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MicroRNA-320c inhibits development of osteoarthritis through downregulation of canonical Wnt signaling pathway

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ABSTRACT

Aims: Osteoarthritis (OA) is a leading cause of deformity in aging people. Emerging evidence suggests that microRNAs and Wnt signaling pathway are associated with its pathogenesis. We aimed to determine whether microRNA-320c inhibits the development of osteoarthritis by suppressing Wnt signaling pathway.

Materials and methods: MiR-320c and β-catenin expression was assessed in human adipose derived stem cells (hADSCs) model of chondrogenesis and in normal and OA primary human chondrocytes. OA chondrocytes were transfected with miR-320c or its antisense inhibitor and β-catenin siRNA respectively. Direct interaction between miR-320c and β-catenin mRNA as well as activity of β-catenin/TCF complex were confirmed by luciferase reporter assay. Mmu-miR-320-3p agomir was intra-articularly injected in collagenase-induced OA mouse model. OA progression was evaluated histologically and immunohistochemically.

Key findings: MiR-320c was decreased and β-catenin was increased in OA chondrocytes and late stage of hADSCs chondrogenesis. Overexpression of miR-320c and knockdown of β-catenin had similar effects that the cartilage-specific genes were elevated and hypertrophy-related genes were down-regulated in OA chondrocytes. Luciferase reporter assay confirm that miR-320c regulated the expression of β-catenin by directly targeting 3′UTR of β-catenin mRNA and decreased the relative transcriptional activity of the β-catenin/TCF complex. Injection of mmu-miR-320-3p attenuated OA progression in the OA mouse model.

Significance: Our results supports that miR-320c can inhibits the degeneration of osteoarthritis chondrocytes via suppressing the canonical Wnt signaling pathway and indicates the potential of miR-320c as a novel therapeutic agent for osteoarthritis treatment.

1. Introduction

Osteoarthritis (OA), characterized with progressive breakdown of articular cartilage, is a common disease of joints. Patients with OA experience severe pain and deformity. The only effective treatment regimen is total joint replacement, which may be dangerous and expensive. Hence, it is necessary to investigate the mechanisms of OA pathogenesis [1]. Increasing evidences suggest the role of microRNAs (miRNAs), small non-coding RNAs, in the development and degeneration of cartilage. miRNAs bind to the 3′-untranslated region (3′-UTR) of target mRNAs and inhibit their translation or increase their degradation [2]. For instance, miR-30a promotes chondrogenesis through targeting Delta-like 4 (DLL4) [3]. In our previous study, we have reported a 3.437-fold upregulation in the expression of miR-320c during chondrogenesis of human adipose-derived stem cells (hADSCs) [4]. We hypothesize that miR-320c may play a key role in chondrogenesis and cartilage degeneration.

The canonical Wnt signaling pathway is critical in the regulation of many biological processes [5]. In the absence of Wnt ligands, a “destruction complex” induces degradation of β-catenin in a phosphorylated manner in the presence of Adenomatous polyposis coli (APC), Axin, and Disheveled (Dvl) via glycogen synthase kinase-3β (GSK-3β) [6]. The binding of Wnt ligands to its transmembrane receptor Frizzled (Fzd) and co-receptors low-density lipoprotein receptor-related protein (LRP)-5/6 results in the inhibition of GSK-3β, and consequently increases the level of β-catenin. β-Catenin translocates into the nucleus and binds to T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) transcription factors to promote the expression of Wnt target genes [7]. In many studies, canonical Wnt signaling has been demonstrated as an essential factor for chondrogenesis and hypertrophic differentiation of chondrocytes [8–10]. Inhibition of Wnt signaling may increase the expression of chondrogenic SOX9 and type 2 collagen

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alpha 1 (COL2A1) and ameliorate OA [11,12].

In our study, we found that miR-320c has the potential to regulate the expression of β-catenin using miRNA target prediction algorithms. Given the role of miRNAs and canonical Wnt signaling in the regulation of chondrogenesis and cartilage homeostasis, the aim of our study is to examine whether miR-320c regulates cartilage development and degeneration by restricting canonical Wnt signaling in chondrocytes.

2. Methods

2.1. Ethics

This study adhered to the standards of the Ethics committee on Human Experimentation at the First Affiliated Hospital of Sun Yat-Sen University, China (IRB:2014C-028) and the Helsinki Declaration (2000). All participants provided informed consent.

2.2. Culture of hADSCs

The adipose tissue was obtained from the donors who underwent elective liposuction or other abdominal surgery. Three samples of adipose tissues were included in the study (mean age: 21 years, range: 16–30 years). The method of hADSC isolation was described in our preciously study [13]. hADSCs were cultured in Alpha-modified Eagle's medium (α-MEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technology) and 1% penicillin/streptomycin (PS; Gibco Life Technology). Cells were cultured at 37 °C in a 5% CO_2 atmosphere. The culture media were changed every 3 days. The cells were detached after reaching near 80%–90% confluency using 0.05% trypsin/ethylenediaminetetraacetic acid and passaged in culture.

2.3. Induction of chondrogenesis

Chondrogenesis induction was carried out as previously described [4]. Briefly, cultured hADSCs between passages three and five were harvested and resuspended in an incomplete chondrogenic medium (194 mL human adipose mesenchymal stem cells chondrogenic differentiation basal medium, 20 μL dexamethasone, 600 μL ascorbate, 2 mL of ITS [insulin, transferrin, selenium] supplement, 200 μL sodium pyruvate, 200 μL proline; Cyagen, Guangzhou, China) at a density of 10^5 cells/μL. A 12.5-μL droplet was carefully dotted on the center of each well of 24-well plates. hADSCs were allowed to adhere for 70 min at 37 °C, followed by the addition of 500 μL of complete chondrogenic medium (addition of 5 μL TGF-β3 to incomplete chondrogenic medium) to each well. Complete medium was changed every 3 days. Samples were collected for experiments at different time points.

2.4. Primary human chondrocyte isolation and culture

OA cartilage samples were obtained from patients with OA ($n = 6$, mean ± standard deviation [SD] age: 64.17 ± 3.06, male: 1, female: 5) that underwent total knee replacement surgery. Normal cartilage samples were obtained from patients ($n = 6$, mean ± SD age: 20.17 ± 6.37, male: 4, female: 2) that underwent amputation surgery because of malignant bone tumors not involving the knee joint. Primary human chondrocytes (PHCs) were isolated as previously described [14]. OA cartilage was isolated from the areas of gross erosion, while normal cartilage was isolated from the areas with surface regularity. The chondrocytes were seeded into flasks containing Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco Life Technology) supplemented with 5% FBS and 1% PS at 37 °C in a humidified atmosphere of 5% CO_2, and the medium was changed every 3 days.

