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*Original Article*

# MicroRNA-497-5p attenuates IL-1β-induced cartilage matrix degradation in chondrocytes via Wnt/β-catenin signal pathway

Liying Hou[1], Hui Shi[4], Mingming Wang[5], Jinghua Liu[2], Guoqiang Liu[3]

*Departments of [1]Orthopeadic Surgery, [2]Integrated Traditional Chinese and Western Medicine, [3]Traumatology, The Second People's Hospital of Dongying, Dongying, Shandong, China; [4]Department of Bone and Joint Surgery, Binzhou Meidical University Hospital, Binzhou, Shandong, China; [5]Department of Orthopeadic Surgery (C), The People's Hospital of Binzhou, Binzhou, Shandong, China*

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**Abstract:** Osteoarthritis (OA) is a degenerative joint disease. Degradation of extracellular matrix (ECM) in chondrocytes is closely related to joint destruction in OA progression. MicroRNAs (miRNAs) have been reported to play important roles in progression of OA. However, the roles of miR-497-5p in OA process and its underlying mechanism remain not been well established. Chondrocytes were obtained from articular cartilage and stimulated with IL-1β. The expression of miR-497-5p and Wnt3a was detected by qRT-PCR. Western blot analysis was performed to measure the proteins of Wnt3a, collagen II, aggrecan matrix metalloproteinase (MMP) 13 and ADAMTS4. Cell apoptosis was detected by flow cytometry. The putative binding sites of miR-497-5p and Wnt3a were predicted by Targetscan and verified through luciferase report assay. We found that miR-497-5p expression was reduced and Wnt3a expression was enhanced in OA cartilage and IL-1β-stimulated chondrocytes. Moreover, Wnt3a was a direct target of miR-497-5p, and expression of miR-497-5p was negatively correlated with Wnt3a level in OA cartilage. Furthermore, overexpression of miR-497-5p prominently increased the expression of cartilage matrix molecules collagen II and aggrecan, and reduced the expression of matrix-degrading enzymes MMP13 and ADAMTS4 while overexpression of Wnt3a reversed these effects, whereas addition of DKK-1attenuated the Wnt3a-mediated functions in IL-1β-stimulated chondrocytes. In conclusion, miR-497-5p attenuated IL-1β-induced cartilage matrix degradation in chondrocytes via Wnt/β-catenin signal pathway, providing a potential therapeutic target for treatment of OA.

**Keywords:** Osteoarthritis, miR-497-5p, Wnt/β-catenin, extracellular matrix

## Introduction

Osteoarthritis (OA) is the most prevalent chronic joint disease in the elderly, and it is one of the main causes of the health and economic burden in the elderly population around the world [1]. Articular cartilage consists of a small amount of chondrocytes and a large number of extracellular matrices (ECM). OA is characterized by degradation of ECM macromolecules and reduced expression of chondrocytes proteins, which results in severe joint pain, limitation of movement, and joint inflammation [2, 3]. Chondrocytes are the only cells found in the articular cartilage and are responsible for the synthesis and turnover of ECM, which play a critical role in joint function [4]. However, the underlying molecular mechanisms regulating chondrogenesis during OA progression remains largely unknown.

ECM is mainly composed of type II collagen, aggrecan and other matrix macromolecules to maintain the cartilage structure and the homeostasis of the extracellular environment [5]. It has been reported that the pro-inflammatory cytokine interleukin (IL)-1β plays a key role in the pathogenesis of OA, and it can inhibit cell proliferation, decrease the synthesis of aggrecan and type II collagen, and induce the release of matrix metalloproteinase (MMP) 13 in chondrocytes, and cause the insufficient synthesis of chondrocytes ECM [6, 7]. However, their effects in chondrocyte ECM degradation have not been fully established.

MicroRNAs (miRNAs) are small non-coding RNAs with about 22 nucleotides in length, which negatively regulate gene expression by preventing mRNA translation and promoting mRNA degradation [8, 9]. Recent studies have shown that miRNAs are associated with many human diseases, including OA [10, 11]. For instance, overexpression of miR-210 could suppress the activation of NF-κB signaling pathway by targeting DR6 in OA [12]. Previous documents showed that miR-497-5p was involved in the progression of many cancers, such as hepatocellular carcinoma and osteosarcoma [13, 14]. A previous report revealed that the expression of miR-497-5p was downregulated in human OA chondrocytes treated with IL-β [15]. However, the exact roles of miR-497-5p in progression of OA and its underlying mechanism remain poorly understood.

The emerging evidence has suggested that many miRNAs can regulate the Wnt signaling pathway in number of diseases [16]. Wnt proteins are approximately 40 kDa in size and contain many conserved cysteines, and the Wnt3a gene is a vital member of the Wnt ligand family and exerts its effect via activating the canonical Wnt signaling pathway [17, 18]. The canonical Wnt/β-catenin signaling widely participate in variety of cellular process, including cell proliferation, cell survival, and migration, and is inappropriately activated in many cancers and other diseases [19, 20]. This signaling pathway functions are mediated through β-catenin, and the β-catenin acts as intracellular signal transduction in the Wnt/β-catenin signaling pathway [21, 22]. Recent studies have shown that Wnt/β-catenin signaling also involved in OA progression [23, 24]. However, little is known about its possible use in OA treatment.

