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Targeted inhibition of β-catenin by miR-320 and decreased MMP-13 expression in suppressing chondrocyte collagen degradation

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Abstract. – OBJECTIVE: Wnt/β-catenin pathway plays a critical role in modulating embryonic development, cell growth, and differentiation. The over-expression of β-catenin activates this pathway and up-regulates expression of matrix metalloproteinase-13 (MMP-13), and promotes matrix degradation and occurrence of osteoarthritis (OA). This study aims to explore the effect of miR-320 expression in OA chondrocyte and underlying mechanisms.

PATIENTS AND METHODS: Chondrocyte tissues from OA patients and normal individuals were collected for the detection of expression levels of miR-320, β-catenin, MMP-13, and alpha-1 chain of type II collagen (COL2A1). Dual luciferase reporter assay was performed to test targeted regulation between miR-320 and β-catenin. IL-1β was used to simulate *in vitro* cultured chondrocytes, which were transfected with miR-320 mimic and/or si-β-catenin, followed by quantification of miR-320, β-catenin, MMP-13, and COL2A1.

RESULTS: In chondrocytes of OA patients, expression of microRNA (miR)-320 is decreased. Bioinformatics analysis revealed complementary binding sites between miR-320 and β-catenin. Compared to control group, increasing levels of β-catenin and MMP-13 expression with reduction of miR-320 and COL2A1 expressions were observed in OA chondrocytes. Transfection of miR-320 mimic and/or si-β-catenin depressed expression of β-catenin and MMP-13 inside chondrocytes, accompanied with elevation of COL2A1 expression.

CONCLUSIONS: MiR-320 expression in OA chondrocyte is decreased, accompanied with up regulation of β-catenin and MMP-13. MiR-320 can inhibit β-catenin and MMP-13 expressions, elevates COL2A1 expression, which provides novel insights for the treatment of osteoarthritis.

Key Words:
MicroRNA-320, Wnt/β-catenin, MMP-13, Osteoarthritis.

Introduction

Osteoarthritis (OA), also named as degenerative arthritis or aged arthritis, represents a type of chronic degenerative bone joint disease featured with degradation of joint cartilage or attachment site of joint boundary ligament, and reactive bone hyperplasia or osteophyte formation that can be related with aging, obesity, and trauma[1,2]. Canonical Wnt/β-catenin signal pathway was firstly recognized in embryonic formation and development. Its participation in regulation of multiple biological processes includes cell growth, differentiation, inflammatory immunity, and tissue repair[3]. Previous finding indicated its correlation with functional stability of bone or chondrocyte tissues[4]. Wnt/β-catenin signal pathway has been shown to be essential for human osteoblast development, as potentiation of its activity can increase osteoblastic activity, whilst over-activation could destruct chondrocyte tissues for inducing OA[5]. The over-expression of β-catenin leads to over-activation of Wnt/β-catenin pathway, and plays a significant role in up-regulating expression of matrix metalloproteinase-13 (MMP-13)[6,7] or facilitating degradation of chondrocyte extracellular matrix (ECM) or inducing OA[8]. MicroRNA is one small molecule of non-coding RNA with 21-24 nucleotides in eukaryotes. It can bind with 3'-untranslatied region (3'-UTR) of target gene mRNA via complete or incomplete binding manners[9]. Meng et al[10] showed significantly lowered miR-320 expression in OA patient's chondrocytes, indicating the role of miR-320 down-regulation in OA pathogenesis. We investigated if miR-320 played a role in regulating OA pathogenesis and the related mechanism.

