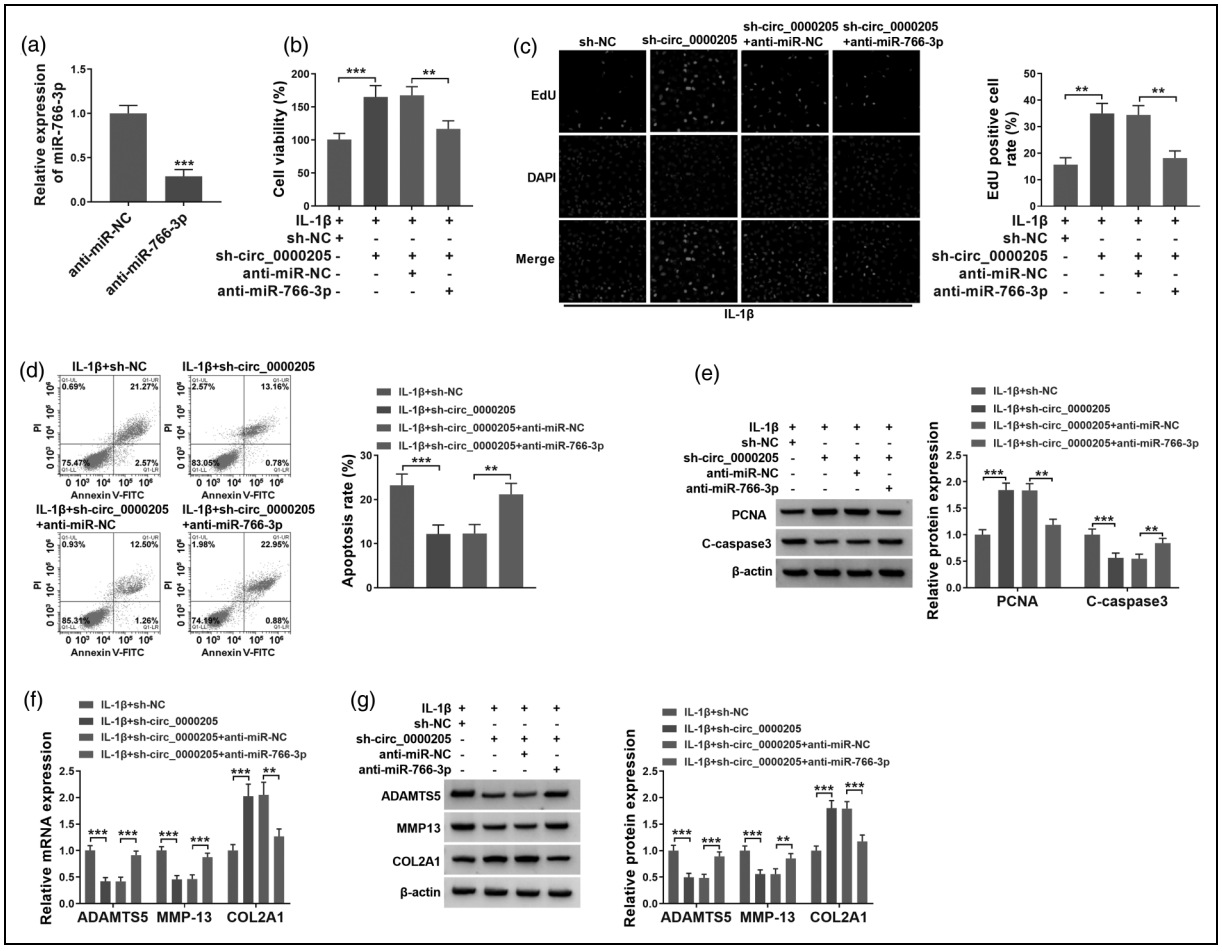

Figure 5. The effect of miR-766-3p knockdown on circ_0000205-silenced chondrocytes under IL-1β condition. IL-1β-induced human primary chondrocytes were pre-transfected with shRNAs and anti-miRNAs, and this experiment was split into four different groups: IL-1β + sh-NC, IL-1β + sh-circ_0000205, IL-1β + sh-circ_0000205 + anti-miR-NC, IL-1β + sh-circ_0000205 + anti-miR-766-3p. **(**a) QRT-PCR was used to confirm the knockdown efficiency of anti-miR-766-3p. (b) Cell viability was analyzed by MTT assay. (c) EdU positive cell rate was examined by EdU assay. (d) Flow cytometry was conducted to analyzed apoptosis rate. (e, g) The protein levels of PCNA, C-caspase3, ADAMTS5, MMP13, and COL2A1 were explored by Western blot. (f) The mRNA levels of ADAMTS5, MMP13 and COL2A1 were confirmed by qRT-PCR. $^{**}P < 0.01$ and $^{***}P < 0.001$. Experiments were carried out three times, with at least three replicates of each reaction.