In this study, we detected the expression levels of miR-497-5p and Wnt3a in OA cartilage tissues and cells. Moreover, we investigated the relationship of miR-497-5p and Wnt3a, and explored their possible effects on ECM degradation in IL-1β-stimulated chondrocytes, and might provide a novel approach for the treatment of human OA.

## Materials and methods

### *Tissue samples*

Human cartilage samples were obtained from 12 osteoarthritis patients who accepted total joint replacement, and normal human cartilage samples were collected from 10 traumatic amputees without rheumatoid arthritis or OA. All patients with OA were diagnosed according to the American College of Rheumatology (ACR). All samples were collected from the Second People's Hospital of Dongying. The written informed consent was received from all patients, and this work approved by the Ethics Committee of the Second People's Hospital of Dongying.

### *Cell culture and transient transfection*

After collecting the cartilage specimens, the tissues were chopped finely with a scalpel blade. Cartilage small slices were sequentially digested with trypsin (0.1%; Sigma, St. Louis, MO, USA) at 37°C for 10 min. After removing the trypsin solution, the tissue slices were incubated with 0.04% collagenase type II (CLS-2; Worthington, Lakewood, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 5% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) for 16 h at 37°C. Then the sample was filtered through a 100-μm cell strainer (Falcon; Becton-Dickinson Labware GmbH, Heidelberg, Germany) to remove the undigested cartilage and centrifuged at apace of 1000 r/min for 5 min. The supernatant was discarded and the cells were maintained in culture medium consisting of DMEM with 10% FBS at 37°C in an incubator supplemented with 5% $CO_2$.

MiR-497-5p mimics, miR-negative control (miR-NC), miR-497-5p inhibitor, miR-NC inhibitor, Wnt3a overexpression vector (Wnt3a) and empty vector were obtained from GenePharma (Shanghai, China). Chondrocytes were transfected with oligonucleotides or vector using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) for 48 h, following the manufacturer's protocol. After that, the medium would be discarded, and chondrocytes were treated with IL-1β for 24 h.

### *qRT-PCR*

Total RNA was isolated from cartilage samples or chondrocytes utilizing the Trizol reagent

(Invitrogen) referring the manufacturer's instructions. First strand complementary DNA (cDNA) was synthesized by reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) or microRNA reverse transcription Kit (Thermo Fisher Scientific). Subsequently, qRT-PCR analysis was performed using the quantitative SYBR Green PCR kit (Toyobo, Tokyo, Japan) on an ABI 7005HT fast real-time PCR system (Applied Biosystems, Foster City, CA, USA).The primers of miR-497-5p, Wnt3a, U6, and GAPDH were purchased from Sangon Biotech (Shanghai, China) and listed below: miR-497-5p (sense, 5'-CCTTCAGCAGCACACTGTGG-3'; antisense, 5'-CAGTGCAGGGTCCGAGGTAT-3'); Wnt3a (sense, 5'-CTCCTCTCGGATACCTCTTAGTG-3'; antisense, 5'-GCATGATCTCCACGTAGTTCCTG-3'); U6 (sense, 5'-ATTGGAACGATACAGAGAAGATT-3'; antisense, 5'-GGAACGCTTCACGAATTTG-3'); GAPDH, (sense, 5'-GACTCATGACCACAGTCCATGC-3'; antisense, 5'-AGAGGCAGGGATGATGTTCTG-3'). The relative expression level of miR-497-5p or Wnt3a was normalized to U6 or GAPDH, respectively, and calculated using the $2^{-\Delta\Delta Ct}$ method.

*Western blot assay*

Cartilage samples and chondrocytes were lysed in RIPA lysis buffer (Sigma) containing protease inhibitor (Sigma) for 30 min on the ice. Subsequently, total protein concentration was determined using BCA Protein Assay Kit (Pierce Rockford, IL, USA). Next, equal amount of total protein was loaded on SDS-PAGE (8%-12%) gels for electrophoresis, and then the gels were transferred to PVDF membrane (Millipore Corp, Atlanta, GA, USA). After blocking for 2 h in TBST containing 5% (w/v) non-fat milk, the membranes were incubated overnight at 4°C with primary antibodies against Wnt-3a, collagen II, aggrecan, MMP 13, ADAMTS4, β-catenin, and GAPDH (1:1000; Abcam, Cambridge, UK). After that, the membranes were incubated with HRP-conjugated secondary antibodies (1:4000, Sangon Biotech) for 2 h at room temperature. Finally, the band signals were visualized by chemiluminescence (ECL) reagent. The densitometry of the bands were quantified using the Image J software and normalized to GAPDH.

*Apoptosis assay*

To explore the apoptosis-induced effect of miR-497-5p in chondrocytes, the ratio of apoptosis was detected by flow cytometry with Annexin V-FITC/PI apoptosis kit (Sigma). Briefly, chondrocytes were seeded into six-well plates and treated with miR-497-5p mimics, miR-NC mimics, miR-497-5p inhibitor, and miR-NC inhibitor for 48 h. After that, chondrocytes were collected and stained with Annexin V-FITC and PI for 15 min in the darkness. Finally, cell apoptosis was examined by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA), and the apoptosis rate was analyzed by the flow cytometer software.