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Patients and Methods

Clinical Information

A total of 48 OA patients who received whole knee joint replacement surgery in the No. 89 Hospital of the People's Liberation Army of China from December 2015 to June 2016 were recruited. Tibia samples were collected during the surgery. All cases fitted diagnostic guideline of OA as stipulated by American Rheumatology Society, including: (1) Persistent knee joint pain within one month; (2) X-ray showed formation of boundary osteophyte; (3) Clear and thick joint fluid in at least two assays, with <2000 WBC per mL; (4) Age ≥ 40 years; (5) Duration of morning stiffness ≤ 30 min; (6) Sound of friction during joint movement. Knee OA can be diagnosed when satisfying (1)(2), or (1)(3)(5)(6), or (1)(4)(5)(6). OA caused by infection, tumor or rheumatoid disease were excluded. OA patients were classified into grade I (N=25) and grade II (N=23) based on Kellgren-Lawrence imaging guideline. Another 20 patients who received post-traumatic amputation were recruited as the control group to collect tibia tissues. Patients with diabetes or tumors were excluded. The collection of all tissue samples obtained informed consents from patients. This study has been reviewed and approved by the Ethical Committee of the No. 89 Hospital of the People's Liberation Army of China.

Major Reagent and Materials

Dulbecco's Modified Eagle's Medium (DMEM) /F12 culture medium, fetal bovine serum (FBS), penicillin-streptomycin, 0.25% trypsin, TRIzol, and type II collagenase were purchased from Gibco (Rockville, MD, USA). X-tremeGENE siRNA transfection reagent was from Roche (Basel, Switzerland). ReverTra Ace quantitive Reverse Transcript-Polymerase Chain Reaction (qRT-PCR) kit and SYBR dye was obtained from Toyobo (Osaka, Japan). MiR-320 nucleotide fragment was designed and synthesized by Ruibo Bio (Beijing, China). Rabbit anti-β-catenin polyclonal antibody, mouse anti-MMP-13 monoclonal antibody, rabbit anti-COL2A1 polyclonal antibody, and rabbit anti-β-actin polyclonal antibody were bought from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP) conjugated secondary antibody was acquired from Zhongshan Jinqiao (Shanghai, China). IL-1β was provided from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dual-luciferase reporter assay system and pGL3-promoter plasmids were all purchased from Promega (Madison, WI, USA).

Separation and Culture of Chondrocytes

Collected cartilage tissues were transferred to bio-safety cabinet. Chondrocytes were separated under sterile conditions. In brief, cartilage tissues were cut into pieces with 1-3 mm^3 size, and were mixed with 0.25% trypsin. After digestion at 37°C for 30 min, tissues were further digested in 0.2% type II collagenase at 37°C for 2.5 h. Tissue debris were filtered out by cell mesh. Chondrocytes were re-suspended in DMEM/F12 culture medium containing 10% FBS and 1% streptomycin-penicillin, and were cultured in a chamber with 5% CO_2 at 37°C. Culture medium was changed every 2-3 days. Experiments were performed when cells reached 70-80% confluence.

Luciferase Reporter Gene Plasmid Construction

Using human embryonic kidney (HEK) 293 genomic DNA as the template, full-length fragment of wild type or mutant forms of 3'-UTR of β-catenin gene was amplified and were cloned into pGL-3M plasmid. Recombinant plasmid was then used to transform DH5α competent cells. Positive clones with correct sequences were screened out by sequencing and were named as pGL3-β-catenin-3'UTR-wt and pGT-β-catenin-3'UTR-mut.

Luciferase Reporter Gene Assay

X-tremeGENE was used to co-transfect HEK293 cells with 100 ng pGL3-β-catenin-3'UTR-wt plasmid (or pGL-3-β-catenin-3'UTR-mut), and miR-320 mimic. After 48 h continuous incubation, dual-luciferase assay was performed. In brief, culture medium was discarded. Cells were washed in phosphate buffer solution (PBS) with the addition of 100 μL Passive Lysis Buffer. After 15 min culture, the mixture was centrifuged at 1000 rpm for 5 min. 50 μL cell lysate was mixed with 50 μL luciferase substrate. Activity of luciferase was measured immediately. The enzymatic reaction was stopped in 50 μL Stop & Glo, followed by quantification of sea pansy luciferase activity. The relative expression level of reporter gene was calculated as the ratio of luciferase activity against sea pansy luciferase activity. Oligonucleotide sequences used were: scramble NC, 5'-UUCUC CGAAC GUGUC ACGUU U-3'; miR-320 mimic, 5'-AAAAG CUGGG UUGAG AGGGC GA-3'.

