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circRNA.33186 Contributes to the Pathogenesis of Osteoarthritis by Sponging miR-127-5p

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Introduction

Osteoarthritis (OA) is the most prevalent age-related joint disorder in the elderly, leading to chronic pain, stiffness, and disability.Chronic inflammation, progressive destruction of articular cartilage, and subchondral bone sclerosis are primarily characteristics of OA.Currently, there are no effective disease-modifying therapies available for OA because of limited understanding of its pathogenesis. Thus,joint replacement remains the primary treatment for patients with advanced OA.Chondrocytes are the only resident cells in the articular system and are critical for maintaining the dynamic equilibrium between anabolism and catabolism in the extracellular matrix (ECM).Several risk factors such as abnormal mechanical stress and proinflammatory cytokines have been shown to reduce chondrocytes and degrade the ECM in cartilage. Although increasing efforts have been dedicated to revealing the pathological process of OA,the molecular mechanisms remain elusive. Thus, there is an unmetmedical need to find novel drug targets to develop more effective therapeutics.

Circular RNAs (circRNAs) are a class of non-coding RNAs characterized by covalently closed loop structures with neither 50 to 30 polarity nor a polyadenylated tail.They are produced by precursor mRNA back-splicing and widely expressed in mammals with highly conserved, stable, and tissue-specific patterns.There has been accumulating evidence suggesting that circRNAs are critically involved in various diseases, such as diabetes,cardiac fibrosis and carcinomas. However, their potential roles in OA pathogenesis are poorly understood.MicroRNAs (miRNAs) are non-coding, single-stranded RNAs that are 19–25 nt long, which suppress protein expression by directly
binding to the 30 UTR of the target mRNAs. Recent studies have shown that miRNAs are also involved in the development and progression of OA.Interestingly, circRNAs could function as miRNA sponges by competitively interacting and suppressing their downstream functions.Thus, revealing the roles of circRNAs and their potential miRNA regulators is critical for understanding the molecular mechanisms of OA and identifying new biomarkers or therapeutic targets for OA.

The previous study found that circRNAs are differentially expressed in chondrocytes after interleukin-1b (IL-1b) treatment,suggesting that they might be potential regulators of OA.In this study, we identified a circRNA derived from the Umad1 gene, circRNA.33186, which is significantly upregulated inIL-1b-treated chondrocytes and cartilage tissues of the destabilized medial meniscus (DMM)-induced OA mouse model.circRNA.33186 regulates chondrocyte functions, including ECM catabolism, proliferation, and apoptosis. Silencing of circRNA.33186 in vivo markedly alleviated OA by acting as a sponge of miR-127-5p and promoted its cartilage-protecting function. Taken together, our findings reveal a fundamental role of circRNA.33186 in the progression of OA and provide a potential drug target in OA therapy.

MATERIALS AND METHODS

1.Animal Experiments

Adult male C57BL/6 mice were used to induce an OA model by DMM surgery.

(1) μCT (ITS analyses)

(2) Histology： Safranin O/Fast Green staining

2.Primary Chondrocyte Isolation and Culture, circRNA, and miRNA Transfection

(1) μCT (ITS analyses)

