

Silencing of Wnt5a prevents interleukin-1 β -induced collagen type II degradation in rat chondrocytes

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Abstract. Osteoarthritis (OA) is a joint disease, and few treatments to date have been able to delay OA progression. The degradation of collagen type II (COL2) in the cartilage matrix is an important initiating factor for OA progression; the upregulation of Wnt5a protein activates COL2 degradation. In the present study, small interfering RNA of Wnt-5a was delivered by a lentiviral vector (LV-Wnt5a-RNAi) to silence Wnt-5a mRNA and prevent COL2 degradation. To determine the function of LV-Wnt5a-RNAi, the OA chondrocyte model (OA-like chondrocytes) were constructed using interleukin (IL)-1 β . Detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), Wnt-5a mRNA in the OA-like chondrocytes were upregulated in a time-dependent manner, indicating that OA-like chondrocytes were successfully constructed. The bioactivity of OA-like chondrocytes was determined using Live-Dead staining, and the result illustrated that the OA-like chondrocytes stimulated with IL-1 β for 6 h remained viable, and these were used in Wnt5a silencing. The OA-like chondrocytes were divided into three groups: Group I, cultivated with common medium; group II, cultivated with common medium supplemented with empty lentiviral vector; group III, cultivated with common medium supplemented with LV-Wnt5a-RNAi. The efficiency of LV-Wnt5a-RNAi transfection was determined using fluorescence microscopy, the result of which indicated that LV-Wnt5a-RNAi could efficiently be transfected into the OA-like chondrocytes. The LV-Wnt5a-RNAi efficiency for the Wnt5a mRNA silencing was determined using RT-qPCR. The result illustrated that the mRNA of Wnt5a in group III was significantly lower in group I compared with that in group II ($P < 0.05$), indicating that the LV-Wnt5a-RNAi could successfully

silence Wnt5a mRNA. To further verify whether the silencing of Wnt5a mRNA could prevent COL2 degradation, western blotting and immunohistochemical analyses were performed. The results demonstrated that COL2 in group III was significantly higher compared with that in groups I and II ($P < 0.05$), which illustrated that the silencing of Wnt5a mRNA could prevent COL2 degradation. In conclusion, LV-Wnt5a-RNAi was formed successfully and could efficiently silence Wnt5a mRNA expressed by OA-like chondrocytes. In addition, the silencing of Wnt5a mRNA could prevent the degradation of COL2 in OA-like chondrocytes, confirming that LV-Wnt5a-RNAi may be used as a novel tool for OA treatment.

Introduction

Osteoarthritis (OA) affects ~10% of the world population >60 years old; it is difficult to treat joint disease and it is associated with a heavy financial burden on families and society (1). In developed countries, the cost of OA treatment represents 1.0-2.5% of the gross domestic product per year (2). To date, the treatment of OA is primarily based on symptom management, such as the use of non-steroidal anti-inflammatory drugs (NSAIDs) to relieve pain (3). However, few treatments have the proven ability to delay OA progression (4).

Gene therapy may be a useful method to delay OA progression (5), and it has been studied for nearly 20 years (6). The transferred gene could deliver gene products to the local area of the joint in a sustained manner, which has fewer extra-articular adverse effects (7). Currently, the most prominent transgenes used in OA gene therapy are transforming growth factor β 1 (TGF- β 1) (8) and insulin-like growth factor-1 (IGF-1) (9), which primarily promote the regeneration of cartilage. However, small interfering RNA (siRNA), which could silence the effect of specific mRNA (10), may be another important tool in OA gene therapy. The present study aimed to explore the biological effect of Wnt5a-specific siRNA in OA.

It is understood that OA results from cartilage degeneration. Specifically, the destruction of collagen type II (COL2) in the cartilage matrix is an important initiating factor for cartilage degeneration and OA progression. COL2 forms the skeletal structure of cartilage, which provides structural and biochemical support (11). As a result of the poor self-repair ability of cartilage, preventing COL2 degradation in cartilage injury is the key factor in inhibiting further cartilage degeneration and