*Dual-luciferase reporter assay*

As suggested by the bioinformatics software (TargetScan), Wnt3a was a direct target of miR-497-5p. The sequences of wild type Wnt3a (Wnt3a-WT) and mutant Wnt3a (Wnt3a-MUT) containing binding sites of miR-497-5p were synthesized and inserted into pGL3 plasmids (Promega, Madison, WI, USA). The Wnt3a-WT or Wnt3a-MUT reporter vector co-transfected with miR-497-5p mimics or miR-NC mimics into chondrocytes for 48 h. Subsequently, the relative luciferase activity was detected in cell lysates by a dual-luciferase assay kit (Promega).

*Statistical analysis*

All experiments were expressed as the mean ± standard deviation (SD) from at least three independent experiments. Student's *t* test was used to assess the significant difference between two groups. The correlation between miR-497-5p and Wnt3a was evaluated by Pearson correlation coefficient. All results were analyzed by GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA). $P<0.05$ was commonly considered as statistically significant.

## Results

*MiR-497-5p was downregulated and Wnt3a was upregulated in OA cartilage*

To explore the potential roles of miR-497-5p and Wnt3a in OA, qRT-PCR and Western blot analysis were performed to confirm the expression of miR-497-5p and Wnt3a in cartilage. Results showed that the expression of miR-497-5p was evidently downregulated in OA cartilage (n=12) compared to normal cartilage (n=10) (**Figure 1A**). In addition, the expression

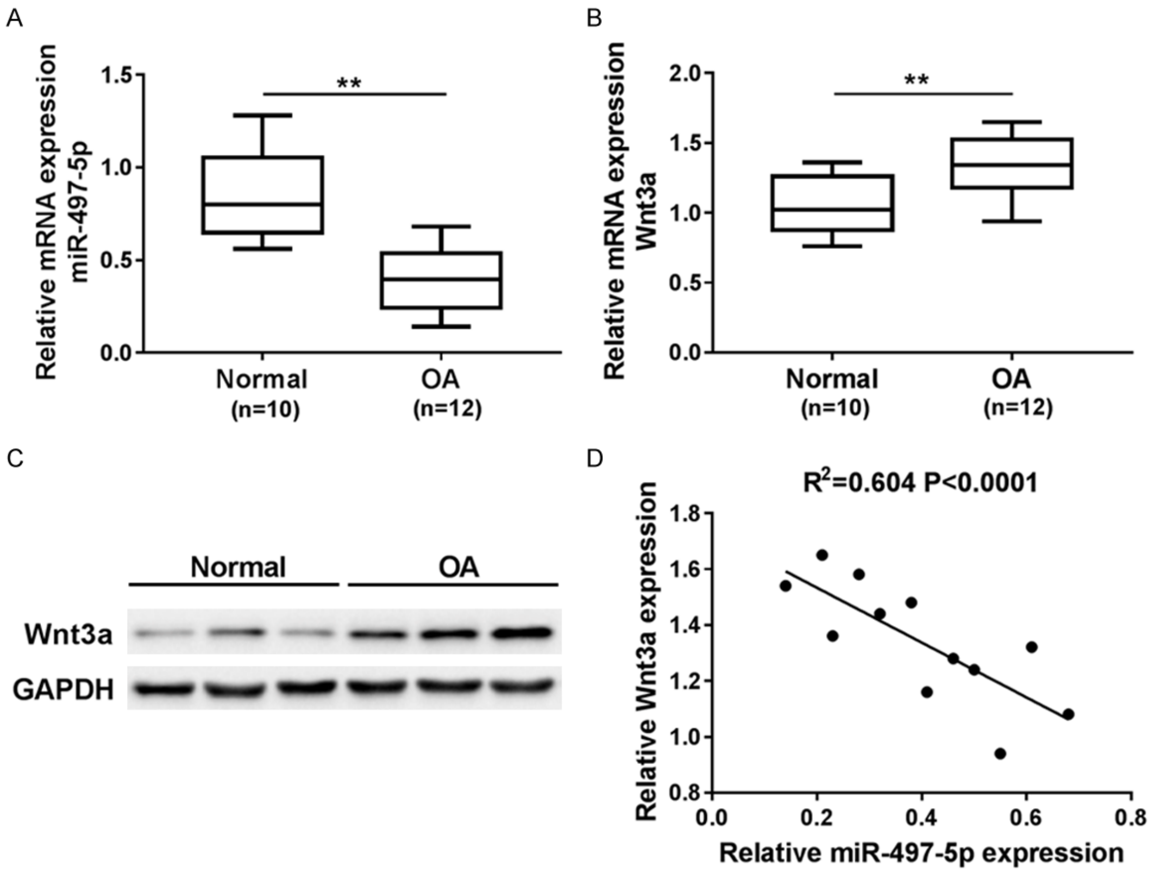


**Figure 1.** MiR-497-5p was downregulated and Wnt3a was upregulated in OA cartilage. A and B. The expression levels of miR-497-5p and Wnt3a were measured in OA cartilage and normal cartilage by qRT-PCR. C. The protein of Wnt3a was detected by Western blot. D. The correlation between miR-497-5p expression and Wnt3a expression in cartilage samples was analyzed. Data are presented as the mean ± SD, and *p* values were determined by two-sample independent *t*-tests $^{**}P<0.01$.

level of Wnt3a was obviously higher in OA cartilage than normal cartilage (**Figure 1B**). Similarly, the protein of Wnt3a was also upregulated in OA cartilage compared to normal cartilage (**Figure 1C**). Moreover, correlations between levels of miR-497-5p and Wnt3a were analyzed using Pearson correlation coefficient, and we found that expression of miR-497-5p was negatively correlated with Wnt3a expression in OA cartilage (**Figure 1D**). These findings showed that miR-497-5p was greatly decreased and Wnt3a was notably increased in OA cartilage.