(2) Histology： Safranin O/Fast Green staining

Results

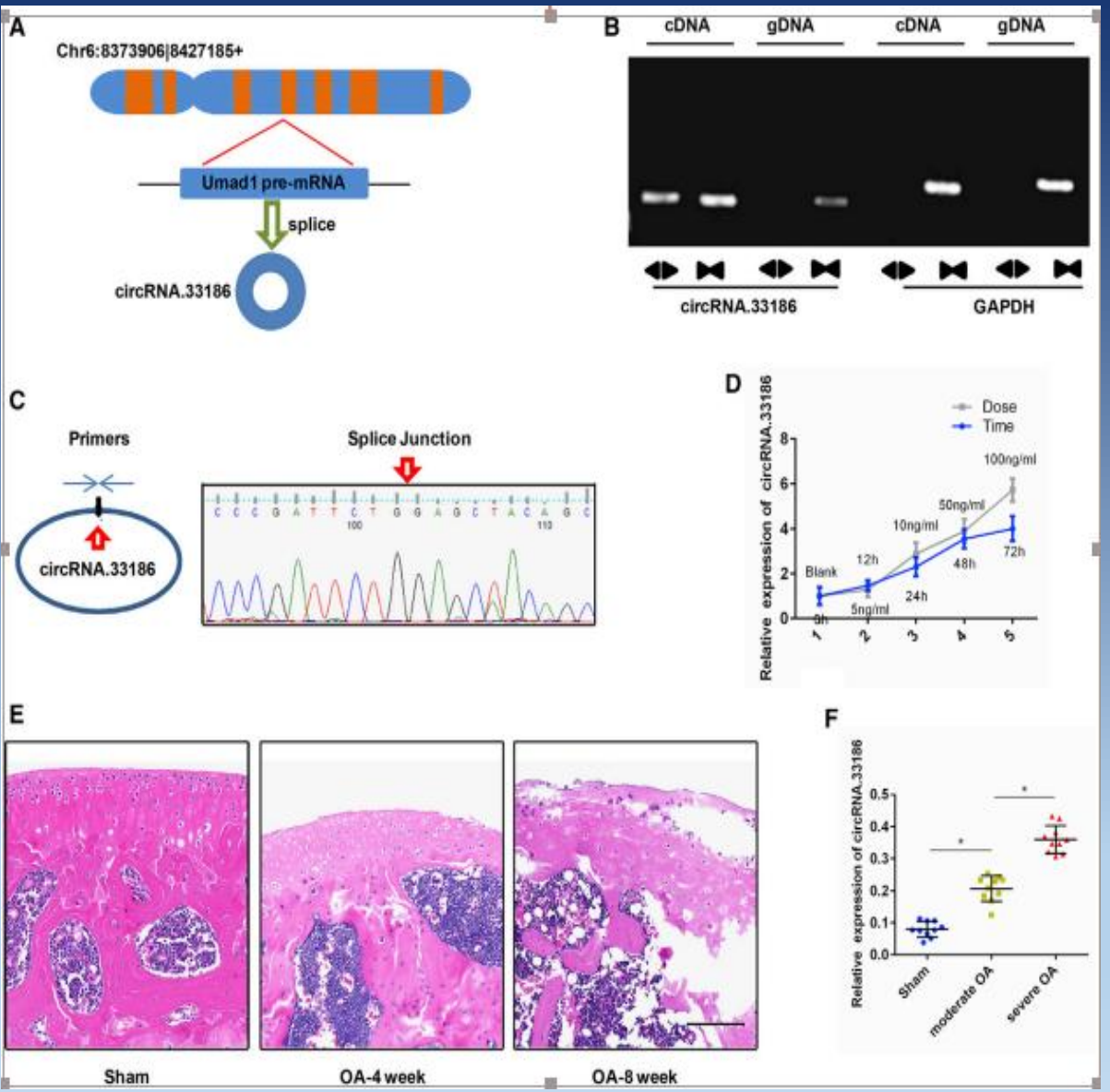

Figure 1. Characterization and Expression Analysis of circRNA.33186

(A) Schematic diagram of circRNA.33186 formed by back-splicing from the mouse Umad1 gene at chromosome 6.

(B) Divergent primers detected circular RNAs in complementary DNA (cDNA), but not in genomic DNA (gDNA).

(C) Sanger sequencing showed the back-splice junction (arrow) of circRNA.33186.

(D) qRT-PCR analysis of circRNA.33186 expression in primary chondrocytes stimulated with 10 ng/mL IL-1b for 0, 12, 24, 48, or 72 h and at 0, 5, 10, 50, or 100 ng/mL for 24 h.

(E) Representative pictures of articular cartilage stained by H&E. Scale bar, 100 mm.

(F) qRT-PCR analysis of circRNA.33186 expression in DMM-induced OA cartilage tissues (n = 10). *p < 0.01.