contrarily, COL2A1 expression played the opposite way to ADAMTS5 (Figure 7f and 7g). These outcomes demonstrated that the suppressive role of circ_0000205 knockdown in IL-1β-induced injury in primary chondrocytes could also be partially abated by restoring ADAMTS5. Therefore, circ_0000205 might alter IL-1β-induced chondrocyte function through regulation of ADAMTS5.

Discussion

CircRNAs received an increasing attention regarding their role in OA progression and treatment. It was reported that oxidative stress, inflammation, ECM degradation, autophagy, and apoptosis in chondrocytes were overall controlled by dysregulation of several circRNAs, such as circRSU1,[24] ciRS-7,[25] and circANKRD36.[26] Here, we identified that circ_000205 was up-regulated in OA tissues and might be a contributor in IL-1β-induced OA transformation in primary chondrocytes via circ_0000205/miR-766-3p/ADAMTS5 competing endogenous RNA (ceRNA) pathway by regulating the proliferation, apoptosis and ECM degradation (Figure 8).

According to the literature by Li et al.[8] 4 circRNAs (including two novel circRNAs based on circBase database) were significantly down-regulated in intact versus damaged cartilage in OA patients. Thus, in this study, we investigated the two known circRNAs (circ_0000205 and circ_0016733) in this group of OA cartilages versus normal control cartilages. As a result, circ_0016733 expression was not significantly altered in our specimens (data not shown), while circ_0000205 was significantly up-regulated in OA tissues. Moreover, the parental gene of