*MiR-497-5p was decreased and Wnt3a was increased in IL-1β-stimulated chondrocytes*

To further explore the effects of miR-497-5p and Wnt3a in OA, the expression levels of miR-497-5p and Wnt3a were determined in IL-1β-stimulated chondrocytes by qRT-PCR and Western blot analysis. Our results suggested that the abundance of miR-497-5p was reduced in a dose-dependent manner in IL-1β-stimulated chondrocytes (**Figure 2A**). According to **Figure 2B**, the expression of miR-497-5p was downregulated in a time-dependent manner in chondrocytes induced with IL-1β (5 ng). Moreover, the expression of Wnt3a was elevated in a dose-dependent manner in IL-1β-stimulated chondrocytes (**Figure 2C**). Furthermore, the expression of Wnt3a also increased in a time-dependent manner in chondrocytes induced with IL-1β (5 ng) (**Figure 2D**). Similarly, the protein of Wnt3a was markedly upregulated in chondrocytes induced with IL-1β (**Figure 2E**). Thus, these findings proved that miR-497-5p was downregulated and Wnt3a was upregulated in IL-1β-stimulated chondrocytes *in vitro*.

*Overexpression of miR-497-5p inhibited ECM degradation in IL-1β-stimulated chondrocytes*

To explore the involvement of miR-497-5p in the regulation of IL-1β-induced expressions of

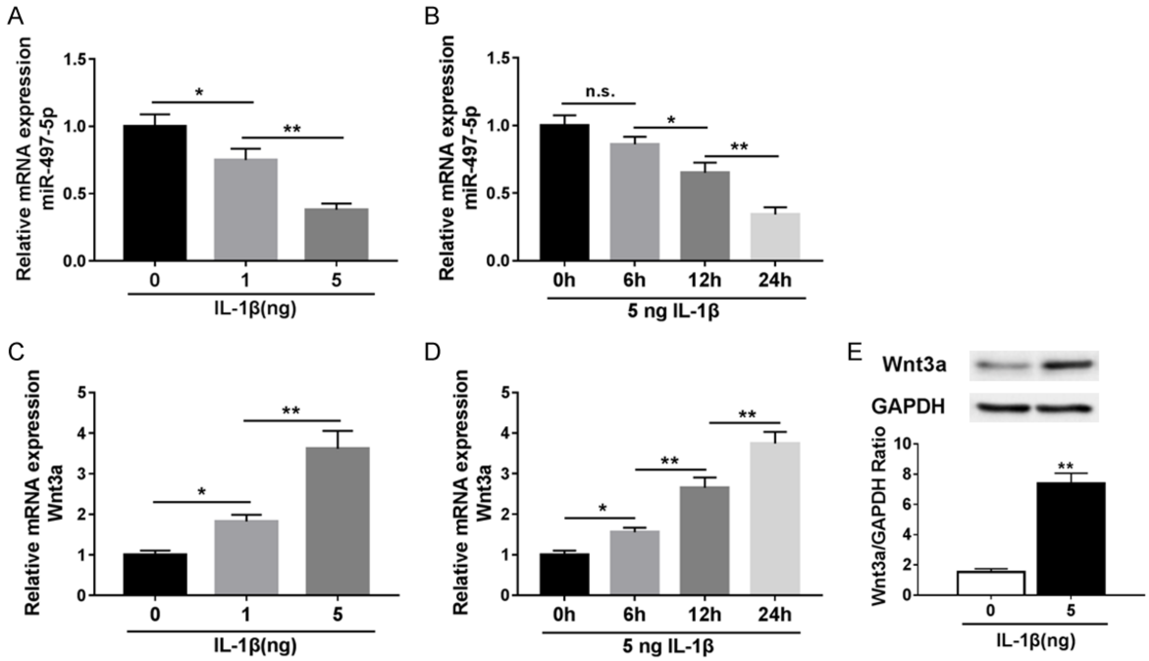


**Figure 2.** MiR-497-5p was reduced and Wnt3a was enhanced in IL-1β-stimulated chondrocytes. A-D. The expression levels of miR-497-5p and Wnt3a were determined in IL-1β-stimulated chondrocytes by qRT-PCR. E. The protein level of Wnt3a was detected IL-1β-stimulated chondrocytes by Western blot. Data are presented as the mean ± SD of three independent experiment, and *p* values were determined by two-sample independent *t*-tests. *$P<0.05$, **$P<0.01$.

matrix-degrading enzymes and matrix molecules, chondrocytes were transfected with miR-497-5p mimics or inhibitor. Analysis of qRT-PCR indicated that transfection of miR-497-5p mimics led to a significant increase of miR-497-5p abundance in chondrocytes and its knockdown showed an opposite effect (**Figure 3A** and **3B**). However, overexpression or inhibition of miR-497-5p had any effect on chondrocytes apoptosis, indicating miR-497-5p would not affect the viability of chondrocytes (**Figure 3C** and **3D**). Besides, overexpression of miR-497-5p promoted the mRNA and protein levels of type II collagen (collagen II) and aggrecan while inhibited the mRNA and protein levels of MMP13 and ADAMTS4 in IL-1β-stimulated chondrocytes, whereas inhibition of miR-497-5p caused opposite effects (**Figure 3E** and **3F**). These data clearly showed that miR-497-5p might play a critical role in OA.