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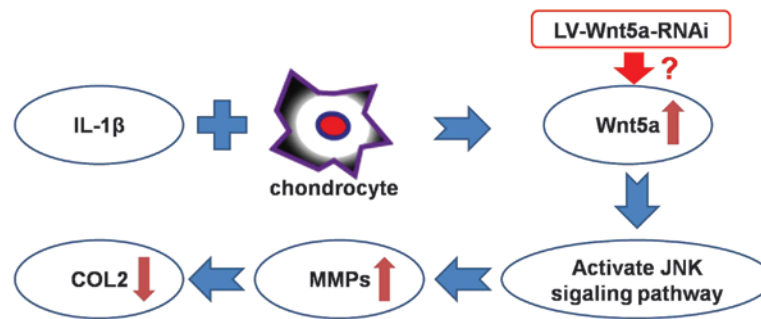


Figure 1. Schematic illustration of the working hypothesis for LV-Wnt5a-RNAi. IL-1 β upregulates the Wnt5a protein, and the Wnt5a protein activates the JNK signaling pathway, inducing the upregulation of MMPs. Ultimately, the MMPs result in COL2 degradation, thus inducing osteoarthritis. The LV-Wnt5a-RNAi was designed in the present study, and is used to silence the Wnt5a mRNA with the purpose of preventing COL2 degradation. LV-Wnt5a-RNAi; lentivirus vector-mediated Wnt5a-specific siRNA; MMP, matrix metalloproteinase; IL-1 β , interleukin-1 β ; COL2, collagen type II; JNK, jun amino-terminal kinases.

OA progression (12). Interleukin (IL)-1 β is the most important proinflammatory cytokine in the pathophysiology of OA, which serves a key role in COL2 degradation (13,14). It has been reported that IL-1 β could upregulate the Wnt5a protein, and the Wnt5a protein could activate the Jun amino-terminal kinase (JNK) signaling pathway, inducing the upregulation of matrix metalloproteinases (MMPs) (15,16). Ultimately, the MMPs cause COL2 degradation and destruction, thus inducing OA. That is to say, the Wnt5a protein is the core site of IL-1 β -induced COL2 degradation in chondrocytes (Fig. 1). Consequently, Wnt5a mRNA can be chosen as a therapeutic target of siRNA.

The present study aimed to silence Wnt5a mRNA with lentiviral vector-mediated Wnt5a-specific siRNA (LV-Wnt5a-RNAi) to prevent COL2 degradation. OA-like chondrocyte injury was mimicked using IL-1 β *in vitro*, and LV-Wnt5a-RNAi was used to transfect the OA-like chondrocytes. Following this, the efficiency of Wnt5a mRNA silencing was determined. Finally, the expression of COL2 proteins were determined to assess whether the silencing of Wnt5a mRNA can prevent COL2 degradation *in vitro*.

Materials and methods

Articular chondrocyte culture and identification. Chondrocytes from Sprague-Dawley rats (n=24 rats; age, 9 weeks; weight, 180 \pm 12 g) were harvested according to a previous study (17). These rats were fed with standard laboratory food (containing 1.56% calcium, 0.8% phosphorus and 800 IU/kg vitamin D) and kept under a 12 h light/dark cycle at 24°C and 50% humidity. After sacrifice with an overdose of anesthetic (12 ml/kg 20% urethane; Hengyuan Biological Technology Co., Ltd., Shanghai, China), the cartilage was sliced into small pieces from the femoral trochlea and digested with 2.0 mg/ml type II collagenase (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C for 4 h. Then, the digest solution was filtered with 200 mesh-filtrating screen to remove large fragments. The collected chondrocytes were seeded into culture dishes and cultured with Dulbecco's modified Eagle's medium (DMEM; Hyclone, GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 15% fetal bovine serum (Hyclone), and 50 U/ml penicillin and streptomycin. At 90% confluence, the chondrocytes were passaged; chondrocytes of passage 2 were used, as in a previous study (18). All animal experiments were approved by the ethics committee of Shandong Provincial

Hospital Affiliated to Shandong University, China, and complying with the 'Guide for the Care and Use of Laboratory Animals' published by the National Academy Press (NIH Publication no. 85-23, revised 1996).

To verify that the cultured cells were chondrocytes, the Toluidine Blue staining for sulfated glycosaminoglycans (GAGs) and immunohistochemical staining for COL2 were performed according to previous studies, respectively (19,20). The GAGs and COL2 were specific markers of hyaline cartilage.