Transfection and Grouping of Chondrocytes

In vitro cultured chondrocytes from OA patients were treated with 10 ng/mL IL-1β for 48 h. Cel-

ls were assigned into five groups: scramble NC transfection group; miR-320 mimic transfection group; si-NC transfection group; si-β-catenin group; and miR-320 mimic + si-β-catenin group. Nucleotide fragments used were: si-β-catenin sense: 5'-UGGUU GCCUU GCUCA ACAA-3'; si-β-catenin anti-sense: 5'-ACCAA CGGAA CGAGU UGUU-3'; si-NC sense: 5'-UUCUC CGAAC GUGUC ACGUU U-3'; si-NC anti-sense, 5'-ACGUG ACACG UUCGG AGAAU U-3'.

qRT-PCR for Gene Expression Assay

Cartilage tissues were homogenized in liquid nitrogen. TRIzol reagent was added to lyse cells. After layering, RNA precipitation, elution and re-suspension, RNA was obtained. ReverTra Ace qPCR RT Kit synthesized cDNA from RNA by reverse transcription. Using cDNA as the template, PCR amplification with the addition of SYBR fluorescent dye. Data were collected for analysis. PCR conditions were: 95°C for 15 s, followed by 60°C 30 s and 74°C 30 s. 40 cycles were performed on ABI ViiA TM7 fluorescent PCR cycler (Waltham, MA, USA). Primer sequences used for PCR were: miR-320P_F: 5'-ACACT CCAGC TGGGA AAAGC TGGGT TGAGA-3'; miR-320P_R: 5'-ACACT CCAGC TGGGT CGCCC TC -3'; U6P_F: 5'-ATTGG AACGA TACAG AGAAG ATT-3'; U6P_R: 5'-GGAAC GCTTC ACGAA TTTG-3'; β-cateninP_F: 5'-AGGAC CACCG CATCT CTACA T-3'; β-cateninP_R: 5'-GCAGT TTTGT CAGTT CAGGG A-3'; COL2A1P_F: 5'-TGGAC GCCAT GAAGG TTTTC T-3'; COL2A1P_R: 5'-TGGG AGCCA GATTG TCATC TC-3'; MMP-13P_F: 5'-CCAGA CTTCA CGATG GCATTG-3'; MMP-13P_R: 5'-GGCAT CTCCT CCATA ATTTG GC-3'; β-actinP_F: 5'-GAACC CTAAG GCCAA C-3'; β-actinP_R: 5'-TGTCA CGCAC GATTT CC-3'.

Western Blot

Cartilage tissues were mixed with homogenizing buffer for tissue lysate. Protein supernatant was prepared after 12 000 rpm centrifugation for 10 min. Bovine serum albumin (BSA) approach was used to test protein quantity and quality. After boiling for 5 min in 4X loading buffer, 50 μg protein samples were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (2.5-3 h), and were transferred to polyvinylidene difluoride (PVDF) membrane in a Bio-Rad (Hercules, CA, USA) Wet transfer chamber (300 mA current, 90 min time). The membrane was blocked in 5% defatted milk powder for 60 min, followed by primary antibody (anti-β-catenin at 1:400, anti-MMP-13 at 1:200, and COL2A1 at 1:200 or anti-β-actin at 1:500) incubation at 4°C for 12 h. By Phosphate-Buffered Solution Tween-20 (PBST) washing (3 times), HRP-labeled secondary antibody (anti-mouse or anti-rabbit at 1:8 000 dilution) was added for 60 min incubation under room temperature. After PBST rinsing three times, electrochemiluminescence (ECL) reagent was added for 1-3 min incubation in the dark. The membrane was then exposed in the dark and scanned for data analysis using Quantity One software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

SPSS18.0 (SPSS Inc., Chicago, Il, USA) was used for data analysis. Measurement data were presented as mean±standard deviation (SD). The Student *t*-test was used to compare measurement data between groups. Differences between multiple groups were compared using analysis of variance with Tukey's post-hoc test. A statistical significance was defined when $p<0.05$.

