Abstract. Osteoarthritis (OA) is a chronic condition caused by cartilage degradation, and there are currently no effective methods for preventing the progression of this disease; gene therapy is a relatively novel method for treating arthritis. Decreased collagen type II (Col2) expression within the cartilage matrix is an important factor for the development of OA, and Wnt3a serves a significant role in cartilage homeostasis. The present study assessed whether Wnt3a knockdown promoted Col2 expression in chondrocytes. Lentivirus-introduced small interfering RNA was used to knock down the expression of Wnt3a in primary rat chondrocytes, and then IL-1β treatment was used to establish an OA chondrocyte model. The expression of target genes (Wnt3a, Col2, MMP-13 and β-catenin) was analyzed using reverse transcription-quantitative PCR, western blotting and immunocytochemistry. There was significantly less MMP-13 and β-catenin expression in the Wnt3a knockdown cells compared with the other controls. Col2 expression was significantly higher in the Wnt3a-knockdown cells compared with the control cells, indicating that knockdown of Wnt3a may promote Col2 expression. Consequently, Wnt3a was indicated to be an important factor in cartilage homeostasis, and Wnt3a knockdown may serve as a novel method for OA therapy.

Introduction

Osteoarthritis (OA) is a chronic refractory joint disease that places a heavy burden on health care systems, and there were $2046 in annual incremental expenditures for osteoarthritis in the USA. The annual incidence rate of OA was 0.18% worldwide in 2017 (1,2). Traditional treatments are primarily based on symptomatic management and include non-steroidal anti-inflammatory drugs and mind-body approaches (3,4). However, these therapies do not prevent OA deterioration.

Gene therapy has potential for treating OA. OA progression is closely associated with the TGF-β family (TGF-β1, TGF-β2 and TGF-β3), MAPK and Wnt signaling pathways (5-8). IL-1β is a major pro-inflammatory cytokine involved in the process of chondrocyte degradation that modulates the expression of Wnt-related proteins in chondrocytes (9,10). Wnt3a activates mesenchymal stem cell populations in autografts and modulates the articular chondrocyte phenotype (11,12). Wnt3a also promotes chondrocyte differentiation through Ca2+/calmodulin-dependent protein kinase II and proliferation through β-catenin (13). Overexpression of Wnt3a can stimulate the expression of various matrix metalloproteinases (MMPs), including MMP-1, MMP-2, MMP-3 and MMP-13, and can significantly reduce the expression of cartilage matrix molecules, such as collagen type II (Col2) and aggrecan (14,15). However, to the best of our knowledge, the effect of Wnt3a knockdown on gene expression in chondrocytes has not yet been assessed.

OA arises from cartilage cataplasia (5). Col2 forms the backbone for the construction of cartilage, which has structural and biochemical properties (16,17). A decrease in Col2 abundance may initiate and promote chondrocyte hypertrophy and OA progression (18,19). IL-1β can upregulate MMPs in the chondrocyte matrix, and MMPs can cause Col2 degradation (20). In addition, Wnt3a expression is closely associated with the expression of MMPs in synovial cells in vitro (15). These findings suggest that Wnt3a may serve an important role in chondrocyte degradation. Therefore, it was hypothesized that knockdown of Wnt3a may be a potential therapeutic intervention for management of OA (Fig. 1).

The present study aimed to silence Wnt3a mRNA with lentiviral vector-mediated Wnt3a-specific siRNA (LV-Wnt3a-RNAi) to promote COL2 expression. First, a cell model of osteoarthritis was established using interleukin-mediated mechanisms in vitro. And then OA-like chondrocytes were transfected with LV-Wnt3a-RNAi. Finally, it was determined whether silencing Wnt3a mRNA could promote COL2 expression by determining the expression of COL2 proteins.
Materials and methods

Animals. A total of five male Sprague-Dawley rats (age, 8 weeks; weight, 200±25 g) were obtained from the Animal Experiment Center of Shandong University (Jinan, China) and allowed to acclimate for 6 days before surgery. All operations were performed under anesthesia induced by 5% isoﬂurane in 1 l/min oxygen and maintained with 2.5% isoﬂurane in 0.5 l/min oxygen for the duration of endotracheal intubation and catheterization. Euthanasia was used in accordance with AVMA Guidelines for the Euthanasia of Animals (https://www.avma.org/sites/default/files/2020-02/Guidelines-on-Euthanasia-2020.pdf). The best efforts were made to minimize suffering and distress. Animals were housed at home temperature, relative humidity ~40-70% and a 12-h light/dark cycle, and were provided fresh air, food and water ad libitum.