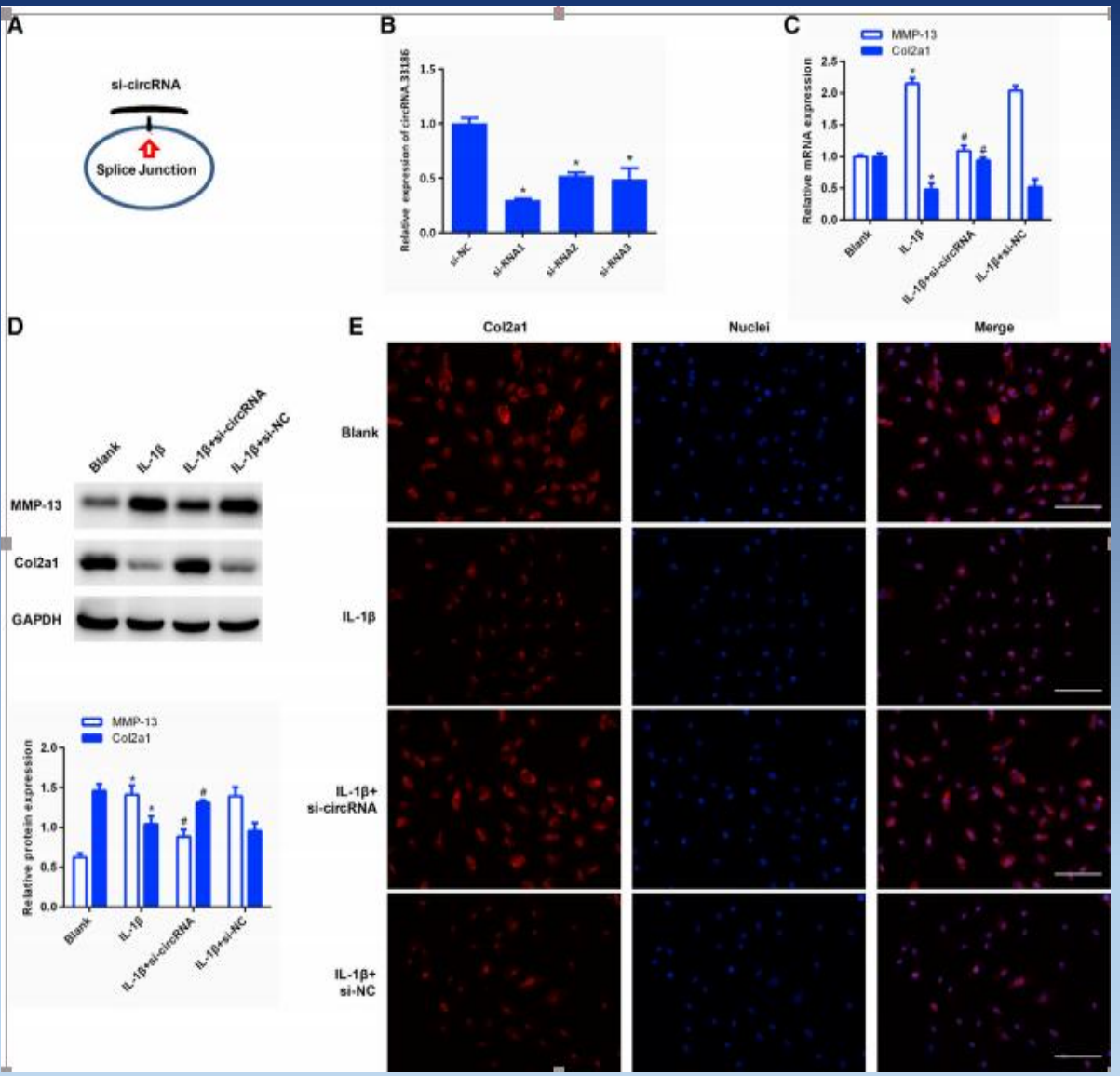

Figure 2. Effects of circRNA.33186 on Type II Collagen and MMP-13 Expression in IL-1b-Induced Chondrocytes Chondrocytes were transfected with si-circRNA or si-NC, and then treated with 10 ng/mL IL-1b for 24 h. (A and B) Schematic illustration of si-circRNA target sites (A) and expression analyses of circRNA.33186 knockdown efficiency by three different si-circRNAs in chondrocytes (B). (C) Effects of circRNA.33186 inhibition on Col2a1 and MMP-13 mRNA levels were determined by qRT-PCR. (D) Effects of circRNA.33186 inhibition on Col2a1 and MMP-13 protein levels were determined by western blot. (E)Effects of circRNA.33186 inhibition on Col2a1 protein levels were determined by immunofluorescence staining. Images were acquired by confocal microscope. Scale bars,200 mm. *$p < 0.01$ compared with blank; $^{\#}p < 0.01$ compared with IL-1b group.

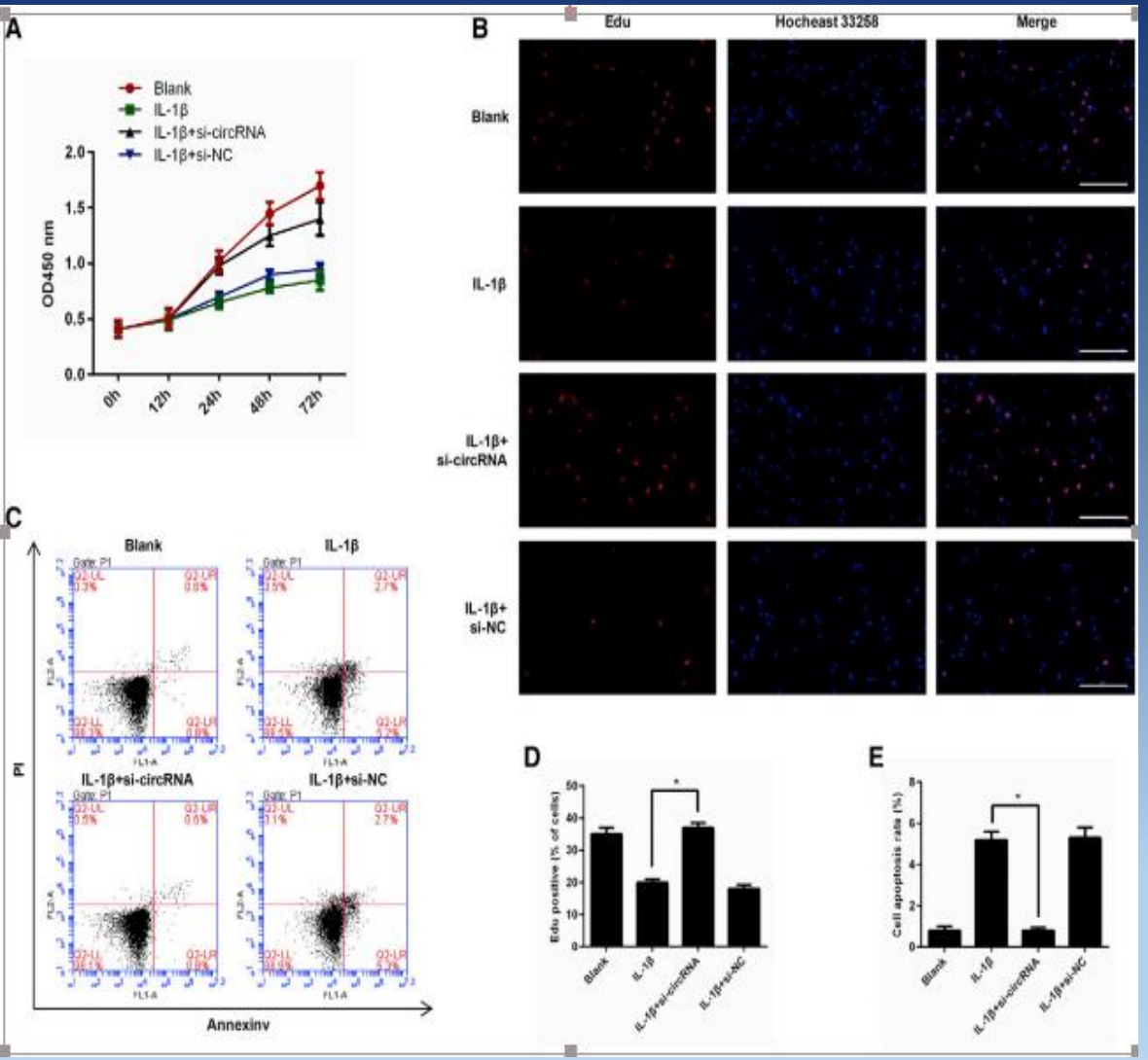

Figure 3. Effects of circRNA.33186 on Proliferation and Apoptosis in IL-1b-Induced Chondrocytes