Results

Reduced MiR-320 and Elevated β-Catenin Expressions in Cartilage of OA Patients

qRT-PCR results showed that miR-320 expression in cartilage tissues of OA patients was significantly reduced compared to those in controlled cartilage tissues ($p<0.05$) (Figure 1A), whilst β-catenin mRNA level was statistically increased ($p<0.05$) (Figure 1B). Moreover, in OA patients, MMP-13 mRNA level in their cartilage tissues was significantly elevated compared to that in control tissues ($p<0.05$) (Figure 1C). Conversely, COL2A1 mRNA expression was statistically declined ($p<0.05$) (Figure 1D). Western blot obtained consistent results that protein levels of β-catenin and MMP-13 in OA cartilage tissues were remarkably elevated (Figure 1E), whilst COL2A1 protein was decreased (Figure 1E). These results suggested that down-regulation of miR-320 with rise of β-catenin were correlated with OA onset.

miR-320 Targeted and Inhibited β-Catenin Expression

Online prediction of the target gene by microRNA.org showed the targeted binding site between miR-320 and 3'-UTR of β-catenin (Figure 2A). Transfection of miR-320 mimic significantly decreased relative luciferase activity in HEK293 cells previously transfected with pGL3-β-caten-

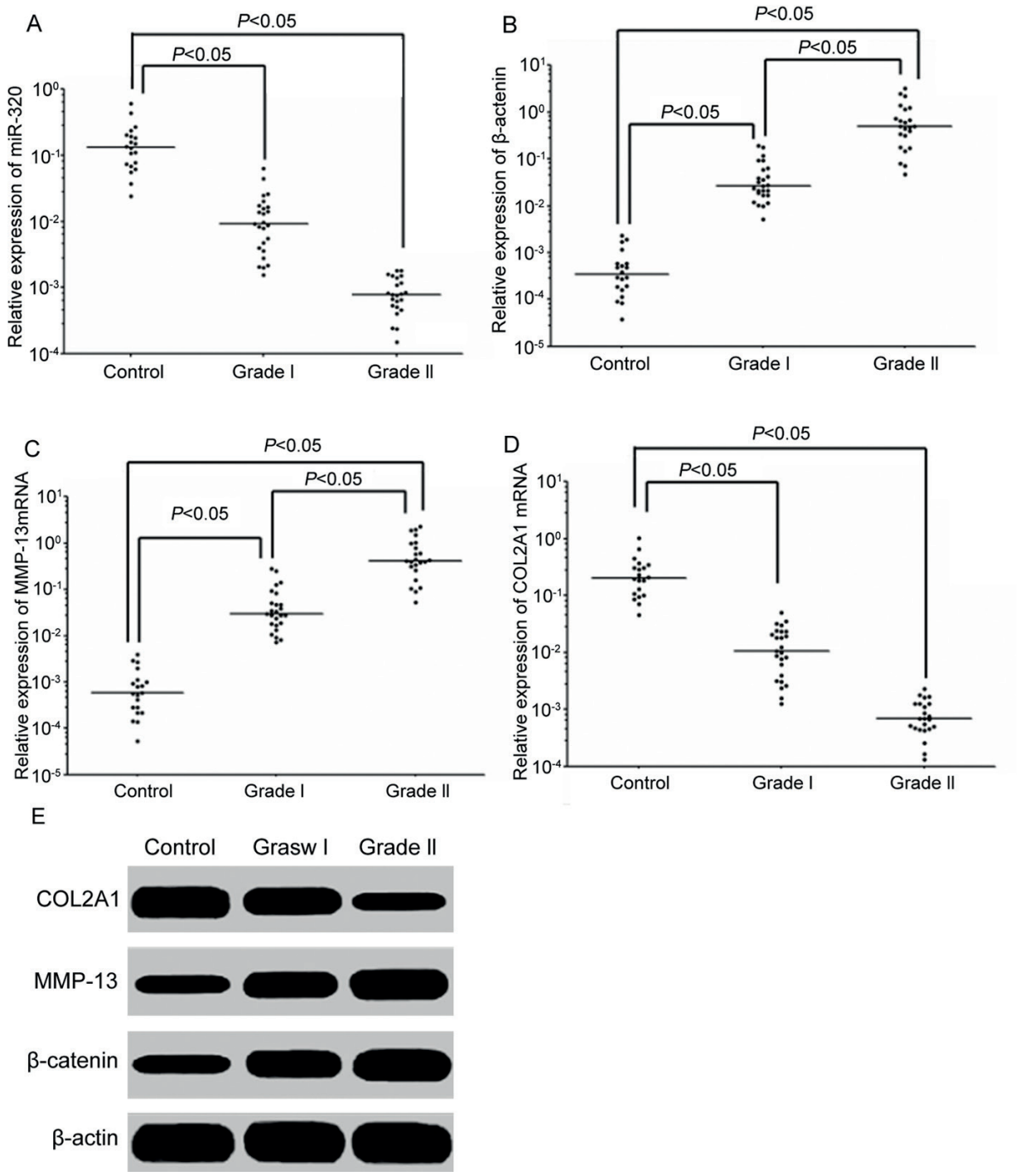

Figure 1. Lower miR-320 and elevated β-catenin expressions in cartilage tissues of OA patients. ***A,*** qRT-PCR for miR-320 expression. ***B,*** qRT-PCR for β-catenin mRNA. ***C,*** qRT-PCR for MMP-13 mRNA. ***D,*** qRT-PCR for COL2A1 mRNA. E, Western blot for protein expression.

in-3'-UTR-wt plasmid (p<0.05). The relative luciferase activity in HEK293 cells transfected with pGL3-β-catenin-3'-UTR-mut plasmid, however, was not changed by miR-320 mimic transfection (p>0.05). The transfection of scramble NC had no significant effects on relative luciferase activity in HEK293 cells expressing both plasmids (p>0.05). These results indicated that miR-320 could specifically targeted on 3'-UTR of β-catenin-gene and regulated its expression (Figure 2B).