Volume-controlled ventilator (Anhui Zhenghua Biological Instrument Equipment Co., Ltd.) was used before endotracheal intubation and abdominal aorta catheterization via the femoral artery. Arterial blood pressure, respiration rate and heart rate were monitored continuously by telemetry devices (Anhui Zhenghua Biological Instrument Equipment Co., Ltd.). Euthanasia was performed using 100% CO2 at a rate of 70% chamber volume displacement per minute. After heartbeat and respiration ceased, arterial blood pressure and heart rate were monitored for a further 2 min to conﬁrm death. All the animal’s vital signs were extinct after a minimum of 22 min and a maximum of 27 min of breathing carbon dioxide. The animal experiments were approved by the Ethics Committee of Shandong Provincial Hospital, Cheeloo College of Medicine, Shandong University (Shandong, China), and were performed in accordance with the Guide for the Care and Use of Laboratory Animals (21).

Primary chondrocytes were isolated from the articular cartilage of euthanized rats. A total of five rats were used. Briefly, cartilage tissue from femoral trochlea was cut into pieces and digested with 2.0 mg/ml collagenase type II (Gibco; Thermo Fisher Scientiﬁc, Inc.) at 37°C for 30 min with shaking, followed by incubation at 37°C in a water bath for 4 h. Samples were then sifﬁed three times through 200-mesh (SolelyBio-mall), and chondrocytes were collected, seeded in 60-mm culture dishes at a density of 8x10^5 cells per dish and cultured in antibiotic-conditioned medium (penicillin: 100 U/ml; Streptomycin: 100 µg/ml; DMEM; Invitrogen; Thermo Fisher Scientiﬁc, Inc.) supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientiﬁc, Inc.), and at 5% CO2 and 37°C. After the cells had grown to 90% conﬂuence, the chondrocytes were subcultured; passage-2 cells were used for all subsequent experiments.

Chondrocyte isolation and identiﬁcation. Chondrocyte identiﬁcation was performed by detecting suﬁlated glycosaminoglycans using toluidine blue staining (MilliporeSigma). Briefly, cells were ﬁxed in 4% paraformaldehyde at room temperature for 0.5 h. After three washes with PBS and blocking at room temperature for 5 min in 0.1% BSA (Beyotime Institute of Biotechnology), 0.2% Triton (cat. no. T9284; MilliporeSigma) in PBS was used to permeabilize the cells at room temperature for 0.5 h. After three washes with PBS, the cells were incubated with allophycocyanin-conjugated goat anti-rabbit secondary antibody (1:500; cat. no. ab130805; Abcam) and DAPI (MilliporeSigma) for 1 h at room temperature. Cells were observed visually using a ﬂuorescence microscope (magnification, x200).

Establishment of an in vitro OA model. To construct an in vitro OA model, chondrocytes were cultured in DMEM without serum at 37°C for 24 h and then with 10 ng/ml IL-1β (MilliporeSigma) at 37°C for 1, 2 or 6 h. MMP-13 was revealed to be highly expressed in the chondrocytes stimulated with IL-1β for 6 h, which conﬁrmed that the OA model was successfully established.

Lentivirus packaging and transfection. Lentivirus vector-mediated Wnt3a-specific RNA interference (LV-Wnt3a-RNAi; cat. no. GL27282; SS Sequence, GGA  ACU ACG UGG AGA CUG) was obtained from Shanghai GeneChem Co., Ltd., and used according to the manufacturer’s recommendations. Lentiviral vector used was GFP-tagged and ﬂuorescence staining was used to observe transfection. A preliminary experiment was ﬁrst performed to determine the duration of transduction into OA-like chondrocytes, and MOI. OA-like chondrocytes were assigned to three groups: i) Cells incubated in complete media at 37°C for 48 h (Control group); ii) cells incubated in complete media with the empty vector at 37°C for 48 h (Empty vector group); and iii) cells incubated in complete media supplemented with LV-Wnt3a-RNAI at 37°C for 48 h (LV-Wnt3a group). The amount of vector added to empty vector group was the same as that added to LV-Wnt3a group, several multiplicity of infection (MOI=20, 40, 60 and 100) confirm the chondrocyte properties, expression of Col2 protein in chondrocytes was determined using immunofluorescence. Briefly, samples were ﬁxed in 4% paraformaldehyde at room temperature for 0.5 h. After three washes with PBS and blocking at room temperature for 5 min in 0.1% BSA (Beyotime Institute of Biotechnology), 0.2% Triton (cat. no. T9284; MilliporeSigma) in PBS was used to permeabilize the cells at room temperature for 20 min, after which the cells were washed with PBS and incubated overnight at 4°C with an anti-Col2 antibody (1:500; cat. no. SAB4500366; Calbiochem; Merck KGaA) in PBS + 0.1% BSA. After three washes with PBS, the cells were incubated with allopachocyanin-conjugated goat anti-rabbit secondary antibody (1:500; cat. no. ab130805; Abcam) and DAPI (MilliporeSigma) for 1 h at room temperature. Cells were observed visually using a ﬂuorescence microscope (magnification, x200).