Chondrocytes were transfected with si-circRNA or si-NC, and then treated with 10 ng/mL IL-1b for 24 h. (A) The effect of circRNA.33186 on cell proliferation in vitro was determined by CCK8 assay. (B and D) Representative photomicrographs of EdU staining (B) and quantitative data showing the percentage of EdU-positive cells in different treatment groups (D). Blue: Hochest labeling of cell nuclei; Red: EdU labeling of nuclei of proliferative cells. Scale bars, 100 mm. (C and E) The effect of circRNA.33186 on cell apoptosis was measured by flow cytometric analysis (C), and the results of flow cytometric analysis are presented as percentages of positive mean values ± SD.*p < 0.01

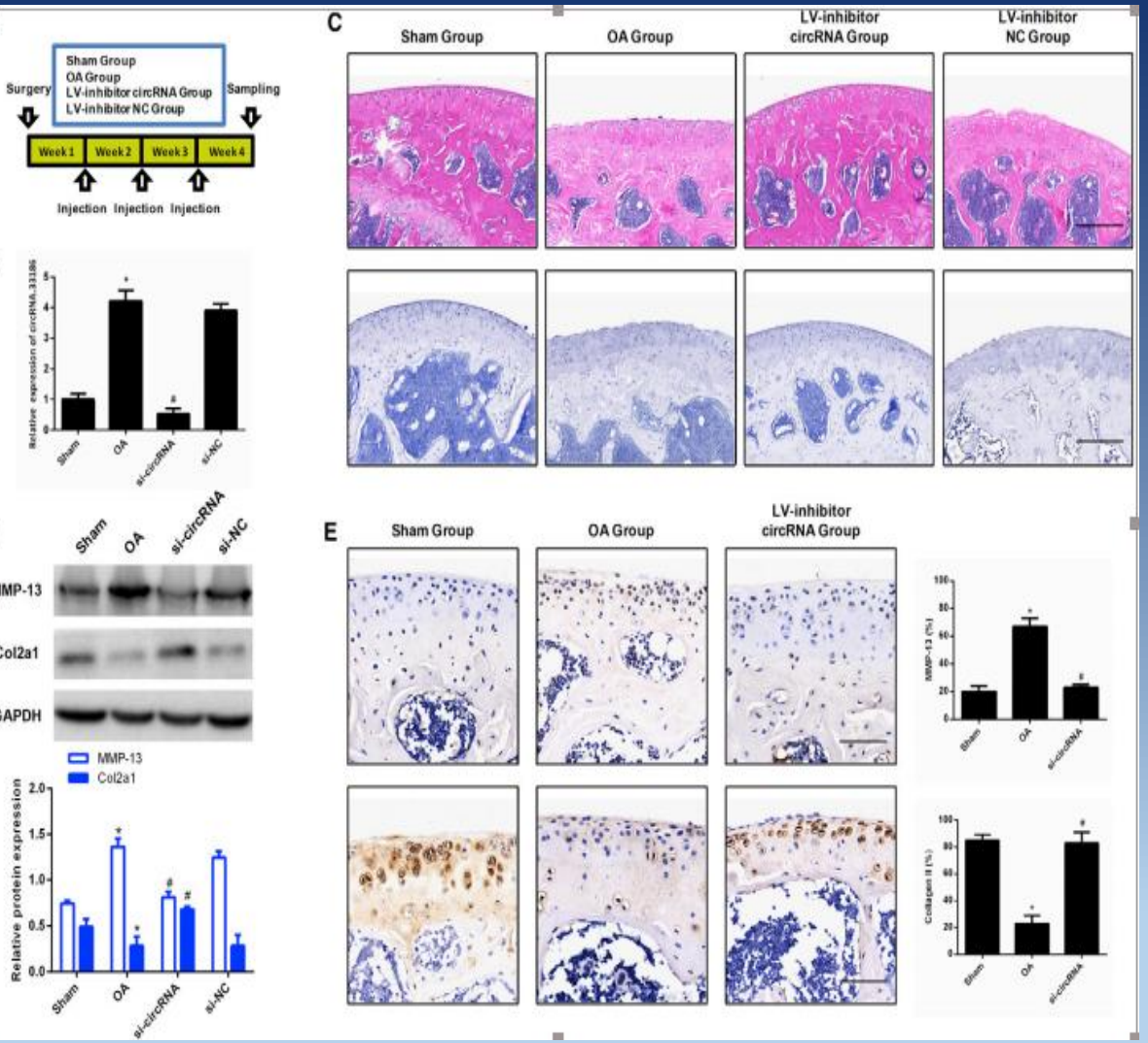

Figure 4. Silencing of circRNA.33186 In Vivo Alleviates DMM-Induced OA

(A) Schematic diagram illustrating the design of the OA therapeutic experiment targeting circRNA.33186. (B) qRT-PCR analysis of circRNA.33186 expression in knee articular cartilage from OA mice in different groups after surgery (n = 6). (C) The articular cartilage at the distal femur metaphysis was stained with H&E and toluidine blue. Scale bars, 200 mm. (D) Western blot analysis of Col2a1 and MMP13 expression in knee articular cartilage from OA mice in different groups. (E) Expression of Col2a1 and MMP13 were observed by immunohistochemistry staining in a DMM-induced OA mice model. Scale bars, 100 mm. *p < 0.01 compared with Sham group; #p < 0.01 compared with OA group