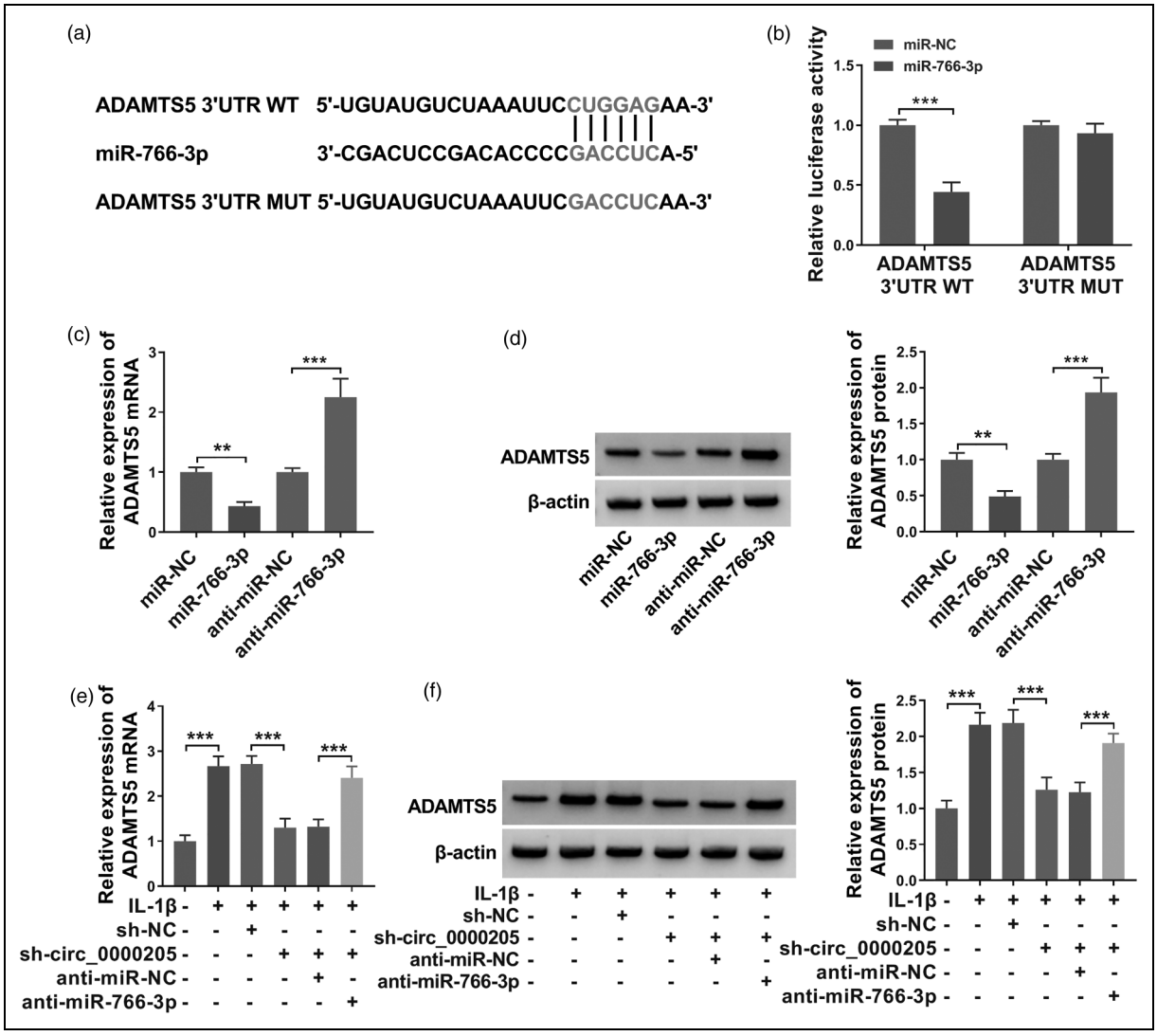

Figure 6. The identification of ADAMTS5 as a target for miR-766-3p. (a) The putative binding sites of miR-766-3p and ADAMTS5 are presented. (b) The prediction sites were identified by dual-luciferase reporter assay by co-transfecting ADAMTS5 3'UTR WT/MUT vectors and miRNAs. (c) The mRNA level of ADAMTS5 was confirmed by qRT-PCR, and (d) its protein level was detected by Western blot in primary chondrocytes transfected with miRNAs or anti-miRNAs. (e) The mRNA level of ADAMTS5 was confirmed by qRT-PCR and (f) its protein level was detected by Western blot in IL-1β-induced primary chondrocytes transfected with shRNAs and anti-miRNAs. $^{**}P < 0.01$ and $^{***}P < 0.001$. Experiments were carried out three times, with at least three replicates of each reaction.

circ_0000205, the amino acid Trp-Asp (WD) repeat domain 37 (WDR37), was one of the differentially expressed 3 transcriptional signatures in the blood of OA patients, and their gene alterations might indicate the heterogeneity of OA profiles which was potentially related to disease progress and the severity of clinical symptoms.[27] However, circ_0000205 was still a largely unknown gene and its role in human cells remained to be illuminated. Therefore, we selected this circRNA for further study in IL-1β-induced OA model in chondrocytes. Besides, up-regulation of circ_0000205 was observed in IL-1β-induced human primary chondrocytes, and its knockdown via shRNA could mitigate IL-1β-induced proliferation inhibition, apoptosis, and ECM degradation. This functional experiment indicated a protective role of circ_0000205 knockdown in IL-1β-induced OA development in chondrocytes. Furthermore, this finding might be the first evidence illuminating the involvement of circ_0000205 in chondrocyte injury in OA, suggesting circ_0000205 as a new regulator for OA.

MiRNAs were aberrantly expressed in various pathological and physiological processes in OA.[28] Hence, how miR-766-3p orchestrated the gene network in OA chondrocytes should be elucidated. Mechanically, circ_0000205