Table 1
Primers for quantitative real-time polymerase chain reaction (qRT-PCR).

Gene		Primer sequence (5′-3′)
has-GAPDH	F	GCACCGTCAAGGCTGAGAAC
has-GAPDH	R	ATGGTGGTGAAGACGCCAGT
has-SOX9	F	AGCGAACGCACATCAAGAC
has-SOX9	R	CTGTAGGCGATCTGTTGGGG
has-COL2A1	F	TGGACGATCAGGCGAAACC
has-COL2A1	R	GCTGCGGATGCTCTCAATCT
has-ACAN	F	GTGCCTATCAGGACAAGGTCT
has-ACAN	R	GATGCCTTTCACCACGACTTC
has-RUNX2	F	CACTGGCGCTGCAACAAGA
has-RUNX2	R	CATTCCGGAGCTCAGCAGAATAA
has-COL10A1	F	CATAAAAGGCCCACTACCCAAC
has-COL10A1	R	ACCTTGCTCTCCTCTTACTGC
has-MMP3	F	CGGTTCCGCCTGTCTCAAG
has-MMP3	R	CGCCAAAAGTGCCTGTCTT
has-MMP13	F	TCCTGATGTGGGTGAATACAATG
has-MMP13	R	GCCATCGTGAAGTCTGGTAAAAT
has-ADAMTS4	F	GGTCAAGGTCCCATGTGCAAC
has-ADAMTS4	R	GAATGCGGCCATCTTGTCATC
has-U6	F	CTCGCTTCGGCAGCACA
has-U6	R	AACGCTTCACGAATTTGCGT
has-320c	F	AAAAGCTGGGTTGAGAGGGT
has-CTNNB1	F	AAAGCGGCTGTTAGTCACTGG
has-CTNNB1	R	CGAGTCATTGCATACTGTCCAT
has-AXIN2	F	AGCCAAAGCGATCTACAAAAGG
has-AXIN2	R	AAGTCAAAAACATCTGGTAGGCA
has-c-MYC	F	GTCAAGAGGCGAACACACAAC
has-c-MYC	R	TTGGACGGACAGGATGTATGC
has-LEF1	F	TGCCAAATATGAATAACGACCCA
has-LEF1	R	GAGAAAAGTGCTCGTCACTGT
has-JUN	F	TCCAAGTGCCGAAAAAGGAAG
has-JUN	R	CGAGTTCTGAGCTTTCAAGGT
has-Oct-4	F	CTTGAATCCCGAATGGAAAGGG
has-Oct-4	R	CCTTCCCAAATAGAACCCCCA

2.5. RNA extraction, reverse transcription, and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated using miRNeasy Mini Kit (QIAGEN, CA, USA) and cDNA was synthesized using the PrimeScript® miRNA cDNA Synthesis Kit (TAKARA Biotechnology, Japan). RT-qPCR was performed using SYBR® Premix Ex Taq™ II (TAKARA Biotechnology) on CFX96 real-time qPCR machine. The transcript levels of mRNA were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and miRNAs were normalized to the small U6 RNA. The mRQ 3′ Primer (Clontech) was used as the reverse primer for miR-320c. The specific primers used for analyses are listed in Table 1. The method of $2^{-\Delta\Delta Ct}$ was used for calculating relative gene expression. All experiments were performed in triplicates.

2.6. Transfection

PHCs were transfected with a mimic of miR-320c (RiboBio, Guangzhou, China) at 50 nM concentration, while an inhibitor of miR-320c was used at 100 nM concentration. Nonspecific microRNA (miR-control; RiboBio) was used as a control. PHCs were also transfected with 50 nM of si-catenin beta 1 (siCTNNB1; RiboBio) and 50 nM of siNC as negative control. Lipofectamine® 2000 Transfection Reagent (Gibco Life Technologies) was used to transfect cells.

2.7. Western blot analysis

The method of protein isolation and western blotting was previously described [15]. The nuclear proteins were isolated using a Nuclear Extraction kit (CW0199, CoWin Biosciences). Briefly, proteins (20 μg) were separated with 8–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride membranes (Millipore, Bedford USA). Membranes were