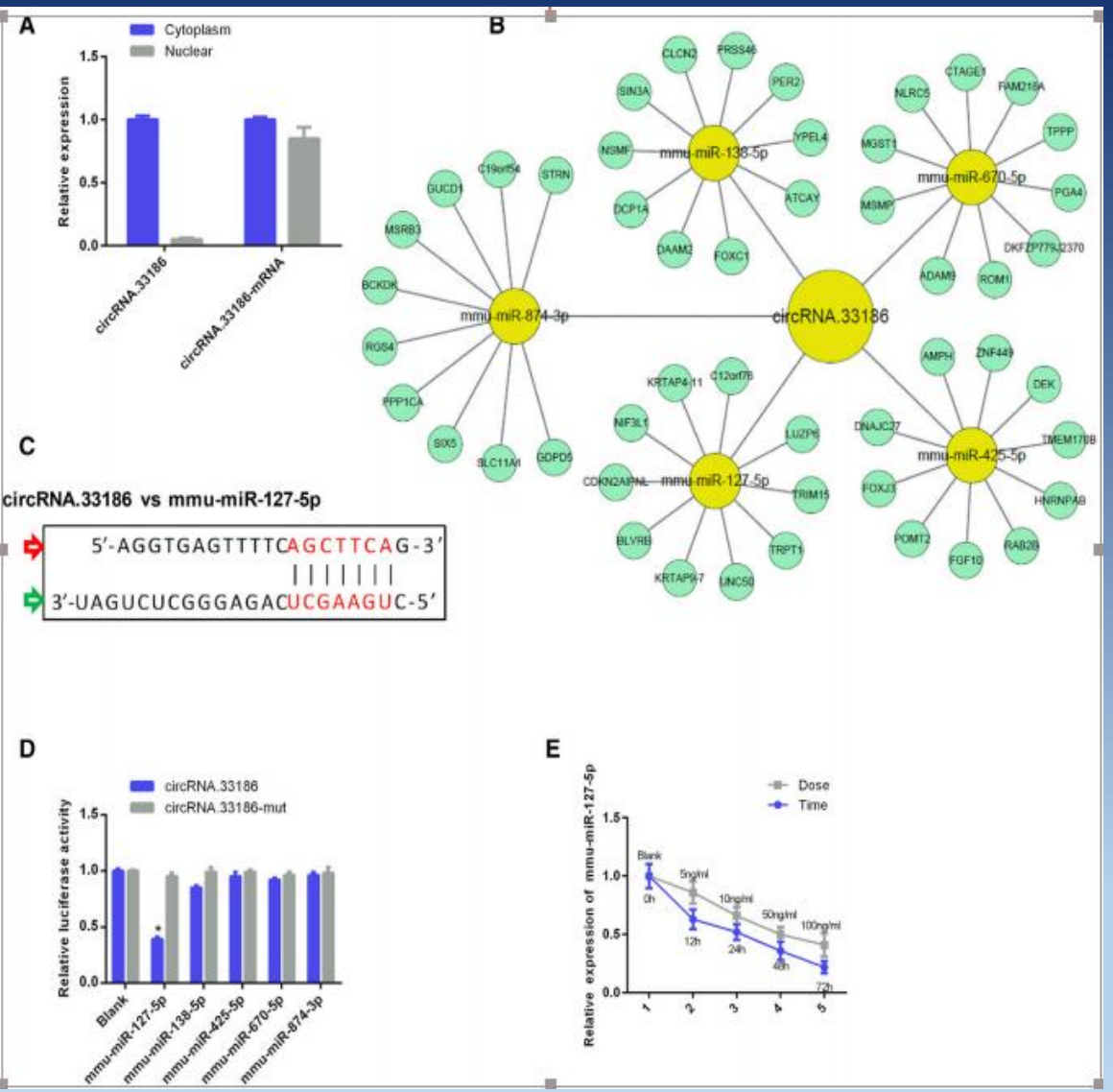

Figure 5. circRNA.33186 Is a Sponge of miR-127-5p
(A) Relative expression of circRNA.33186 and circRNA.33186 mRNA in the cytoplasm and nucleus of chondrocytes were determined by qRT-PCR. (B) Targeted microRNAs matching the circRNA.33186 UTRs predicted by both TargetScan and miRanda. (C) Putative miR-127-5p binding site in the 30 UTR of circRNA.33186 predicted by TargetScan and miRanda. The red arrow indicates circRNA.33186, and the green arrow indicates mmu-miR-127-5p. (D) Luciferase reporter assay for circRNA.33186 or circRNA.33186 mutant in HEK293T cells co-transfected with five miRNA mimics. (E) Time- (10 ng/mL IL-1b for 0, 12, 24, 48, or 72 h) and dose (0, 5, 10, 50, or 100 ng/mL for24 h)-dependent downregulation of miR-127-5p expression in primary chondrocytes. *p < 0.01.