MiR-320 Up-Regulation Inhibited Expression of MMP-13 and β-Catenin in Chondrocytes

Transfection of miR-320 mimic and/or β-catenin siNRA significantly lowered MMP-13 and β-catenin expression in chondrocytes ($p<0.05$) (Figure 3A and 3B). Meanwhile, COL2A1 expression was remarkably elevated (Figure 3A and 3B), indicating that miR-320 up-regulation could suppress Wnt/β-catenin signal pathway activity, down regulate MMP-13 expression, and weaken the inhibitor effects on COL2A1throuhg increasing COL2A1 expression.

Discussion

OA is a type of degenerative cartilage joint disease. In aged people, fewer feeding vessels under cartilage cause lower elasticity, friction and structural destruction as a result of disorders in synthesis and metabolism of extra-cellular matrix of cartilage tissues[11]. OA is featured as degenerative change of joint cartilage and subchondral bones, joint pain, difficulty in activity, and osteophyte formation in clinics, with aged people as the major patient population[12]. OA has relatively higher incidence worldwide. It is estimated that the overall OA incidence was 15%, with 20-30% increments by every 10 years age[13]. There have been currently about 150 million OA patients in China, leading to severe influences on labor capability and life quality[14]. The morbidity of OA reaches 53% eventually, forming a major reason of working ability deprivation and mobility disorder[15,16]. The core pathological change of OA includes defects in joint cartilage and osteophyte formation, both of which are results under combined effects including imbalance of catabolic/anabolic metabolism of joint chondrocytes and cartilage matrix, and focal inflammatory response inside joint cavity, resulting in joint swelling/pain, activity restriction and joint malformation[17-19]. OA is implicated with a complicated pathogenesis, although age is strongly involved, other factors still contribute significantly to OA onset.