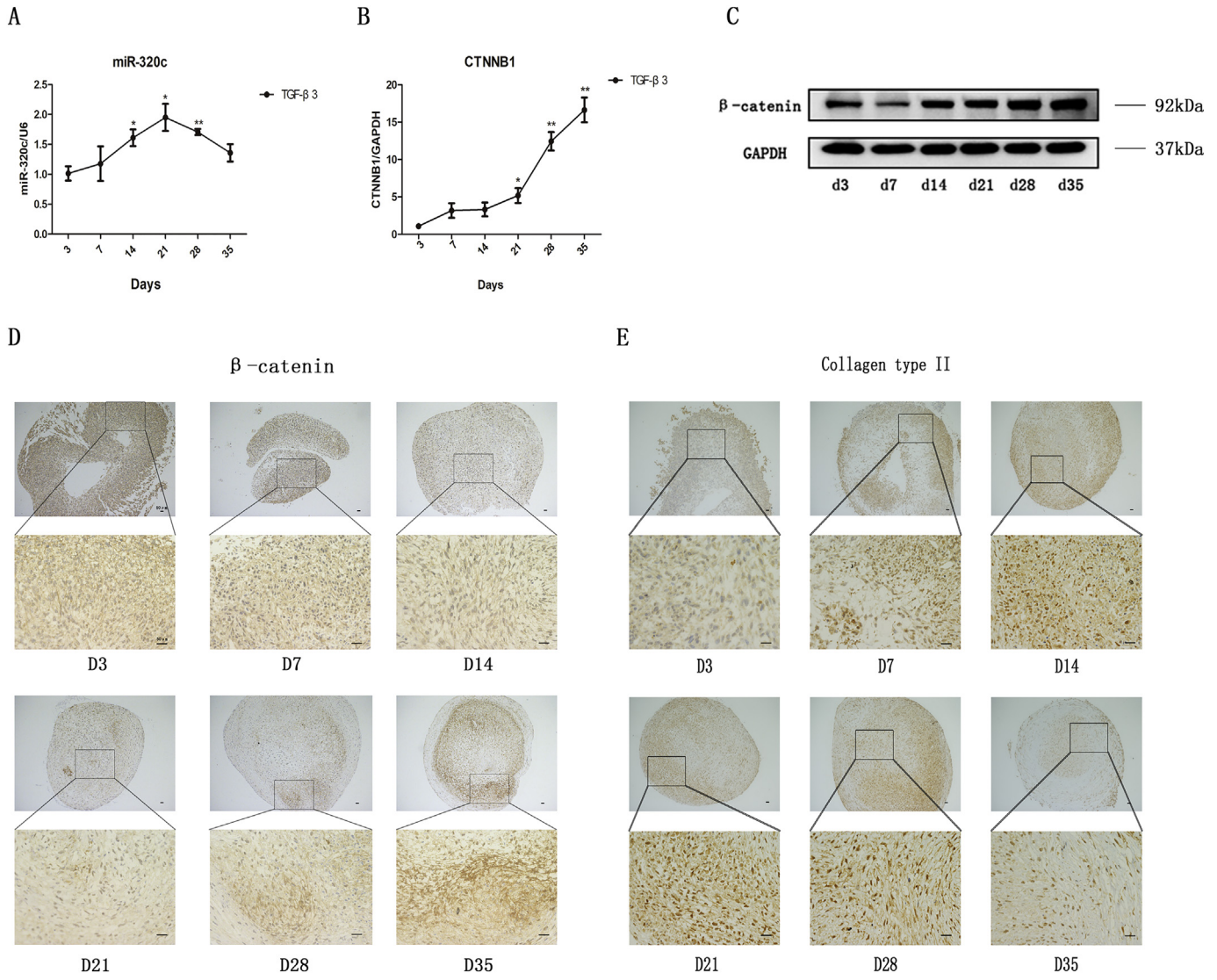

Fig. 1. Analysis of the relative expression levels of miR-320c and β-catenin during the chondrogenesis of hADSCs. hADSCs were induced to chondrogenesis with TGF-β3 for 3, 7, 14, 21, 28 and 35 days. Gene expression of miR-320c (A) and CTNNB1 (B) were determined by qRT-PCR, the protein level of β-catenin was estimated by western blotting (C). hADSCs chondrogenesis at day 3, 7, 14, 21, 28 and 35 were stained with immunohistochemistry of β-catenin and collagen type II (D, E). Upper panels: 100× magnification, lower panel: magnified view of the area enclosed by black boxes, 400×. Scale bar: 50 μm. The data shown representative results of three independent experiments in samples from three different donors. $*p < 0.05$, $**p < 0.01$.

incubated overnight at 4 °C with primary antibodies specific to β-catenin, Runt-related transcription factor 2 (RUNX2), matrix metalloproteinase 3 (MMP3), GAPDH, proliferating cell nuclear antigen (PCNA) (1:1000 dilution, Cell Signaling Technology), SOX9 (1:2000 dilution, Millipore), COL2A1, and MMP13 (1:1000 dilution, Abcam). Following incubation, membranes were treated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3000 dilution, Cell Signaling Technology) at room temperature for 1 h. Protein bands were detected by ChemiDoc Touch (BIO-RAD).

2.8. Immunohistochemical analysis, immunofluorescence and in situ hybridization

Immunohistochemical analysis and in situ hybridization were performed as previously described [16,17]. Briefly, human miR-320c and mmu-miR-320-3p probes were used (Exiqon, Invitrogen, Shanghai, China). For immunohistochemical analysis, sections were deparaffinized and rehydrated with standard xylene-to-ethanol washes and blocked in phosphate-buffered saline (PBS) plus 0.025% Tween-20 and 10% FBS. After blocking, the sections were overnight incubated with primary antibodies specific to β-catenin (1:100 dilution, Cell Signaling Technology), aggrecan, and COL2A1 (1:100 dilution, Abcam) at 4 °C. Negative controls were prepared by substituting primary antibodies with PBS. The sections were incubated for 30 min with secondary HRP-conjugated anti-rabbit IgG (cell signaling technology, Boston, USA) and stained with 3,3′-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. For immunofluorescence, after deparaffinization and rehydration, sections were blocked in BSA buffer for 30 min. After that, the sections were incubated with primary antibody specific to β-catenin (1:300 dilution, Servicebio, China) overnight at 4 °C. Then, the sections were washed with PBS and incubated with Cy3 conjugated goat anti-mouse IgG (1:300 dilution, Servicebio, China) for 50 min at RT. After incubation, the sections were stained with DAPI and mounted with antifade mounting medium. Normal mouse IgG was used instead of a primary antibody on the negative control section. Stained sections were examined with NIKON ECLIPSE C1 confocal laser scanning microscope.