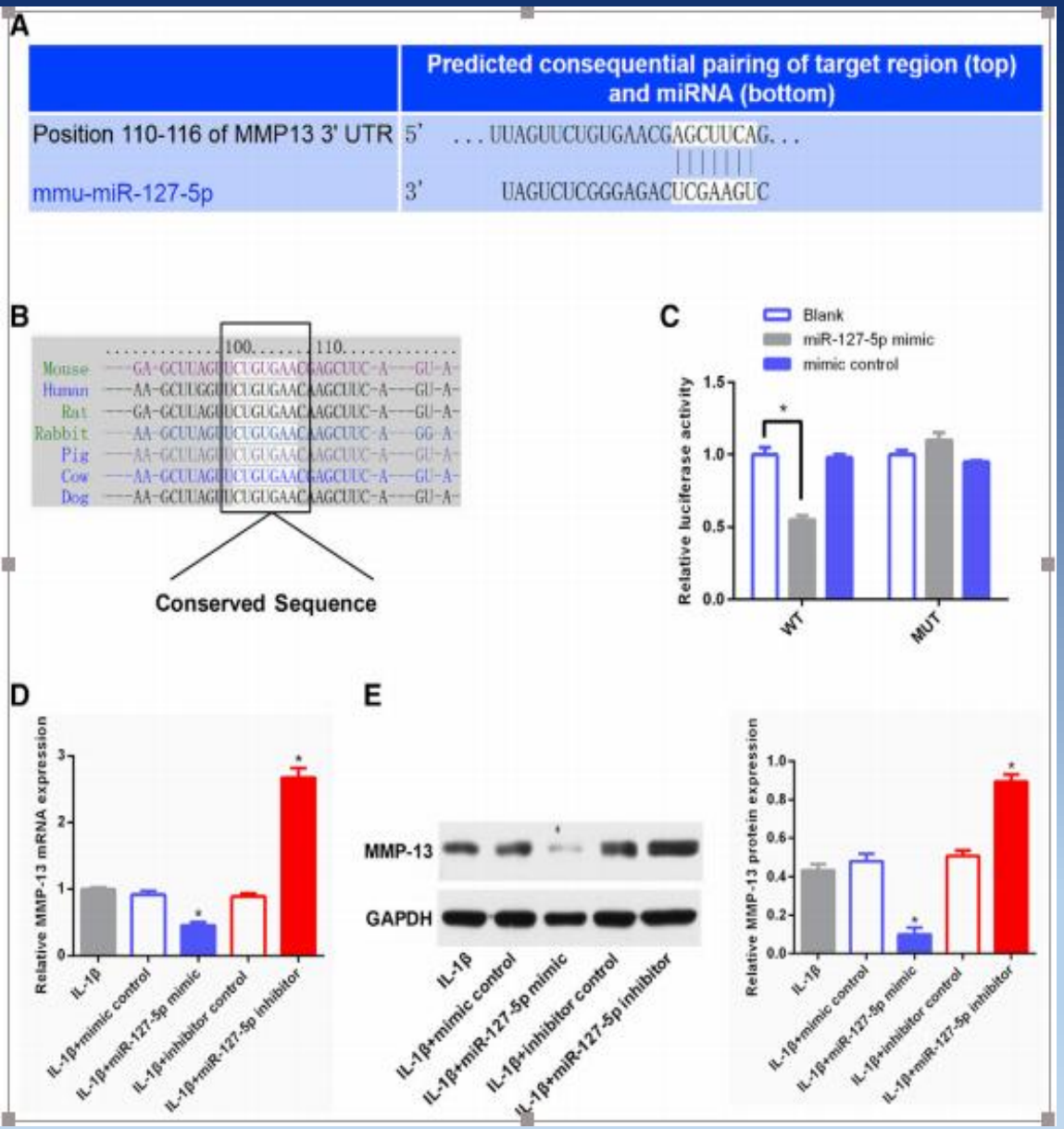

Figure 6. MMP-13 Is a Direct Target of miR-127-5p
(A) Putative miR-127-5p binding site in the 30 UTR of MMP-13 mRNA.
(B) Sequence alignment of a putative miR-127-5p binding site within the 30 UTR of MMP-13 mRNA shows a high level of sequence conservation and complementarity with miR-127-5p.
(C) Interaction between miR-127-5p and MMP-13 was verified by lucif_x0002_erase report assay.
(D and E) The effects of miR-127-5p on MMP-13 expression in chondrocytes were analyzed by qPCR (D) and western blot (E). *p < 0.01.