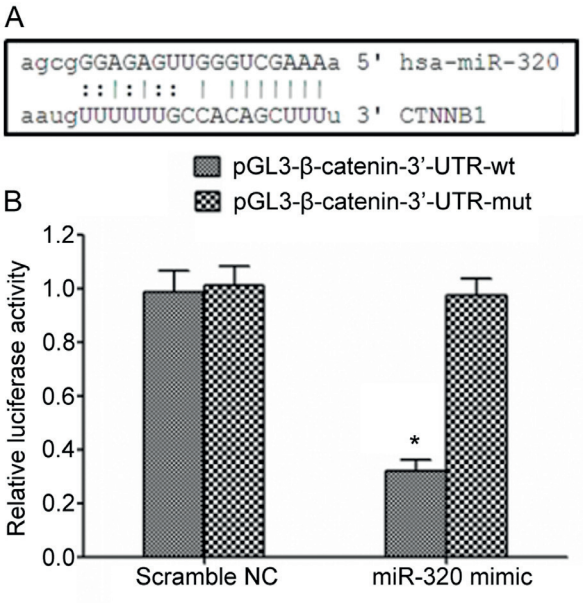

Figure 2. MiR-320 targeted and inhibited β-catenin expression. ***A,*** Binding sites between miR-320 and 3'-UTR of β-catenin mRNA. ***B,*** Dual luciferase reporter gene assay.

Canonical Wnt/β-catenin signal pathway is correlated with tissue/organ formation, cell growth/differentiation[20]. Canonical Wnt/β-catenin signal pathway consists of Wnt protein, transmembrane protein frizzled (Frz), co-receptor LRP5/6, disheveled protein (Dsh), axin, glycogen synthase kinase-3β (GSK-3β), adenomatous polyposis coli (APC), β-catenin, T-cell factor/lymphoid enhancing factor (TCF/LEF)[21]. In the absence of Wnt/β-catenin signal pathway activating factors, β-catenin can form complexes with Axin, APC, and GSK-3β. It is then phosphorylated by CK1 and GSK-3β, and free cytosolic GSK-3β level is decreased. When Wnt/β-catenin signal pathway is activated, Wnt protein binds with extracellular domain of Frz protein. It further recruits and activates Dsh, denatures the axin-GSK-3β-APC-β-catenin complex, thus eliminates phosphorylation between β-catenin and CK1 or GSK-3β. β-catenin accumulates inside cytoplasm, followed by nuclear translocation, and binds with transcriptional factor TCF/LEF to facilitate transcription and expression of downstream target genes[22].

Matrix metalloproteinases (MMPs) belong to proteinase superfamily with important roles in degradation of extracellular matrix, and they are involved in important physiology and pathology processes including embryonic development, osteogenesis and cartilage development[23], and tumor invasion or metastasis[24]. MMPs mainly consists of collagenase (MMP-1, -8 and -13), matrix lyse (MMP-3, -7 and -11) and gelatinase (MMP-2, -9). Previous findings showed that abnormally elevated MMPs in focal region of cartilage tissues is one critical reason causing imbalance in synthesis and degradation of extracellular matrix. MMP-13 represents a potent enzyme specifically targeted on type II collagenase, which is the most