To mimic the OA-like chondrocyte injury *in vitro*, IL-1 β was used, as previously described (21). The chondrocytes of passage 2 were cultivated with serum-free DMEM for 24 h, and stimulated with medium containing 10 ng/ml IL-1 β for 0, 1, 6, 12 or 24 h, respectively, as previously described (22).

To assess whether the OA-like chondrocytes were successfully generated, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to determine the gene expression of Wnt5a at different time points. Briefly, total RNA was harvested from chondrocytes of different groups using 1 ml TRIzol reagent (Takara Bio, Inc., Otsu, Japan). Subsequently, 1 μ g total RNA was reverse transcribed in each group using PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio, Inc., Otsu, Japan), under incubation conditions of 37°C for 15 min, followed by 85°C for 5 sec. The primer sequences of Wnt5a and β -actin are reported in Table I. RT-qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the amplification program as follows: Initial denaturation at 95°C for 10 min; followed by denaturation at 95°C for 5 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec for a total of 40 cycles; and a final extension at 60°C for 1 min. The total PCR reaction volume was 15 μ l, including 1 μ l cDNA, 7.5 μ l SYBR® Green PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan), 1 μ l of each primer and 5.5 μ l sterile water. The expression of Wnt5a relative to β -actin was calculated with the $2^{-\Delta\Delta C_t}$ method (23) using SPSS v. 19.0 software (IBM SPSS, Armonk, NY, USA). This procedure was performed in triplicate for each gene, with each reaction also performed without reverse transcriptase as a negative control.

To verify that the OA-like chondrocytes were viable and could be used in the present study, Live-Dead staining was performed according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.) and as previously described (24). Briefly, the OA-like chondrocytes were

Table I. Primer sequence of reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence
Wnt5a	
Forward	5'-TGTGGTTTAATGGTGCCTGA-3'
Reverse	5'-TTCGTCGTGCTCAAGGTATG-3'
β -actin	
Forward	5'-CTAAGGCCAACCGTGAAAAG-3'
Reverse	5'-AACACAGCCTGGATGGCTAC-3'

cultivated with reagent containing 2 mM calcein AM and 4 mM ethidium homodimer-1 for 30 min at 37°C. After washing with phosphate-buffered saline (PBS), the specimens were assessed using a fluorescence microscope with 488 or 568 nm excitation. Live cells were colored green by calcein AM, while dead cells were colored red by ethidium homodimer-1.

Lentiviral vector packaging and transfection. The siRNA of Wnt-5a was designed and packaged into the LV-Wnt5a-RNAi by Genechem Co., Ltd. (Shanghai, China). The operation process of lentiviral vector formation followed the recommendations of the manufacturer.

In Wnt-5a mRNA silencing, the passage 2 chondrocytes were stimulated for 6 h with IL-1 β as previously described (25). The OA-like chondrocytes were divided into three groups: Group I, incubated with complete DMEM for 7 days; group II, incubated with complete DMEM supplemented with empty lentiviral vector for 7 days; group III, incubated with complete DMEM supplemented with LV-Wnt5a-RNAi for 7 days. The concentration of empty lentiviral vector in group II was the same with the concentration of LV-Wnt5a-RNAi in group III, which ensured that the multiplicity-of-infection (MOI) was 50.

Western blotting analysis of COL2. Western blotting analysis was performed to determine whether the silencing of Wnt5a mRNA could prevent COL2 degradation in OA-like chondrocytes. Protein was harvested from the cells using lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and the protein concentration was determined using a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Protein (30 μ g from each sample) was run on 10% sodium dodecyl sulfate-polyacrylamide gels and electrotransferred onto nitrocellulose membranes. Subsequently, the membranes were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) for 1 h at room temperature. The blots were probed with monoclonal mouse anti-collagen II (cat. no. CP18; Calbiochem; Merck Millipore; 1:300 dilution) and monoclonal mouse anti-GAPDH (cat. no. AG019; Beyotime Institute of Biotechnology, Haimen, China, 1:500 dilution) antibodies overnight at 4°C, then incubated with a 1:4,000 dilution of goat anti-mouse, conjugated to horseradish peroxidase (HRP) (cat. no. sc-2005; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; 1:1,000 dilution) for 1 h at room temperature. The blots were visualized using the enhanced chemiluminescence method according to the

manufacturer's recommendations (EMD Millipore, Billerica, MA, USA). GAPDH was used as an internal control. Finally, the band density values were quantified with ImageJ v. 1.48 software (www.imagej.nih.gov/ij/).