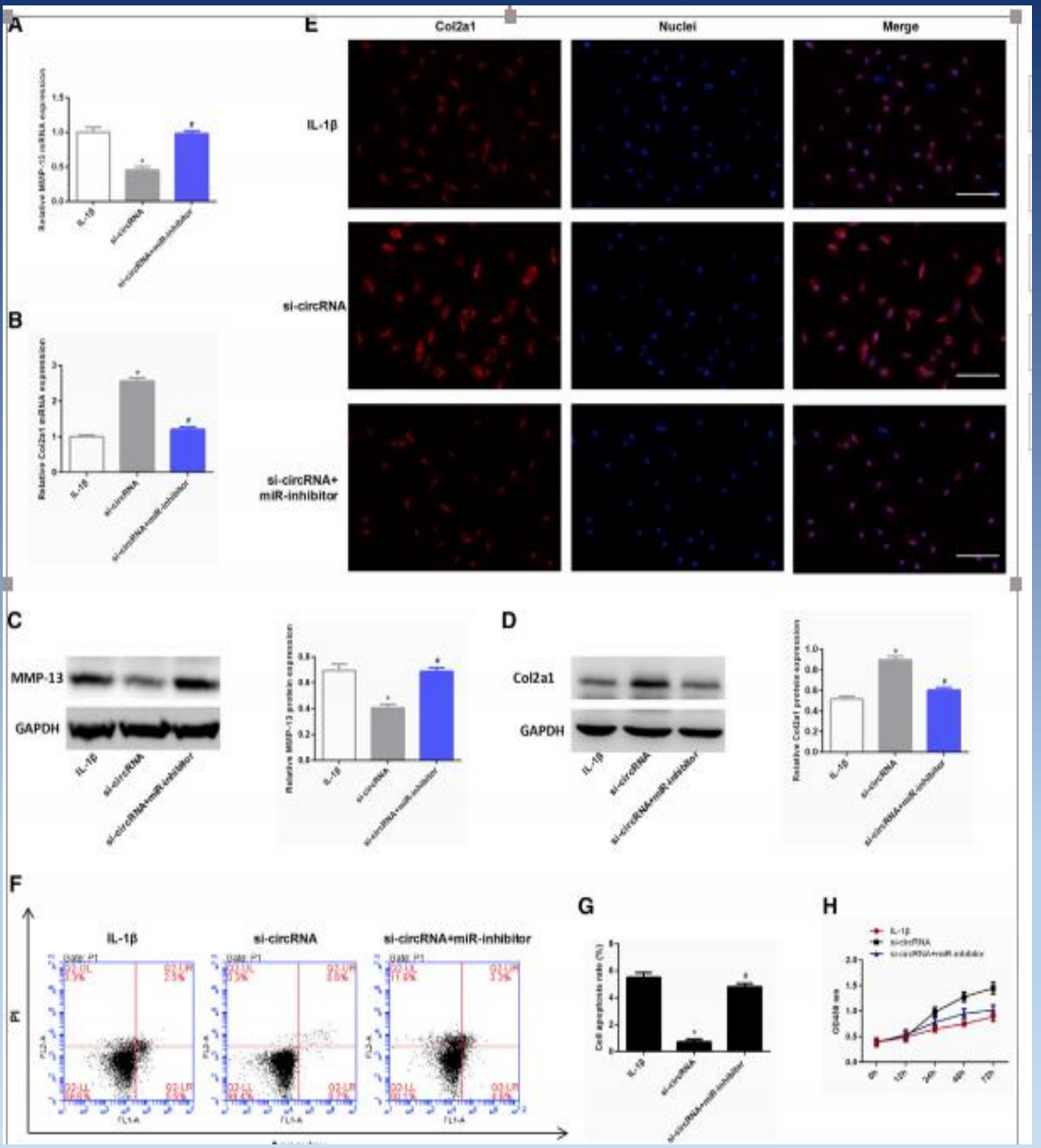

Figure 7. circRNA.33186 Exerts Biological Functions in Chondrocytes via Sponging miR-127-5p

Chondrocytes were transfected with si-circRNA or a combination of si-circRNA and miR-127-5p inhibitor, and then treated with 10 ng/mL IL-1b for 24 h. (A and B) The mRNA levels of MMP-13 (A) and Col2a1 (B) were determined by qRT-PCR. (C and D) The protein levels of MMP-13 (C) and Col2a1 (D) were determined by western blot. (E) The protein levels of Col2a1 were determined by immunofluorescent staining. Scale bars, 20 mm. (F and G) Cell apoptosis was measured by flow cytometric analysis (F), and the results of flow cytometric analysis are presented as percentages of positive mean values ± SD. (H) Cell proliferation in vitro was determined by CCK8 assay. *$p < 0.01$,compared with IL-1b group; #

$p < 0.01$, compared with si-circRNA group

DISCUSSION

Although considerable amounts of studies have been designed to un_x0002_ravel the pathology of OA, the medical treatment of OA still focuses on relieving symptomatic synovial joint pain.3 Therefore, it is urgent to explore the molecular mechanisms underlying OA progression and identify novel drug targets for OA therapy.circRNAs are a class of newly discovered non-coding RNAs, and increasing evidence shows they could be used as diagnostic biomarkers and therapeutic targets for various diseases. For example,Li et al.20 found that hsa_circ_0004277 could be used as a biomarker for acute myeloid leukemia. Zhu et al.21 reported that a circRNA,hsa_circ_0013958, might be a potential novel biomarker for lungFigure 6. MMP-13 Is a Direct Target of miR-127-5p(A) Putative miR-127-5p binding site in the 30 UTR ofMMP-13 mRNA. (B) Sequence alignment of a putative miR-127-5p binding site within the 30 UTR of MMP-13 mRNA shows a high level of sequence conservation and complementarity with miR-127-5p. (C) Interaction between miR-127-5p and MMP-13 was verified by luciferase report assay. (D and E) The effects of miR-127-5p on MMP-13 expression in chondrocytes were analyzed by qPCR (D) and western blot (E). *p < 0.01.adenocarcinoma.

With respect to therapeutic functions, Wang et al.9 revealed that a heart_x0002_related circRNA acts as a positive regulator to inhibit cardiac hypertrophy and heart failure.Cheng et al.22 showed circRNA VMA21 protects against intervertebral disc degeneration through targeting miR-200c and X-linked inhibitor-of-apoptosis protein. However, few reports describe the role of circRNAs in OA.In this study, we found that circRNA.33186 was upregulated in IL-1b-treated chondrocytes and cartilage tissues of the DMM-induced OA model, and the expression level was positively correlated with cartilage degeneration, indicating that circRNA.33186 may be associated with the development and progression of OA. More importantly, upregulation of circRNA.33186 was determined at an early stage during OA development, suggesting the possibility that circRNA.33186 could be used as a diagnostic biomarker for this disorder. Further experiments showed that knockdown of circRNA.33186 corrected the imbalance between anabolic and catabolic factors (e.g., type II collagen and MMP-13), promoted cell growth, and inhibited cell apoptosis in IL-1b-treated chondrocytes,demonstrating that circRNA.33186 plays a vital role in OA progres_x0002_sion and may be a therapeutic target