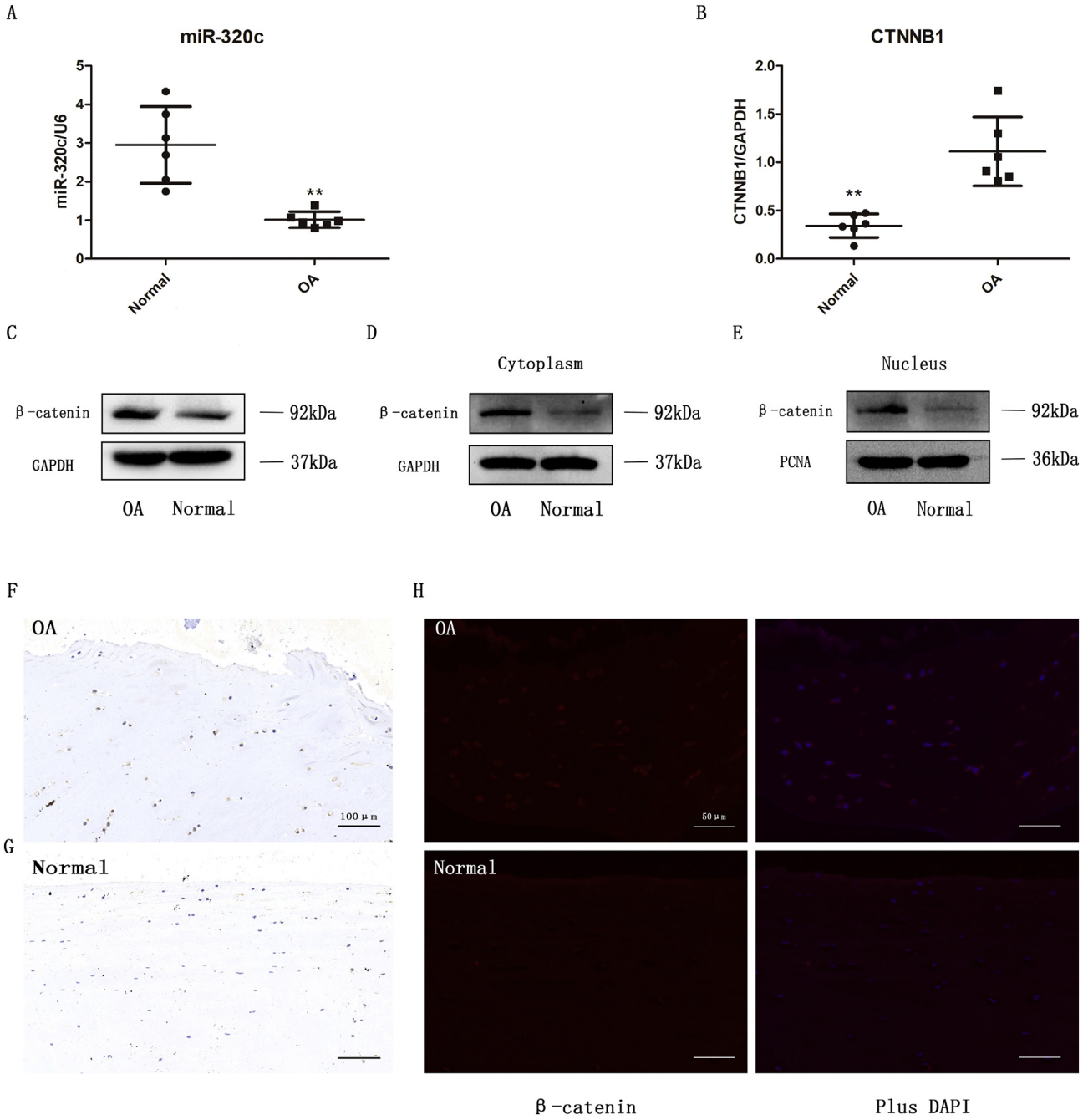

Fig. 2. The expression of miR-320c and β-catenin in OA and normal chondrocytes. The relative level of miR-320c and CTNNB1 mRNA in normal and OA chondrocytes were determined by qRT-PCR (A, B). U6 and GAPDH were detected as endogenous controls. Each dot represents a value of a single experiment of one donor. The bar shows the mean ± SD from six different donors per group. The protein level of β-catenin in normal and OA chondrocytes and their cytoplasm and nucleus were determined by western blotting (C–E), immunohistochemistry (F–G) and immunofluorescence (H). GAPDH as endogenous control and PCNA as nuclear endogenous control. Data shown were representative results from six pairs normal and OA cartilage. The magnification of immunohistochemistry is 180×. Scale bar: 100 μm. Magnification of immunofluorescence is 400×. Scale bar: 50 μm. $*p < 0.05$, $**p < 0.01$.

2.9. Luciferase constructs and reporter assay

The DNA sequences of CTNNB1 3′-UTR were amplified by PCR using the following primers: forward 5′-CTAGTCTAGATAATGTTTTTTGCCACAGCTTTTGC-3′ and reverse 5′-CCGGAATTCAGTCACTCCCAAAATCCATTTGTAT-3′. The seed sequences were mutated using standard PCR techniques with the following primers: forward 5′-CTAGTCTAGATAATGAAAAAAGGAGTCGAAAAGCAACTTAATACTCAAATGAGT-3′ and reverse 5′-CCGGAATTCAGTCACTCCCAAAATCCATTTGTAT-3′. The amplified DNA sequences were inserted into the pGL3-3′UTR Vector (Synbio Technologies) to generate CTNNB1 3′-UTR or mutated (MUT) CTNNB1 3′-UTR luciferase vectors. For the reporter assay, 2.0×10^4 PHCs were cultured in a 48-well plate in 200 μL of culture medium. After 24 h, cells were transfected with 100 ng of has-miR-320c mimic or miR-Control (Synbio Technologies). The cells were co-transfected with 100 ng of vector with the wild-type or mutant 3′-UTR for CTNNB1 gene. After 48 h, the luciferase activity was measured using the Dual-Luciferase® Report Assay System 10-Pack (Promega). Firefly luciferase activity was normalized to the corresponding *Renilla* luciferase activity. Luminescence was measured using EnSpire® Multimode