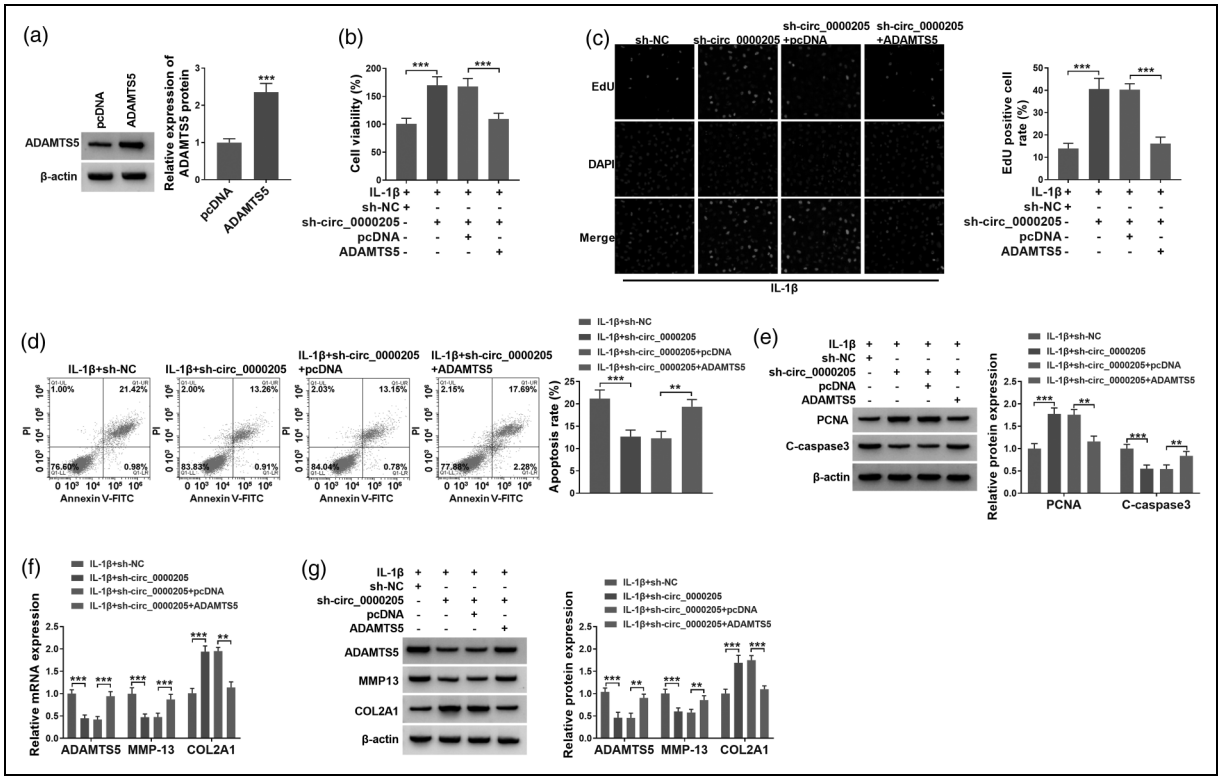

Figure 7. The effect of ADAMTS5 overexpression on circ_0000205-silenced chondrocytes under IL-1β condition. IL-1β-induced human primary chondrocytes were pre-transfected with shRNAs and overexpression vectors, and this experiment was divided into four different groups: IL-1β + sh-NC, IL-1β + sh-circ_0000205, IL-1β + sh-circ_0000205 + pcDNA, IL-1β + sh-circ_0000205 + ADAMTS5. (a) ADAMTS5 protein level was detected by Western blot in plasmids-transfected chondrocytes. (b) Cell viability was analyzed by MTT assay. (c) EdU positive cell rate was examined by EdU assay. (d) Flow cytometry was conducted to analyze apoptosis rate. (e, g) The protein levels of PCNA, C-caspase3, ADAMTS5, MMP13, and COL2A1 were explored by Western blot. (f) The mRNA levels of ADAMTS5, MMP13 and COL2A1 were explored by qRT-PCR. $^{**}P < 0.01$ and $^{***}P < 0.001$. Experiments were carried out three times, with at least three replicates of each reaction.

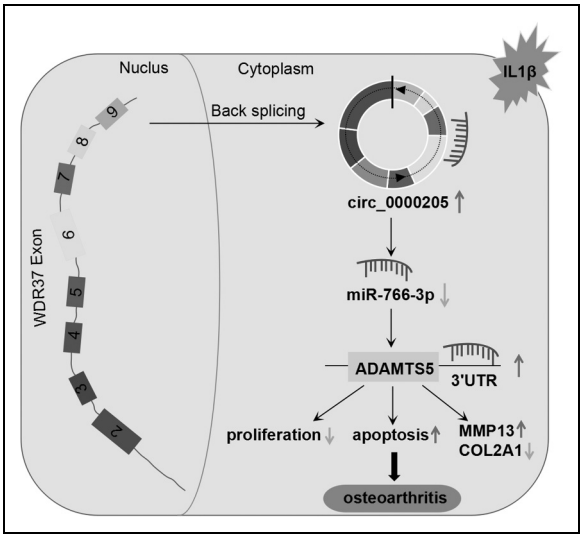

Figure 8. WDR37 exons-derived circ_0000205 could sponge miR-766-3p to regulate ADAMTS5 expression in regulation of IL-1β-induced chondrocyte dysfunction in OA.