*MiR-497-5p directly targeted Wnt3a*

To investigate the regulation mechanism of miR-497-5p, bioinformatics software Targetscan was used to predict the target gene. We found that Wnt3a was a putative target of miR-497-5p (**Figure 4A**). Subsequently, the prediction was confirmed by luciferase activity assay. Results indicated that transfection of miR-497-5p mimics dramatically inhibited the luciferase activity in chondrocytes transfected with Wnt3a-WT, whereas the effect could not change the luciferase activity of chondrocytes transfected with Wnt3a-MUT (**Figure 4B**). Moreover, addition of miR-497-5p significantly reduced the mRNA and protein levels of Wnt3a and β-catenin, while miR-497-5p knockdown had an opposite effect in chondrocytes (**Figure 4C-4G**). Thus, our data suggested that Wnt3a was a direct target of miR-497-5p in chondrocytes.

*MiR-497-5p regulated EMC homeostasis via Wnt/β-catenin signaling pathway in IL-1β-stimulated chondrocyte*

To further explore the molecular mechanism of miR-497-5p regulating the biological processes in OA, qRT-PCR and Western blot analysis were used to detect the mRNA and protein levels related to the EMC degradation in chondrocytes transfected with miR-NC mimics, miR-497-5p mimic, miR-497-5p mimic+vector, miR-