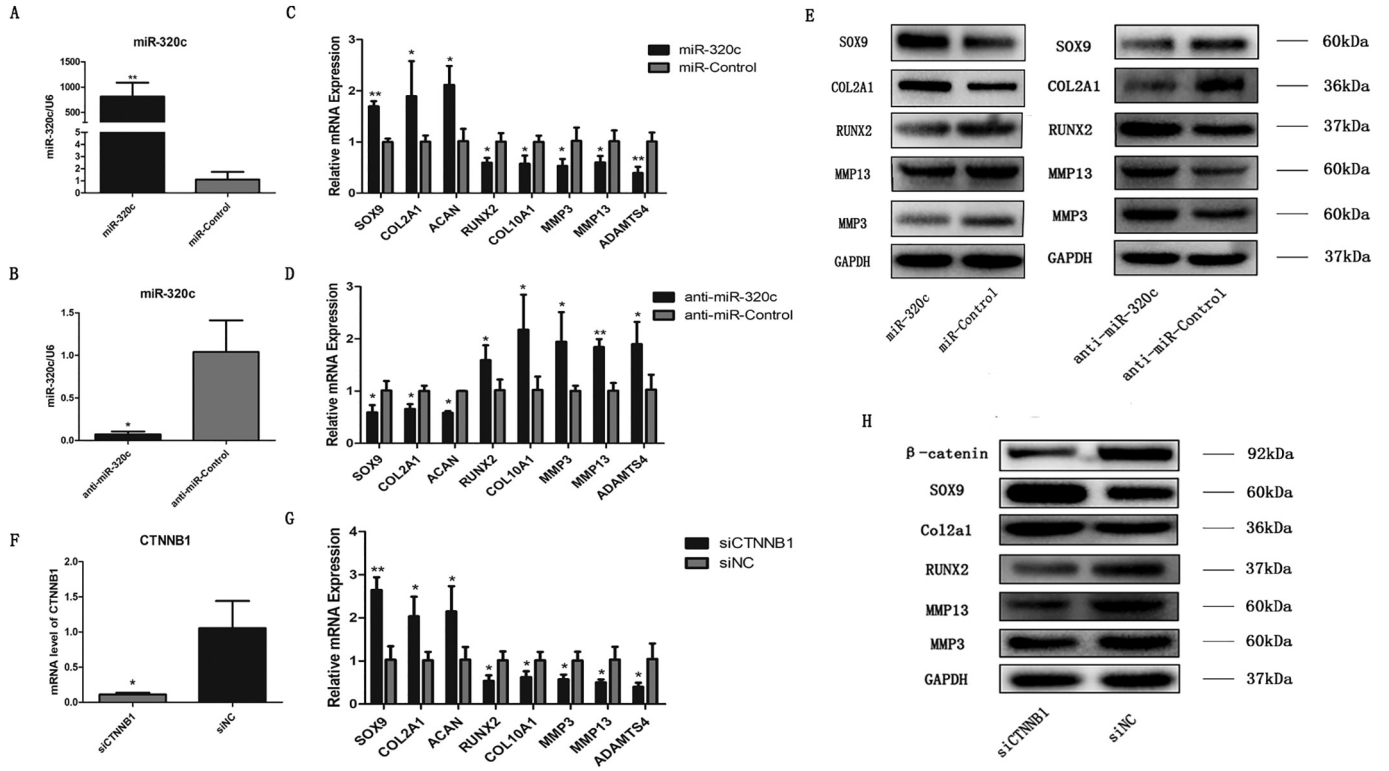

Fig. 3. MiR-320c and β-catenin regulates the expression of cartilage-specific genes in OA chondrocytes. The relative levels of miR-320c, CTNNB1, SOX9, COL2A1, ACAN, RUNX2, COL10A1, MMP3, MMP13 and ADAMTS4 mRNA were measured by qRT-PCR in OA chondrocytes after 48 h transfection with miR-320c, miR-Control, anti-miR-320c, anti-miR-Control and siCTNNB1 (A–D, F, G), GAPDH and U6 as endogenous control. The protein levels of SOX9, COL2A1, RUNX2, MMP13 and MMP3 in OA chondrocytes after 72 h transfection were visualized by western blotting (E, H). GAPDH were detected as internal control. Quantitative data were presented as means ± SD from three independent experiments, $*p < 0.05$, $**p < 0.01$.

Plate Reader (PerkinElmer).

2.10. TOP/FOP flash Wnt reporter assay

The PHCs cultured in 96-well plate were transfected with TOP flash or FOP flash Wnt reporter plasmids (0.2 μg each well) containing wild-type or mutant TCF DNA-binding sites (Miaoling Bioscience and Technology Corporation, China). The cells were also transfected with *Renilla* reporter plasmids (0.004 μg each well). TOP/FOP flash-transfected cells were co-transfected with miR-320c or miR-Control (10 nmol). The reporter activity was measured as described above.

2.11. Collagenase-induced OA model

Twelve-week-old C57B/L10 mice were randomized into four groups as follows: normal ($n = 5$), OA (n = 5), mmu-miR-320-3p (n = 5), and mmu-miR-Control (n = 5). On day 0, collagenase was used to induced OA in all mice except the mice from the normal group. The collagenase-induced OA model was previously described [18,19]. Mice were anesthetized with 4% chloral hydrate (10 mL/kg) through an intraperitoneal injection. The knee joints of mice were subjected to an intra-articular injection of 15 U of collagenase VII (*Clostridium histolyticum*; Sigma-Aldrich) in 10 μL of saline through the patellar ligament on day 0 and 7. In normal group, knee joints were injected with 10 μL of saline without collagenase. On days 14 and 21, mice in mmu-320-3p and mmu-miR-Control groups were injected with 10 μL of mmu-miR-320-3p agomir (5 nmol) or agomir-negative control (5 nmol), respectively. After 4 weeks of injection of agomir, all mice were euthanatized for further analysis.

2.12. Statistical analysis

All experiments were performed with at least three biological replicates. The data were presented as means ± standard deviation (SD). The student *t*-test and Mann-Whitney *U* test were used to identify differences between groups as appropriate. Differences between groups were assessed using one-way analysis of variance (ANOVA) and Kruskal-Wallis tests. Gaussian distribution of the data was confirmed using the Shapiro–Wilk test. p values < 0.05 were considered statistically significant. All analyses were performed using SPSS software, version 13.0 (IBM Corporation, Armonk, NY, USA).

3. Results

3.1. Expression pattern of miR-320c and β-catenin during chondrogenesis of hADSCs

Differentiation of hADSCs into chondrocytes was induced in vitro using transforming growth factor (TGF)-β3. We observed that the levels of miR-320c rapidly increased in chondrogenic hADSCs from day 3 and peak values were observed at day 21, followed by a sharp decrease in the levels of miR-320c at day 28 and 35 (Fig. 1A). An inverse relationship was observed between the expression patterns of miR-320c and β-catenin during the chondrogenic differentiation of hADSCs from 14 to 35 days (Fig. 1B–D). These results suggest that the decrease in the expression of miR-320c and the increase in β-catenin level may be related to the degeneration of chondrocytes, while β-catenin expression may be affected by miR-320c. Chondrocyte differentiation was verified from the increased expression of COL2A1 during early chondrogenesis and decreased expression during late-stage chondrogenesis (Fig. 1E).