Figure 1. Putative effects of LV-Wnt3a-RNAi. IL-1β upregulates Wnt3a expression in chondrocytes, which activates the β-catenin-dependent canonical pathway to induce expression of MMPs and reduce Col2 expression. A LV-Wnt3a-RNAi was designed to knock down Wnt3a expression, which is hypothesized to result in an increase in Col2 expression. COL2, collagen type II; IL-1β, interleukin-1β; LV-Wnt3a-RNAi, lentivirus vector-mediated Wnt3a-specific RNA interference; MMPs, matrix metalloproteinases.
values was used. The optimal duration of transduction and MOI was 48 h and 60, respectively, which was consistent with manual recommendations. Stable cell lines were not required for this experiment. Following 72 h of chondrocyte transfection, to determine the transfection efficiency, the expression of Wnt3a mRNA was detected using RT-qPCR.

**Cell viability detection.** To demonstrate that the transfected chondrocytes were viable, Live-Dead staining was performed according to the manufacturer's instructions of LIVE/DEAD Viability/Cytotoxicity Assay kit (cat. no. L3224, Invitrogen; Thermo Fisher Scientific, Inc.) and as previously described (22). Briefly, calcein AM/ethidium homodimer-1 solution containing 2 mM calcein AM and 4 mM ethidium homodimer-1 were added to transfected chondrocytes for 30 min at 37°C. After washing three times with PBS, the samples were observed using a fluorescence microscope with an excitation wavelength of 488 or 568 nm. Live and dead cells appeared green (calcein AM) or red (ethidium homodimer-1), respectively. Overall, ~200 cells were counted manually under a fluorescence microscope, with dead cells and living cells separately counted.

**RT-qPCR.** Cells were lysed using TRIzol® reagent (Thermo Fisher Scientific, Inc.). cDNA was then generated using a PrimeScript™ RT Reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. Table I presents the primer sequences for Wnt3a, MMP-13, Col2, β-catenin and β-actin. RT-qPCR was performed on a LightCycler 480 System (SYBR Green; Roche Diagnostics Ltd.). Amplification was performed according to the manufacturer's instructions Stable cell lines were not required for this experiment. qPCR and western blotting were used to determine the transfection efficiency, the expression of Wnt3a mRNA was detected using the 2^\(-\Delta\Delta Cq\) method (23), and normalized to β-actin. The procedure was repeated at least three times for each gene.

**Western blotting.** Following infection for 24 h, cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology). Protein concentration was measured using a BCA assay (Thermo FisherScientific,Inc.). Subsequently, 10% SDS-PAGE was performed using an SDS-PAGE Gel Preparation Kit (Beyotime Institute of Biotechnology) to resolve the proteins (25 µg from each sample) by molecular weight, which were then transferred to PVDF membranes (MilliporeSigma). The membranes were blocked in 5% skimmed milk in TBS + 0.05% Tween (TBST) for 1 h at room temperature. After washing three times in TBST, membranes were incubated overnight at 4°C with one of the following primary antibodies: Rabbit anti-Col2 (1:500; cat. no. SAB4500366; Calbiochem; Merck KGaA), rabbit anti-MMP-13 (1:500; cat. no. MA5-42462; Thermo Fisher Scientific, Inc.), rabbit anti-β-catenin (1:1,000; cat. no. MA5-34961; Thermo Fisher Scientific, Inc.), rabbit anti-Wnt3a (1:1,000; cat. no. 703666; Thermo Fisher Scientific, Inc.) or rabbit anti-β-actin antibody (1:1,000; cat. no. 20536-1-AP; ProteinTech Group, Inc.), followed by incubation for 1 h at room temperature with the goat anti-rabbit HRP secondary antibody (1:5,000; cat. no. sc-2054; Santa Cruz Biotechnology, Inc.). After washing with TBST, blots were visualized using Enhanced Chemiluminescence Reagent (MilliporeSigma), and ImageJ software version 1.8.0 (National Institutes of Health) was used for densitometric analysis.