It is widely accepted that IA injection of drugs, such as corticosteroids and hyaluronic acid, are effective means for the clinical treatment of OA. IA injection of miRNAs as a therapeutic method for OA in animal models has been also described in recent years.23 IA injection of miR-140 alleviates OA progression by modulating ECM homeostasis in rats.24 Silencing of miR-101 via IA injection of adenovirus prevents cartilage degradation by regulating ECM-related genes in a rat model of OA.25 Here we found that IA injection of lentivirus-incorporated sicircRNAs against circRNA.33186 successfully decreased the expression of circRNA.33186 in cartilage tissues of the DMM-induced OA model, and silencing of circRNA.33186 in vivo significantly alleviated DMM-induced cartilage destruction in mice. Moreover, the effect of DMM surgery on the expression of type II collagen and MMP-13 was also reversed by knockdown of circRNA.33186. These results further confirmed the therapeutic potential of circRNA.33186 in OA

Recently, circRNAs have been shown to act as miRNA sponge to regulate gene expression.6,16 Han et al.11 found that circRNA MTO1 directly binds to miR-9 and inhibits miR-9 activity to suppress hepatocellular cancer progression. Zheng et al.17 reported that circHIPK3 acts as the sponge of miR-124 to function as a cell-growth modulator. In the present study, we found that circRNA.33186 was mainly localized in the cytoplasm, suggesting it might function as an miRNA sponge. We then identified a group of miRNAs that might interact with circRNA.33186 by means of bioinformatics analyses, and validated the interacting relationship between circRNA.33186 and miR-127-5p using luciferase activity assays. We also found that the expression level of miR-127-5p was negatively correlated with circRNA.33186. Therefore, these data suggest that circRNA.33186 could directly target miR-127-5p.by functioning as a sponge, and that miR-127-5p may play an important role in chondrocytes.To date, more and more miRNAs have been found to play central roles in the pathogenesis and progression of OA by regulating ECM anabolism and catabolism of chondrocytes.

It has been showed that silencing of miR-34a prevents IL-1b-induced ECM degradation in chondrocytes.30 miR-145 was reported to attenuate tumor necrosis factor alpha (TNF-a-driven cartilage matrix degradation in OA via direct suppression of MKK4.31 Previous study revealed that miR- 127-5p is an important regulator of MMP-13 in human chondrocytes and may contribute to the development of OA.19 MMP-13 is a pivotal catabolic factor of OA that degrades type II collagen, a major component of cartilage ECM in chondrocytes.32–34 Here, our results further confirmed that miR-127-5p could directly target MMP-13 and regu_x0002_late type II collagen expression in IL-1b-treated mouse chondrocytes. Several lines of evidence indicate that circRNA.33186 functions as a sponge of miR-127-5p to regulate OA progression. First, bioinformatics analyses showed that the 30 UTR of MMP-13 and circRNA.33186 contains binding sites for miR-127-5p. Second, luciferase reporter assays verified this prediction. Third, knockdown of circRNA.33186 reduced MMP-13 expression. Finally, inhibition of miR-127-5preversed the effect of circRNA.33186 knockdown.

Taken together, our study indicates that the expression level of circRNA.33186 is upregulated in OA. Silencing of circRNA.33186 markedly alleviates OA progression both in vitro and in vivo by regu_x0002_lating catabolic and anabolic factors, promoting cell growth, and in_x0002_hibiting cell apoptosis of chondrocytes. Also, circRNA.33186 may exert these biological functions by acting as a sponge for miR-127-5p. Our findings demonstrate that circRNA.33186 can be used as a diagnostic biomarker and potential target in OA therapy.Although our study shed light on a new option for OA treatment,further investigations are still necessary. For example, more studies are needed to determine whether circRNA.33186 contributes to OA through other molecules and pathways. Because IA injection of lentivirus infects not only articular cartilage but also the synovium, which plays an important role in OA development, further studies are required to identify the role of circRNA.33186 in synovium tissue, articular chondrocytes, or other cell types. Finally, more effective vec_x0002_tors should be identified for circRNA delivery via joint injection Finding the answers to these questions will be critical for a better understanding of the functions of circRNAs in OA pathogenesis and for offering feasible therapeutic targets for clinical use.

Osteoarthritis (OA), the most prevalent age-related joint disorder, is characterized by chronic inflammation, progressive artic_x0002_ular cartilage destruction, and subchondral bone sclerosis.Accumulating evidences indicate that circular RNAs (circRNAs)play a critical role in various diseases, but the function of circRNAs in OA remains largely unknown. Here we showed that circRNA.33186 was significantly upregulated in IL-1b)-treated chondrocytes and in cartilage tissues of a destabilized medial meniscus (DMM)-induced OA mouse model. Knock down of circRNA.33186 increased anabolic factor (type II collagen) expression and decreased catabolic factor (MMP-13) expression. Knockdown of circRNA.33186 also promoted proliferation and inhibited apoptosis in IL-1b-treated chondrocytes. Silencing of circRNA.33186 in vivo markedly alleviated DMM-induced OA. Mechanistic study showed that circRNA.33186 directly binds to and inhibits miR-127-5p,thereby increasing MMP-13 expression, and contributes to OA pathogenesis. Taken together, our findings demonstrated a fundamental role of circRNA.33186 in OA progression and provide a potential drug target in OA therapy.

THANK YOU