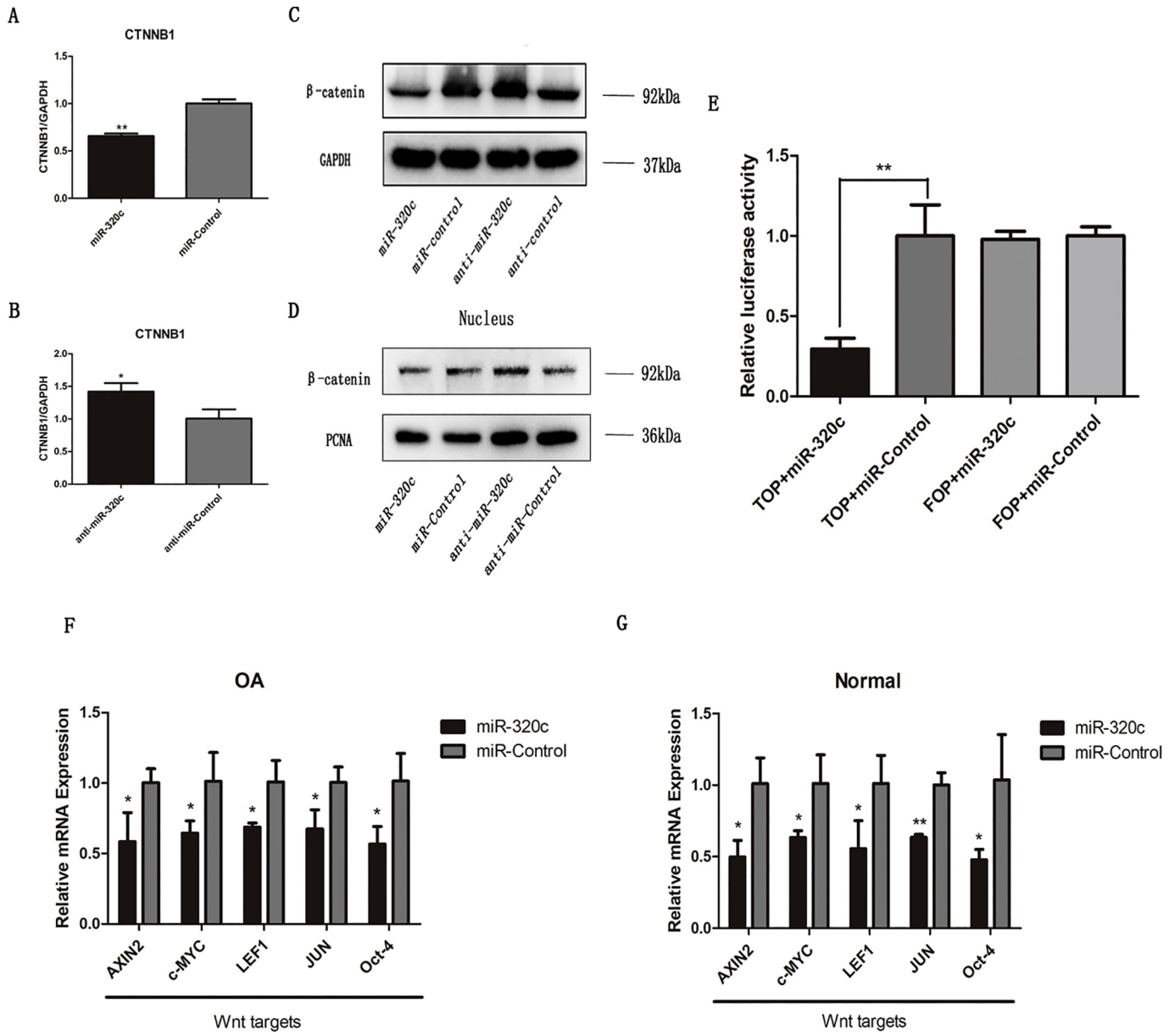

Fig. 4. MiR-320c represses β-catenin translation and Wnt/β-catenin signaling pathway in OA chondrocytes. OA chondrocytes were transfected with miR-320c, miR-Control, anti-miR-320c and anti-miR-Control. The levels of CTNNB1 mRNA were measured by qRT-PCR after 48 h transfection (A–B). The protein levels of β-catenin in OA chondrocytes and nucleus were visualized by western blotting after 72 h transfection (C, D). Luciferase reporter assay of β-catenin-Tcf/LEF activity in PHCs after 48 h transfection of Wnt signaling reporter (TOP flash or FOP flash) with miR-320c or miR-Control, Renilla luciferase activity is internal control (E). qRT-PCR confirmed the differential expression Wnt targets genes in miR-320c and miR-Control overexpressing OA and normal chondrocytes after 48 h transfection (F, G). Quantitative data were presented as means ± SD from three independent experiments, GAPDH or PCNA were used as a normalized control, $*p < 0.05$, $**p < 0.01$.

3.2. Expression level of miR-320c and β-catenin in OA and normal cartilage

To determine the expression level of miR-320c during the progression of OA, we compared its expression level in normal and OA cartilages with qRT-PCR. The expression of miR-320c was significantly decreased in OA cartilage as compared with the normal cartilage (Fig. 2A). This trend was consistent with the result of in situ hybridization, described in our previously study [17]. Furthermore, OA cartilage showed higher levels of β-catenin mRNA and protein than the normal cartilage (Fig. 2B–C), in line with the results of cytoplasmic and nuclear proteins (Fig. 2D–E). Immunohistochemical and immunofluorescence analysis further confirmed this trend (Fig. 2F–H).

3.3. β-Catenin mRNA knockdown induced effects similar to those observed with miR-320c overexpression in OA chondrocytes

To investigate the function of miR-320c in OA cartilage, transfection of miR-320c and anti-miR-320c was performed in OA chondrocytes. Overexpression of miR-320c in OA cartilage resulted in a decrease in the expression of *RUNX2*, *COL10A1*, *MMP3*, *MMP13*, and a disintegrin and metalloproteinase with thrombospondin motifs 4 (*ADAMTS4*) mRNAs and promoted the expression of *SOX9*, *COL2A1*, and aggrecan (*ACAN*) mRNAs (Fig. 3A, C). On the contrary, the suppression of miR-320c expression resulted in an increase in the expression levels of *RUNX2*, *COL10A1*, *MMP3*, *MMP13*, and *ADAMTS4* mRNAs and decreased the levels of *SOX9*, *COL2A1*, and *ACAN* (Fig. 3B, D). Similar trend was observed in western blotting results (Fig. 3E). The decrease in β-catenin expression resulted in effects similar to those observed with

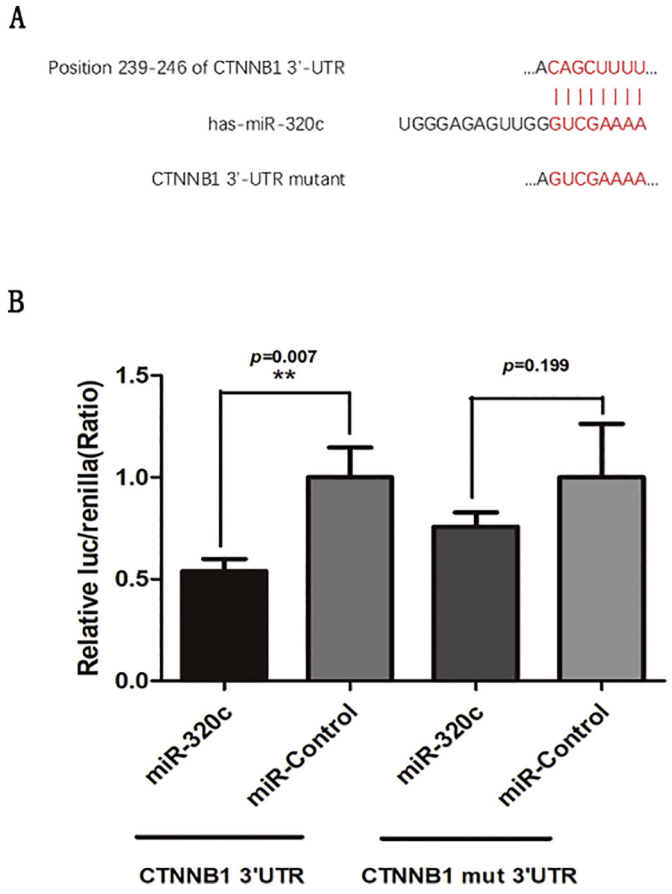

Fig. 5. MiR-320c directly targets CTNNB1. CTNNB1 was predicted targets of miR-320c, alignment of CTNNB1 3′UTR miR-320c were shown (A). The CTNNB1 3′UTR reporter plasmid or a mutant CTNNB1 3′UTR reporter plasmid was co-transfected with miR-320c or miR-Control in PHCs. The cells were harvested after 48 h transfection for luciferase assay (B). Quantitative data were presented as means ± SD from three independent experiments, $*p < 0.05$, $**p < 0.01$.

the overexpression of miR-320c in OA chondrocytes (Fig. 3F–H). These observations indicated that the overexpressed miR-320c and decreased β-catenin levels may result in the inhibition of the degeneration of OA cartilage.