**Immunocytochemistry.** Following fixation with 4% paraformaldehyde at room temperature for 20 min, chondrocytes from the three groups were blocked with 5% BSA at 37°C for 30 min in PBS and incubated overnight at 4°C with a rabbit anti-Col2 antibody (1:500; cat. no. SAB4500366; Calbiochem; Merck KGaA). Samples were then washed three times with PBS, followed by incubation with a goat anti-rabbit HRP secondary antibody (1:5,000; cat. no. sc-2054; Santa Cruz Biotechnology, Inc.) at 37°C for 1 h. After three washes in PBS, immunoreactivity was determined using 3,3'-diaminobenzidine (OriGene Technologies, Inc.). Counterstaining was performed with hematoxylin at room temperature for 15 sec. Stained cells were observed using light microscopy (magnification, x200), and Image-Pro Plus (version 7.0; Media Cybernetics, Inc.) was used to assess integrated optical density of the images and at least three fields were selected for each slide to evaluate the results.

**Statistical analysis.** An analysis of variance was conducted with one-way analysis of variance, followed by Tukey's test for all experiments performed in triplicate. The data is presented as the mean ± standard deviation. The analysis was conducted using SPSS software version 23 (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Identification of chondrocytes and construction of OA-like cells.** Green fluorescence specific to Col2 and toluidine blue-stained cytoplasm as well as Col2-specific green fluorescence expression indicated that the obtained cells were chondrocytes (Fig. 2A); thus, these cells were used in subsequent experiments. qPCR and western blotting were used to
investigate whether the cell model was successfully established after IL-1β stimulation. As shown in Fig. 2D, expression of MMP-13 was significantly higher in the 6 h-group compared with other groups, which indicated that osteoarthritis model was successfully established.

**Transfection of chondrocytes with LV-Wnt3a-RNAi and viability assays.** A total of 48 h after transfection with the lentiviral vector, LV-Wnt3a-RNAi (at MOI=60), transfection was confirmed by fluorescence staining (Fig. 2B). Live-Dead staining was used to assess the viability of transfected chondrocytes, and the results demonstrated that Wnt3a-knockdown chondrocytes remained viable, ~200 cells were counted manually under a fluorescence microscope, with dead cells and living cells separately counted. and the cell survival rate of all the groups was ≥96% (Fig. 2C and Table II). As shown in Fig. 2E, RT-qPCR analysis demonstrated that expression of Wnt3a was significantly lower in the LV-Wnt3a group compared with the control group (non-transfected cells) and empty vector-transfected group. Therefore, the 72 h Wnt3a-knockdown chondrocytes were used in the subsequent experiments.
Wnt3a knockdown increases Col2 expression and decreases MMP-13 and β-catenin expression. RT-qPCR, immunocytochemistry and western blotting were used to investigate whether knockdown of Wnt3a by LV-Wnt3a-RNAi could promote Col2 expression. Results from RT-qPCR analysis demonstrated that expression of Wnt3a was significantly lower in LV-wnt3a group (LV-Wnt3a-RNAi-transfected cells) compared with control group (non-transfected cells) and empty vector group (empty vector-transfected cells) (both P<0.0001; Fig. 3A). In addition, Col2 expression was significantly higher in LV-wnt3a group compared with control group and empty vector group (both P<0.0001), and MMP-13 and β-catenin expression levels were significantly lower in LV-wnt3a group compared with control group and empty vector group (all P<0.0001) (Fig. 3A). Western blotting demonstrated that Col2 protein expression was significantly higher in LV-wnt3a group compared with control group and empty vector group (all P<0.0001) (Fig. 3B). Immunocytochemical analysis demonstrated that Col2 expression was higher in LV-wnt3a group than in control group and empty vector group (Fig. 3C). By contrast, there was no significant difference in

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**Table II. Cell counting of Live-Dead staining.**

| Group name       | First counting | Second counting | Third counting | Dead cell ratio (mean) | Live cell ratio (mean) |
|------------------|----------------|-----------------|----------------|------------------------|------------------------|
| Control          | 7/200          | 8/200           | 7/200          | 3.67%                  | 96.33%                 |
| Empty vector     | 6/200          | 8/200           | 9/200          | 3.83%                  | 96.17%                 |
| LV-wnt3a         | 7/200          | 9/200           | 8/200          | 4.0%                   | 96.0%                  |

LV-wnt3a, lentivirus vector-mediated Wnt3a-specific RNA interference; N/200, number of dead cells/200.
Col2 expression between groups A and B (Fig. 3B). These results of IOD of immunocytochemical images were consistent with the western blotting analysis. Thus, these results indicated that knockdown of Wnt3a promoted Col2 expression in chondrocytes.