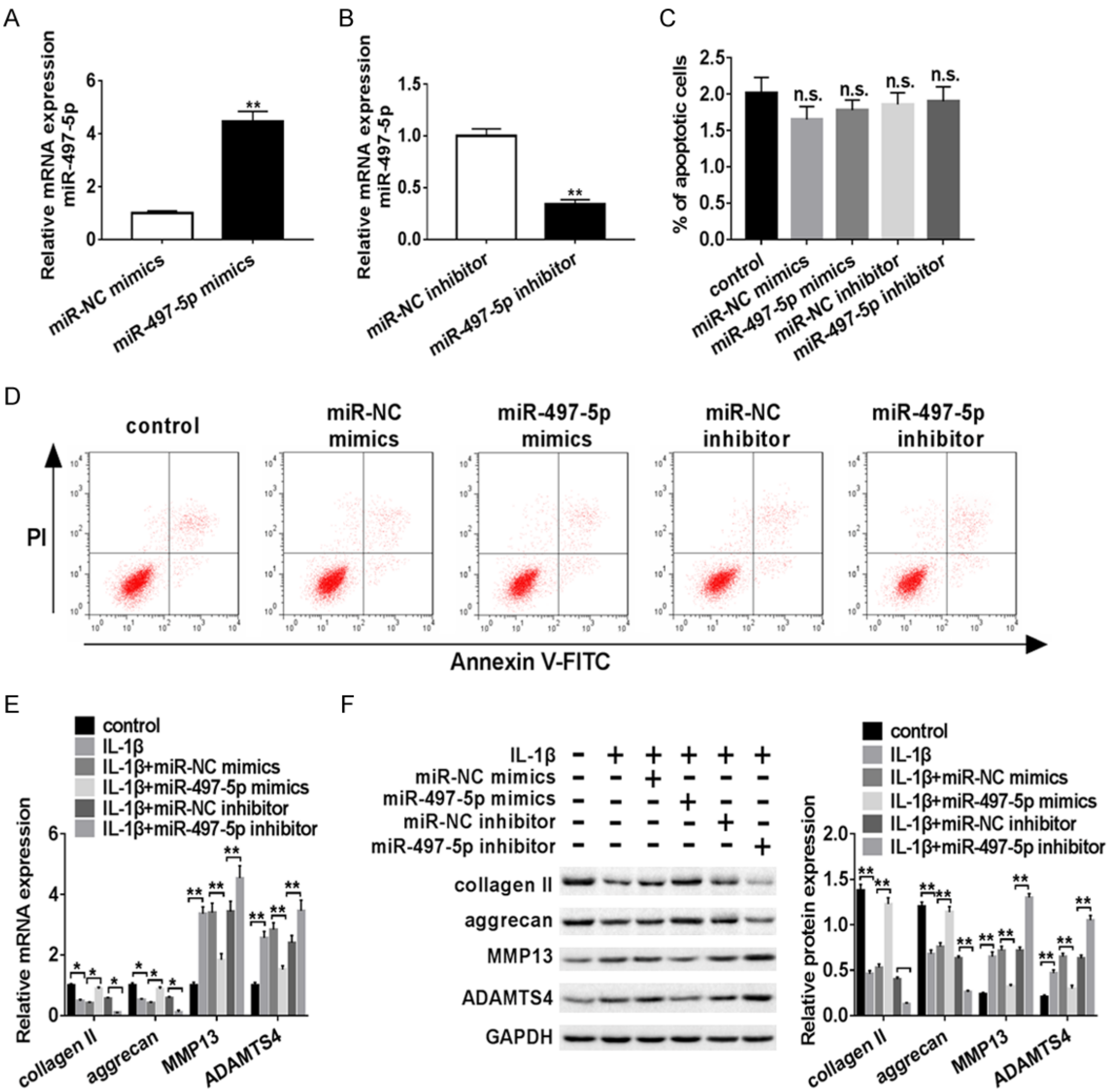


**Figure 3.** Overexpression of miR-497-5p inhibited ECM degradation in IL-1β-stimulated chondrocytes. After transfection with miR-497-5p mimic or inhibitor, chondrocytes were stimulated with 5 ng/ml IL-1β for 24 h. A and B. The expression of miR-497-5p was analyzed using qRT-PCR. C and D. Cell apoptosis was detected by flow cytometry and analyzed by the flow cytometer software. E and F. The mRNA and protein levels of collagen II, aggrecan, MMP13 and ADAMTS4 were assessed by qRT-PCR and Western blot, respectively. Data are shown as the mean ± SD of three independent experiment, and *p* values were determined by two-sample independent *t*-tests **P*<0.05, ***P*<0.01.

497-5p mimic+Wnt3a or miR-497-5p mimic +Wnt3a+DKK-1 and stimulated with IL-1β for 24 h. DKK-1 is an inhibitor of the Wnt/β-catenin receptor, which is added to cells at a concentration of 0.2 μg/ml to block activation of the Wnt/β-catenin signaling pathway. Results indicated that transfection of miR-497-5p prominently increased the mRNA and protein levels of collagen II and aggrecan and reduced the expressions of MMP13 and ADAMTS4 while overexpression Wnt3a reversed the effects, whereas addition of DKK-1weaken the Wnt3a-mediated functions in IL-1β-stimulated chondrocyte (**Figure 5A-5I**). The findings indicated that miR-497-5p could regulate EMC homeostasis through Wnt/β-catenin signaling pathway in IL-1β-stimulated chondrocyte.

## Discussion

OA is a complex inflammatory disease, and there is currently no effective treatment to reverse damaged cartilage. Numerous studies have suggested that aberrant expression miR-