3.4. miR-320c suppresses Wnt/β-catenin signaling pathway by downregulating the expression of β-catenin

As the overexpression of miR-320c in OA cartilage had effects similar to those observed with decreased β-catenin protein, we hypothesized that miR-320c may regulate the expression of β-catenin. To investigate this hypothesis, we transfected miR-320c and anti-miR-320c in OA chondrocytes and observed that β-catenin mRNA and protein expression levels decreased in OA chondrocytes overexpressing miR-320c (Fig. 4A, C). On the contrary, the introduction of anti-miR-320c resulted in an increase in β-catenin mRNA and protein levels (Fig. 4B, C), as observed with nuclear proteins (Fig. 4D). Previous studies have shown that the nuclear translocation of β-catenin induces activation of TCF or LEF and stimulates the transcription of TCF/LEF targets genes [20]. As shown in Fig. 4E, the activity of β-catenin/TCF complex in miR-320c-overexpressing PHCs decreased as compared with the activity reported in miR-Control group, while reporter plasmid with mutated TCF-binding sites (FOP) prevented this change. These results indicate that miR-320c may regulate the protein level of β-catenin in the nucleus and the relative transcriptional activity of β-catenin/TCF complex. To evaluate the effect of miR-320c on Wnt/β-catenin pathway, qRT-PCR was performed in miR-320c-overexpressing OA and normal chondrocytes. As shown in Fig. 4F and G, mRNA transcripts of multiple target genes of Wnt/β-catenin pathway were downregulated in OA and normal chondrocytes overexpressing miR-320c, including axin-related protein 2 (*AXIN2*), *c-MYC*, *LEF1*, *JUN*, and *Oct-4*. These results suggest that miR-320c may suppress the Wnt/β-catenin signaling pathway through the downregulation of β-catenin protein level in nucleus.

3.5. miR-320c directly targets the 3′-UTR of β-catenin mRNA

While our results demonstrate that β-catenin expression was regulated by miR-320c, bioinformatics predictions using TargetScan (http://www.targetscan.org) and miRanda (http://www.microrna.org) revealed that the 3′-UTR of human β-catenin contains a potential binding site for miR-320c (Fig. 5A). We used a luciferase reporter assay to investigate the underlying regulatory mechanism. The wild-type and mutated 3′-UTR of CTNNB1 were transfected in the presence or absence of the overexpressed miR-320c in PHCs. We found that co-transfection of miR-320c and CTNNB1 3′-UTR luciferase reporter plasmids reduced the luciferase activity, while the mutated 3′-UTR sequences prevented the binding of miR-320c to *CTNNB1* mRNA (Fig. 5B). These results demonstrated that miR-320c reduced luciferase activity by binding to the 3′-UTR of β-catenin.

3.6. miR-320-3p promotes cartilage recovery in an OA mouse model

We performed safranin O, collagen II, and aggrecan staining in all groups. Cartilage tissues from the tibial plateau in the normal group presented a smooth surface, regular cellular organization, and normal collagen II and aggrecan content. In contrast, OA group showed degenerative OA changes, including articular cartilage reduction, fibrillation of articular surface, depletion of proteoglycan, collagen II, and aggrecan, and osteophyte formation in the tibial plateau (Fig. 6A). OA group showed higher Osteoarthritis Research Society International (OARSI) grade scores [21] than the normal group (Fig. 6C). Recovery in the content of proteoglycan, collagen II, and aggrecan as well as the smoothness of cartilage surface to some extent and lower OARSI grade scores were observed in mmu-miR-320-3p group than in mmu-miR-Control group (Fig. 6B, D). Furthermore, the β-catenin expression level was lower and miR-320 level was higher in mmu-miR-320-3p group than in mmu-miR-Control group (Fig. 6E). These results indicate that miR-320 exhibits great therapeutic potential in OA.

4. Discussion

MiR-320c has gained attention in recent years owing to its significant regulatory functions in most organs, including tumor, bone, and cartilage [22–27]. In our previous study, we have demonstrated that miR-320c directly inhibits MMP13 expression and regulates chondrogenesis in mouse chondrocytes. In the present study, we demonstrate that miR-320c suppressed Wnt/β-catenin signaling pathway and inhibited degeneration of human chondrocytes by directly targeting β-catenin expression.

The Wnt/β-catenin signaling pathway plays a pivotal role in OA development and progression [28–30]; hence, the molecules involved in this pathway, especially β-catenin, may serve as targets for the therapeutic intervention of OA. Based on the functional information predicted about miR-320c and CTNNB1 by the bioinformatic softwares, we hypothesize that miR-320c may play a critical role in chondrogenesis or pathogenetic process of OA by directly targeting CTNNB1.

We observed decreased levels of miR-320c and increased level of β-catenin in the late stage of chondrogenic differentiation of hADSCs. This observation indicates that the levels of miR-320c and β-catenin may be associated with the pathogenesis of cartilage degeneration. As OA has long been characterized with articular cartilage degeneration, we