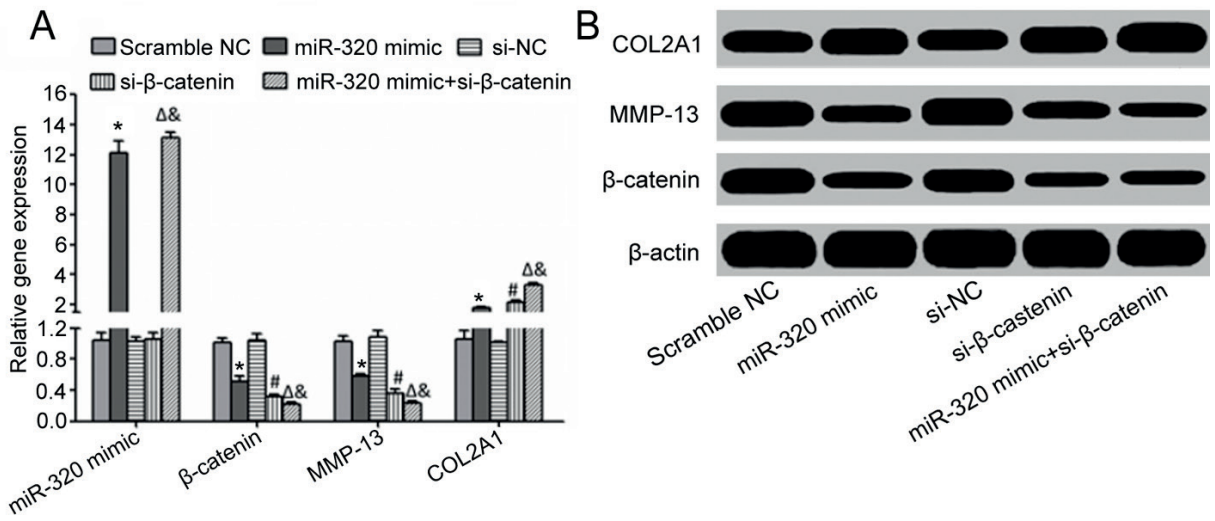

Figure 3. Elevation of miR-320 expression inhibited β-catenin and MMP-13 expression. ***A,*** qRT-PCR for gene expression. ***B,*** Western blot for protein expression. *, $p<0.05$ comparing between miR-320 mimic and scramble NC groups; #, $p<0.05$ comparing between si-β-catenin and si-NC groups; Δ, $p<0.05$ comparing between miR-320 mimic + si-β-catenin and Scramble NC groups; &, $p<0.05$ comparing between miR-320 mimic + si-β-catenin and si-NC group.

featured and abundantly distributed protein inside cartilage matrix, thus degrading extracellular matrix. Moreover, other subtypes of MMPs can only exert degrading effects on type II collagen via MMP-13[25]. A previous study[26] indicated that degradation on major component of joint cartilage, type II collagen, by MMP-13 contributed to OA onset. The over-activation of Wnt/β-catenin signal pathway plays important roles in up-regulating MMP-13 expression[6,7] and facilitating degradation of cartilage matrix and inducing OA[8].

Results from this study showed significantly lower miR-320 expression level in cartilage tissues of OA patients compared to those in control group. Meng et al[10] also found lower miR-320 expression in OA patient's cartilage, as consistent with our study. Compared to control group, OA cartilage tissues also showed remarkable elevation of β-catenin and MMP-13 expressions, whilst COL2A1 expression was suppressed, indicating potential role of β-catenin up-regulation in enhancing MMP-13 expression, degrading COL2A1 and facilitating OA pathogenesis. Zhou et al[27] found similar results showed higher β-catenin and MMP-13 levels in OA cartilage tissues. Liu et al[28] revealed over-activation of Wnt/β-catenin signal pathway and elevated MMP-13 expression in cartilage tissues from OA model rats. Zhu et al[8] reported significantly enhanced MMP-9 and MMP-13 expression in cartilage of transgenic mice with β-catenin over-expression, accompanied with smaller cartilage tissue area and collagen precipitation, OA-like pathogenesis in knee joint tissues. This study indicated the possible correlation between β-catenin and MMP-13 up-regulation in cartilage tissues with OA onset, probably sharing common mechanisms with Zhou et al[27] and Liu et al[28]. This work revealed that the inhibition of β-catenin signal molecules could decrease MMP-13 expression inside cultured chondrocytes, thus suppressing its degradation effects on type II collagen, increasing COL2A1 expression, supporting the result by Tamamura et al[29]. Zhou et al[27] also showed that Tetrandrine could inhibit activity of Wnt/β-catenin signal pathway and MMP-1 expression in cultured chondrocytes under stimulus by IL-1. Importantly, our investigation showed an alternative basis for the regulation of OA with miRNAs besides the inhibitory role of miR-138 via targeting p65[30].

Conclusions

We demonstrated that miR-320 expression was reduced in cartilage tissues of OA patients, with high expression of β-catenin and MMP-13. MiR-320 can inhibit β-catenin and MMP-13 expressions, and elevates the level of COL2A1, which provides fundamental scaffold for the therapy of OA in the future.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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