Immunohistochemical analysis of COL2. To determine the COL2 protein expression in OA-like chondrocytes following the silencing of Wnt5a mRNA, immunohistochemical staining was performed, according to the previous report (26). Briefly, the chondrocytes of the three groups were fixed with 4% para-formaldehyde and blocked with 5% BSA in PBS. Then, the samples were incubated overnight at 4°C with anti-collagen II antibody (as with the Western blot). After three PBS washes, the cells were immersed in polyclonal goat anti-mouse secondary antibody (cat. no. PV-9002, Polink-2 plus Polymer HRP Detection System; Zhongshan Goldenbridge Co. Ltd., Beijing, China) at 37°C for 1 h. Following another round of washing, 3,3'-diaminobenzidine reagent (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) was used to determine the immunoactivity, and then the cell nuclei were counterstained with hematoxylin. The samples were observed under a light microscope, and the integrated optical density of different images were analyzed with Image-Pro Plus version 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. All experiments were performed in triplicate, and the results were analyzed using one-way analysis of variance, followed by Tukey's test. SPSS software version 19.0 (IBM SPSS, Armonk, NY, USA) was used to perform the statistical analysis. The data were presented as mean \pm standard deviation. $P < 0.05$ were considered to indicate a statistically significant difference.

Results

Identification of chondrocytes. As shown in Fig. 2A, the cultured cells of passage 2 were not elongated, which indicated that the cultured cells were chondrocytes. Furthermore, the Toluidine Blue staining confirmed that the passage 2 cells were chondrocytes (Fig. 2B). Consequently, the chondrocytes were successfully cultured, which resulted in approval for the follow-up study.

Properties and bioactivity of OA-like chondrocytes. RT-qPCR was performed to determine whether the OA-like chondrocytes were successfully induced with IL-1 β . The data in Fig. 3A illustrates that the mRNA of Wnt5a was upregulated in a time-dependent manner. The results indicate that the OA-like chondrocytes were successfully induced with IL-1 β .

The bioactivity of OA-like chondrocytes were assessed using Live-Dead staining. As shown in Fig. 3B, although the inflammatory reaction was activated in the OA-like chondrocytes due to the stimulation of IL-1 β , the cells remained viable. From these data, it can be concluded that the OA-like chondrocytes stimulated with IL-1 β for 6 h could be used in subsequent studies of Wnt5a silencing.

LV-Wnt5a-RNAi transfected chondrocytes and Wnt5a mRNA silencing. After 7 days of incubation, the LV-Wnt5a-RNAi

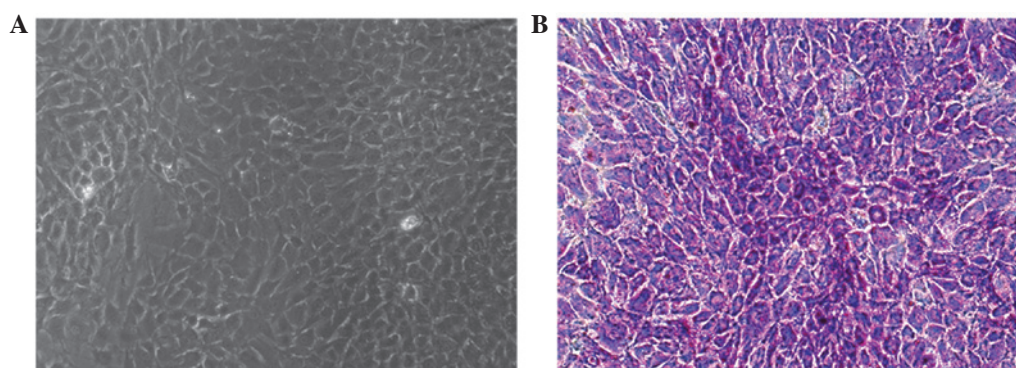


Figure 2. Property validation of cultured chondrocytes. (A) Cultured cells observed under a microscope. The cuboid shape of cells indicated that the cells were chondrocytes. (B) The identification of cell properties with Toluidine Blue staining. The cells were stained with Toluidine Blue, which confirmed that the cultured cells were chondrocytes (magnification, $\times 200$).