was identified to act as a ceRNA for miR-766-3p in regulating the target gene ADAMTS5 and ECM-related proteins COL2A/MMP13, as well as proliferation/apoptosis-related proteins PCNA/C-caspase3. We noticed that miR-766-3p was down-regulated in OA tissues and this could be the first data demonstrating miR-766-3p dys-regulation in OA patients, even though its low expression had been previously reported in IL-1β-induced human chondrocytes.[13] Here, we considered that miR-766-3p silencing was conductive to apoptosis and ECM degradation in IL-1β-induced chondrocytes in spite of circ_0000205 knockdown, In addition, this facilitating role of miR-766-3p silencing in IL-1β-induced chondrocyte apoptosis had been stated by Li et al.[13] and the autophagy was also inhibited by blocking miR-766-3p based on their data.[13] Collectively, up-regulation of miR-766-3p might be one mechanism of the protection in OA-like chondrocytes.

Apoptosis-inducing factor mitochondria-associated 1 (AIFM1) was a functional target of miR-766-3p,[13] and we validated ADAMTS5 as a novel target gene for this miRNA. ADAMTS5, belonging to ADAMTS proteases

that participated in a wide range of activities and functions, was related to the pathogenic progression of cartilage degradation[29] and connected with cartilage loss and aggrecan degradation in osteoarthritic cartilage.[30–32] Functionally, restoring ADAMTS5 could counteract the protective role of circ_0000205 knockdown in IL-1β-induced chondrocyte apoptosis and ECM degradation through serving as miR-766-3p target gene. Additionally, other miRNAs were also declared to target ADAMTS5 in regulation of ECM synthesis in articular cartilage cells, such as miR-1277-5p,[33] miR-92a-3p,[34] and miR-132-3p.[35] MiR-766-3p could modulate the expression of ADAMTS5, as well as MMP13 and COL2A1, suggesting a pivotal role of miR-766-3p in the balance of ECM synthesis and degradation. However, the mechanism for ADAMTS5 in mediating chondrocyte proliferation and apoptosis seemed unclear so far.

This study was a subsequent study of the whole-transcriptome sequencing of knee joint cartilage from OA patients,[8] and further confirmed the dys-regulation and potential role of circ_0000205 in osteoarthritic chondrocytes. This study could be the first evidence of circ_0000205 as an important regulatory circRNA in human diseases, especially in OA. Furthermore, a molecular mechanism underlying osteoarthritic chondrocyte dysfunction was also confirmed, namely the circ_0000205/miR-766-3p/ADAMTS5 ceRNA pathway. Nevertheless, whether targeting circ_0000205 could exert therapeutic effect was undetermined. Moreover, osteoarthritic chondrocytes exhibited many other cell events such as inflammation and autophagy,[36] and these should also be further explored. Last but not the least, the role of circ_0000205 and the presence of circ_0000205/miR-766-3p/ADAMTS5 axis should also be confirmed in the cartilage breakdown in OA model in animals,[25] and if so, the conclusions made in this study would be more convinced. However, the animal model of OA was not included in this study, at least temporarily. Accidently, OA-related miR-671-5p[37] was also indicated to be a potential target for circ_0000205 based on circinteractome prediction and RNA pull-down assay using circ_0000205 probe, suggesting a possible circ_0000205/miRNAs interactions. Besides, according to the targetScan database, both miR-671-5p and the identified miR-766-3p could target multiple common functional genes involved in ECM degradation in osteoarthritic cartilage chondrocytes, such as ADAMTS5, collagens (such as COL27A1) and MMPs (such as MMP2). These outcomes may suggest a complex circ_0000205/miRNAs/mRNAs network. Nevertheless, this ceRNA regulatory network remains to be further functionally validated.

Taken together, our study uncovered that silencing circ_0000205 could potentially promote cell proliferation, and suppress cell apoptosis and ECM degradation of OA-like chondrocytes induced by IL-1β via sponging miR-766-3p and down-regulating ADAMTS5 *in vitro* (Figure 8). Additionally, this paper also revealed a novel circ_0000205/miR-766-3p/ADAMTS5 ceRNA axis underlying chondrocyte dysfunction in OA.

Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request

Consent for publication

Patients agree to participate in this work.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval and consent to participate

The present study was approved by the ethical review committee of Huizhou Central People's Hospital. Written informed consent was obtained from all enrolled patients.

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Supplemental material

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