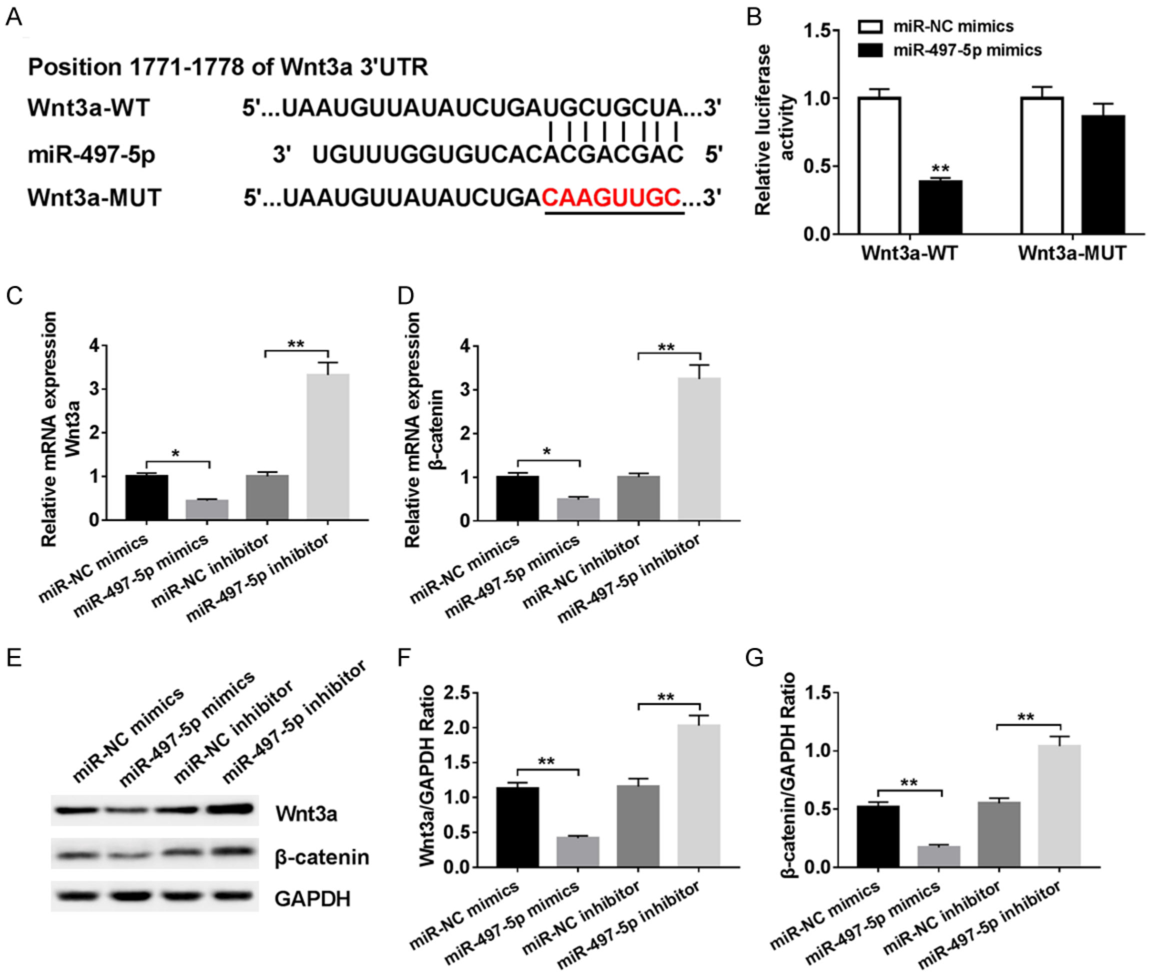


**Figure 4.** Wnt3a was a direct target miR-497-5p. A. The potential binding sites of miR-497-5p and Wnt3a were predicted by Targetscan. B. The luciferase activity was measured in chondrocytes co-transfected with Wnt3a-WT or Wnt3a-MUTT and miR-497-5p mimics or miR-NC mimics. C and D. The expression levels of Wnt3a and β-catenin were detected in chondrocytes transfected with miR-497-5p mimic or inhibitor by qRT-PCR. E-G. The proteins expression of Wnt3a and β-catenin were measured by Western blot. Data are presented as the mean ± SD of three independent experiment, and *p* values were determined by two-sample independent *t*-tests. **P*<0.05, ***P*<0.01.

NAs are closely related to many human diseases, including OA [25, 26]. Therefore, it is particularly important to determine how to regulate miRNA expression in OA. Since IL-1β plays a vital role in the pathogenesis of OA, chondrocytes stimulated with IL-1β *in vitro* has often been used to mimic the microenvironment which occurs in OA [27].

Previous studies indicated that the abundance of miR-497-5p was decreased in various cancer, including breast cancer, cervical cancer and colorectal cancer [28-30]. Moreover, it has been reported that OA relevant gene NOS2 is predicted to be the target of miR-497-5p, and the miR-497-5p is also decreased in OA chondrocytes [15]. Consistent with this study, the level of miR-497-5p in OA cartilage was significantly lower than those in normal cartilage. In addition, the expression level of miR-497-5p was downregulated in a dose/time-dependent manner in chondrocytes induced with IL-1β. Moreover, neither overexpression nor knockdown of miR-497-5p not affected the viability of chondrocytes. Thus, the results showed that miR-497-5p might be a more effective target for OA treatment.

Several researchers have been reported that miRNAs are found to regulate components of the Wnt signaling pathway, and miRNAs and Wnt signaling pathway interact to regulate different biological processes [31, 32]. For example, miR-612 inhibited stemness of hepatocel-