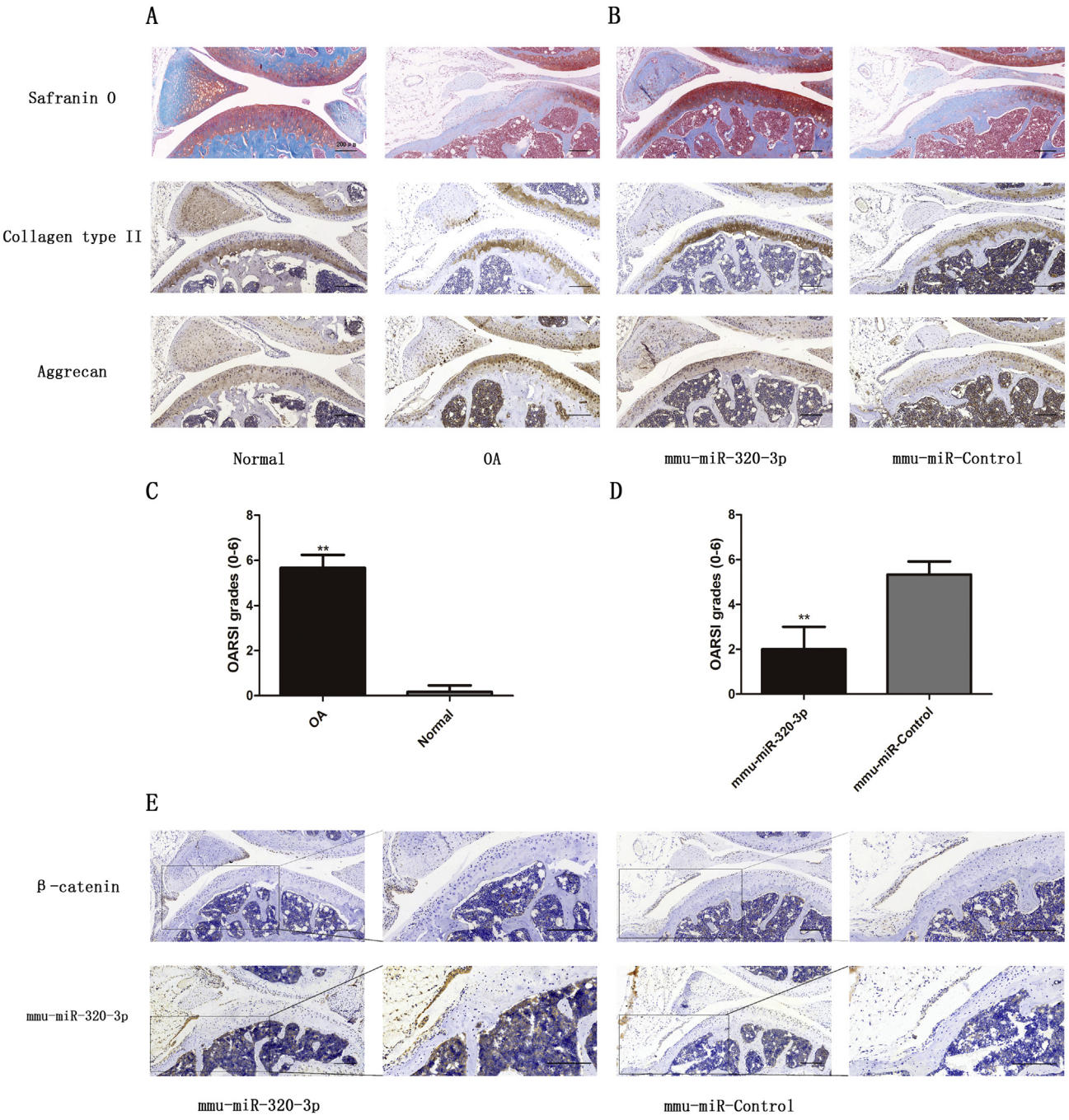

Fig. 6. Mmu-miR-320-3p promotes cartilage recovery in collagenase-induced OA mice. Compared with the normal group, OA group represented irregular cellular organization, reduction of proteoglycan, collagen type II and aggrecan in knee joint by Safranin O/fast green staining and Immunohistochemical analyses (A). Mmu-miR-320-3p group presented a recovery of smooth cartilage surface and content of proteoglycan, collagen type II and aggrecan in OA model mice (B, magnification 80×; Scale bar: 200 μm). OARSI grade scores of three paired representative OA and normal samples (C), three paired representative mmu-miR-320-3p and mmu-miR-Control samples (D). The levels of β-catenin and miR-320-3p in tibial plateau of mmu-miR-320-3p and mmu-miR-Control group were visualized by immunohistochemical analysis and in situ hybridization (E, magnification 80×, 160×). Quantitative data were presented as means ± SD, $*p < 0.05$, $**p < 0.01$.

compared the expression levels of miR-320c and β-catenin between OA chondrocytes and normal chondrocytes. As shown in Fig. 2, OA chondrocytes had low expression of miR-320c but high expression of β-catenin. We investigated the role of miR-320c in OA chondrocytes by overexpressing and inhibiting its expression. miR-320c promoted the expression of cartilage-specific genes and inhibited the expression of hypertrophy-related genes. These results were similar to those of CTNNB1 knockdown experiment in OA chondrocytes. Thus, miR-320c may inhibit the degeneration of chondrocytes by targeting CTNNB1 expression. We also proved that miR-320c may regulate the expression of β-catenin in OA chondrocytes, and used a luciferase reporter assay to clarify the underlying regulatory mechanism. Previous study has illustrated that the accumulation of cytoplasmic β-catenin results in its translocation into the nucleus to become a co-factor of TCF/LEF transcription and promote the expression of Wnt target genes [31]. We proved that the overexpression of miR-320c decreased the nuclear localization of β-catenin and the relative transcriptional activity of the β-catenin/TCF complex in chondrocytes. In OA chondrocytes and normal chondrocytes, the mRNA transcripts of multiple target genes of Wnt/β-catenin signaling pathway were downregulated in response to miR-

320c overexpression. It was previously illustrated that the intra-articular injection of microRNA agomir may alleviate OA progression and that microRNA levels in cartilage significantly increased within 6 weeks of intra-articular injection [32]. To further clarify the function of miR-320c in OA chondrocytes, we intra-articularly administered miR-320-3p agomir, a specially modified miRNA agonist, in a collagenase-induced OA model. The results showed that miR-320-3p decreased the level of β-catenin in OA model, significantly attenuated OA progression, and restored the smoothness of cartilage and contents of proteoglycan, collagen II, and aggrecan.

Previous study clarified that miR-320 can suppress chondrocyte degradation by inhibition of total β-catenin level [33]. However, we know that activated β-catenin were in nucleus. In this study, we examined that miR-320c can inhibits not only β-catenin expression level in nucleus but also transcriptional activity of β-catenin/TCF complex. Combined with the results of downstream genes of Wnt signaling pathway, we demonstrated that miR-320c can suppress the Wnt signaling pathway in chondrocytes, not just total level of β-catenin. Meanwhile, previous study proved the regulatory mechanism between miR-320 and β-catenin by luciferase reporter assay in HEK293 cells [33]. In our study, we demonstrated this process in chondrocytes, which is more reasonable.

The present study has some limitations. We used mmu-miR-320-3p intraarticular injection and did not clarify the regulatory relationship between mmu-miR-320-3p and β-catenin in vitro. However, the bioinformatic predictions revealed that the 3′-UTR of β-catenin contains a potential binding site for miR-320-3p. Furthermore, we demonstrated that miR-320c expression was silenced during the late stage of chondrogenesis and progression of OA, but the exact mechanism needs further clarification.

5. Conclusions

Our results indicate that miR-320c may inhibit the degeneration of OA chondrocytes and attenuate OA progression through the down-regulation of the Wnt/β-catenin signaling pathway. Therefore, miR-320c may serve as a novel therapeutic agent for the treatment of OA.

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Conflict of interest

The authors declare that they have no conflict of interests.

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