Discussion

OA is a common degenerative disease amongst older individuals (>60 years old) and is a significant burden on healthcare resources. Therapeutic regimens have primarily focused on symptomatic relief and typically involve non-steroidal anti-inflammatory drugs and rehabilitation exercises (2,24). There are few treatments available to delay the degeneration of articular cartilage; gene therapy may be an effective treatment method.

Chondrogenic dedifferentiation is closely associated with various signaling pathways, including TGF-β, MAPK and Wnt (7,8,22). Cartilage matrix and chondrocyte degeneration are aggravated by increased levels of IL-1β. For example, in osteoarthritic chondrocytes, IL-1β upregulates MMP-3 and tumor necrosis factor-stimulated gene 6, and downregulates aggrecan (ACAN) (25,26). Similar results were observed for chondrocytes from patients with rheumatoid arthritis. Continuous IL-1β exposure has been shown to efficiently suppress synthesis of Col2 and CoI4, but increase the mRNA levels of CoI1 and CoI3. The differential expression of collagen proteins in cartilage may potentially promote the breakdown of the cartilage matrix (26,27). IL-1β-related pathways are complex; IL-1β can modulate Wnt3a expression, and may therefore induce β-catenin nuclear accumulation to increase expression of MMPs (28,29). Differential expression of MMPs results in the decrease of CoI2 and CoI4, which contributes to OA (30). Overexpression of Wnt3a can induce differential expression of MMPs and can significantly reduce the expression of the cartilage matrix molecules, CoI2 and ACAN (14,15). Therefore, it was hypothesized that Wnt3a knockdown may promote Col2 expression in chondrocytes and the appropriate repair of cartilage matrix.

A Wnt3a-targeting small interfering (si)RNA packaged in a lentiviral vector was used in the present study to knock down Wnt3a. This method was supported by a previous transgenic mouse study that demonstrates the effectiveness of lentiviral vectors to deliver siRNA (31). To explore whether Wnt3a knockdown could increase Col2 expression, the levels of Col2 in chondrocytes were determined. Col2 mRNA and protein expression levels were significantly higher in the Wnt3a-knockdown group compared with the control groups. These results were consistent with the immunocytochemistry results, where the levels of Col2 were markedly higher in the Wnt3a-knockdown group compared with the control groups. Wnt3a-knockout also significantly increased the protein expression of Col2 in OA-like chondrocytes.

The specific molecular mechanisms for this effect can be associated with the β-catenin-dependent canonical Wnt pathway (32). A decrease in Wnt3a levels, combined with a decrease in the expression levels of Frizzled receptors and low-density lipoprotein receptor-related receptor (LRP)5 and LRP6, disrupts the accumulation of β-catenin, which further increases Col2 gene transcription (32,33). Similarly, Held et al (34) reported that canonical Wnt inhibitors can regulate the expression of characteristic chondrocyte genes (Sox9, Col2 and ACAN) by blocking Wnt3a receptors. In in vivo experiments, Bertrand et al (35) came to the same conclusion that Wnt3a can modulate cartilage matrix maker genes (Sox9, Col2 and ACAN) through LRP6 phosphorylation and stabilization of β-catenin. In addition, Thomas et al (36) reported that Wnt3a-loaded exosomes can promote cartilage repair in the knees of mice. These exosomes may regulate circumarticular synovial tissues in addition to cartilage. The precise mechanisms of cross-talk and cross-regulation between different tissues requires further study. In the present study, knockdown of Wnt3a can promote protein expression of Col2 in vitro, which may be an effective therapy for management of OA. One concern about the findings was that transfected viral vector (GFP-tagged) and calcein-AM staining have the same green fluorescence properties, which may affect the intensity of the fluorescence. But it had very little impact on the count of living or dead cells. Furthermore, the present study chose naked eye observation to determine the optimal MOI value. Although visual observation is not very accurate, subsequent transfection efficiency and cell viability confirmed that MOI can be applied. Further animal experiments should be performed to verify the effectiveness of Wnt3a knockdown.

In conclusion, Wnt3a siRNA was successfully packaged into a lentiviral vector and used to effectively knock down Wnt3a expression. Wnt3a-knockdown may delay the degeneration of articular cartilage; thus, LV-Wnt3a-RNAi may be a novel tool for the management of OA.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

SSu conceived and designed the study, and revised the manuscript. SSh drafted the manuscript and performed the experiments. ZM collected and analyzed data. SSu and ZM confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the Ethics Committee of Shandong Provincial Hospital, Cheeloo College of Medicine, Shandong University (approval number 2019-08, Shandong, China) and were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.
Competing interests

The authors declare that they have no competing interests.

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