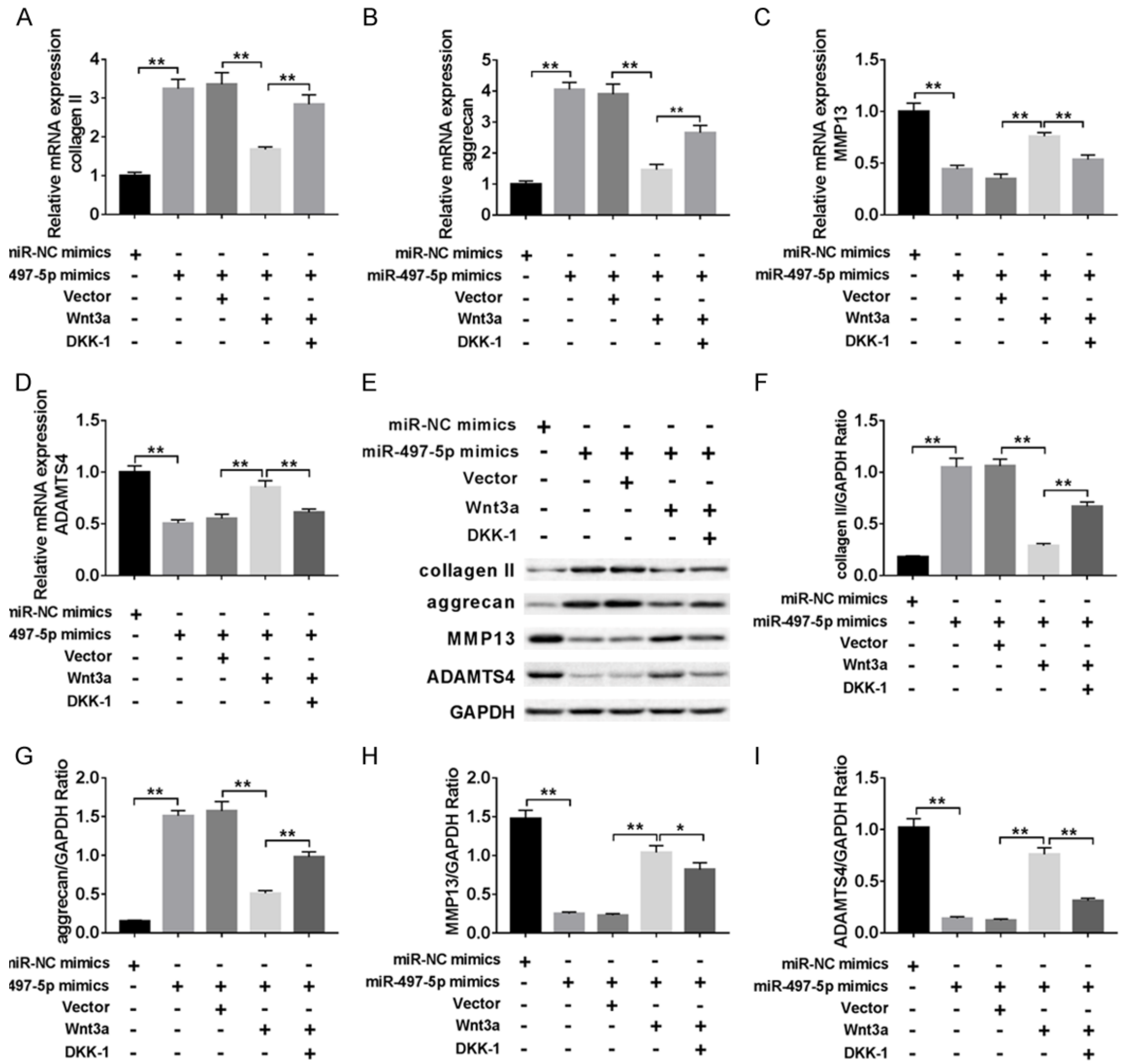

**Figure 5.** MiR-497-5p regulated EMC homeostasis via Wnt/β-catenin signaling pathway in IL-1β-stimulated chondrocyte. After transfection with miR-NC mimics, miR-497-5p mimic, miR-497-5p mimic+vector, miR-497-5p mimic+Wnt3a or miR-497-5p mimic+Wnt3a+DKK-1, chondrocytes were stimulated with 5 ng/ml IL-1β for 24 h. A-D. Collagen II, aggrecan, MMP13 and ADAMTS4 expression levels were assessed by qRT-PCR. E-I. The proteins of collagen II, aggrecan, MMP13 and ADAMTS4 were analyzed by Western blot. Data are expressed as the mean ± SD of three independent experiment, and *p* values were determined by two-sample independent *t*-tests. **P*<0.05, ***P*<0.01.

lular carcinoma by Wnt/β-catenin signaling pathway [33]. Wnt3a is a canonical Wnt ligand, and it has been considered to be an activator of the canonical Wnt signaling pathway [34]. Besides, Wnt3a could induce the accumulation of β-catenin, and the β-catenin is a vital regulator of Wnt pathway [35]. The Wnt/β-catenin signaling pathway is normally activated in many human diseases, including AO [36, 37]. For example, inhibition of EZH2 ameliorates development of OA via the Wnt/β-catenin signaling pathway [38]. Overexpression of miR-1 controls the OA development through targeting FZD7 of Wnt/β-catenin signaling pathway [39]. In our study, we found the expression of Wnt3a was obviously higher in OA cartilage than normal cartilage, the mRNA and protein levels of Wnt3a was increased in IL-1β-stimulated chondrocytes. These results indicated that Wnt/β-catenin signaling pathway might be activated. Besides, correlations between levels of miR-497-5p and Wnt3a were analyzed by Pearson correlation coefficient, and it found that expression of miR-497-5p was negatively correlated with Wnt3a expression in OA cartilage. Moreover, the bioinformatics software predicted that Wnt3a was a direct target of miR-497-5p, and the prediction was confirmed by luciferase activity analysis. Furthermore, addition of miR-497-5p significantly reduced the mRNA and protein levels of Wnt3a and β-catenin, while miR-497-5p knockdown had an opposite effect in chondrocytes. These findings indicated that miR-497-5p might regulate the Wnt/β-catenin signaling pathway in OA.

The emerging evidence has suggested that Wnt signaling pathway can induce the expression of MMP [40]. During OA pathogenesis, chondrocytes produce matrix-degrading enzymes, such as MMP 13 and ADAMTS4, and stop synthesis of cartilage matrix molecules, including type II collagen and aggrecan, destroying the metabolic homeostasis of ECM [41]. Previous reports revealed that the expressions of MMP13 and ADAMTS4 were upregulated in human chondrosarcoma cells [42]. In addition, it has been proved that the expressions of type II collagen and aggrecan were decreased in IL-1β-stimulated chondrocyte [7]. Consistent with prior reports, our results also revealed that IL-1β induced down-regulation of the expression of aggrecan and collagen II, and up-regulation of MMP13 and ADAMTS4 expression in articular chondrocytes. However, overexpression of miR-497-5p prominently increased the mRNA and protein levels of collagen II and aggrecan and reduced the expressions of MMP13 and ADAMTS4 while transfection of Wnt3a reversed the effects, whereas addition of DKK-1 weaken the Wnt3a-mediated effects in IL-1β-stimulated chondrocyte. These data revealed that miR-497-5p regulated EMC homeostasis via Wnt/β-catenin signaling pathway in IL-1β-stimulated chondrocyte.

In conclusion, miR-497-5p was downregulated and Wnt3a was upregulated in OA cartilage and IL-1β-stimulated chondrocytes. Moreover, Wnt3a was a direct target of miR-497-5p, and overexpression of miR-497-5p inhibited the matrix-degrading enzymes and promoted synthesis of cartilage matrix molecules while transfection of Wnt3a reversed the effects, simultaneously, addition of DKK-1 attenuated the Wnt3a-mediated functions in IL-1β-stimulated. Taken together, miR-497-5p ameliorated IL-1β-induced cartilage matrix degradation in chondrocytes via Wnt/β-catenin signaling pathway. These date indicated that miR-497-5p might serve as a new therapeutic target for OA thera.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Guoqiang Liu, Department of Traumatology, The Second People's Hospital of Dongying, 28 Changchun Road, Dawang Town, Guangrao County, Dongying, Shandong, China. Tel: +86-13854698619; E-mail: sva26-14268tuga@163.com

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