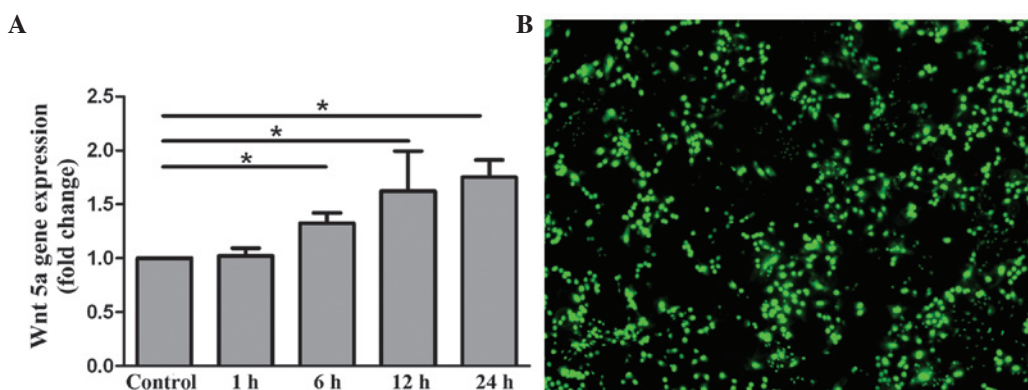


Figure 3. Validation of the OA-like chondrocytes stimulated with IL-1 β . (A) The expression of Wnt5a mRNA following stimulation by IL-1 β . After 6, 12 and 24 h of stimulation with IL-1 β , Wnt5a mRNA levels were significantly higher compared with the control group, which indicated that the OA-like chondrocytes were successfully formed; $P < 0.05$. (B) Live-Dead staining to determine the viability of the OA-like chondrocytes after 6 h of stimulation by IL-1 β . The majority of the cells were viable (green, live cells; red, dead cells), which indicated that the OA-like chondrocytes could be used in the following study (magnification, $\times 40$). OA, osteoarthritis; IL-1 β , interleukin-1 β .

could be transfected into the OA-like chondrocytes with MOI = 50 (Fig. 4A), as determined by fluorescence microscope (Fig. 4B).

RT-qPCR demonstrated that Wnt5a mRNA in group III was significantly lower compared with that in groups I and II ($P < 0.05$), while there was no significant difference between groups I and II (Fig. 4C). In conclusion, the LV-Wnt5a-RNAi used in group III could silence the Wnt5a mRNA expressed by the OA-like chondrocytes.

Preventing COL2 degradation by silencing Wnt5a mRNA. To determine whether the silencing of Wnt5a by LV-Wnt5a-RNAi could prevent COL2 degradation, COL2 proteins expressed in the three groups were assessed using western blotting and immunohistochemical analysis. The results of western blotting indicated that the COL2 content in group III was significantly higher compared with that in groups I and II ($P < 0.05$; Fig. 5A and B), while no significant difference were observed between groups I and II. In the immunohistochemical analysis, the immunoreactivity of group III was the highest among the three groups (Fig. 5C-F); this is consistent with the results of western blotting analysis. From these data, it can be concluded that the silencing of Wnt5a mRNA may prevent COL2 protein degradation.

Discussion

OA is a degenerative joint disease which affects a large number of individuals worldwide (1). In developed countries, the cost of OA treatment is about 1.0-2.5% of gross domestic product per year (2). To date, no highly effective drug can delay OA progression, because the existing treatment of OA is primarily based on symptom management, such as the use of NSAIDs to relieve pain (7). Gene therapy may be a useful method to delay OA progression, which has been studied for >20 years (6). The gene therapy of OA could be more effective and less expensive than the existing method, and be associated with fewer extra-articular adverse effects (10). Currently, the most prominent gene therapy of OA is the transgene of TGF- β 1 (8) and IGF-1 (9), which primarily promote the regeneration of cartilage. However, siRNA may be another useful tool of gene therapy in OA and may silence the biological effects of specific mRNA (10). For example, Chen *et al* (27) used the adenoviral vector-mediated nuclear factor- κ B p65-specific siRNA to alleviate inflammation of the synovium in OA.

It is understood that IL-1 β is the most important proinflammatory cytokine in the pathophysiology of OA. IL-1 β may upregulate the Wnt5a protein, and therefore activate the JNK signaling pathway to increase the expression of MMPs.

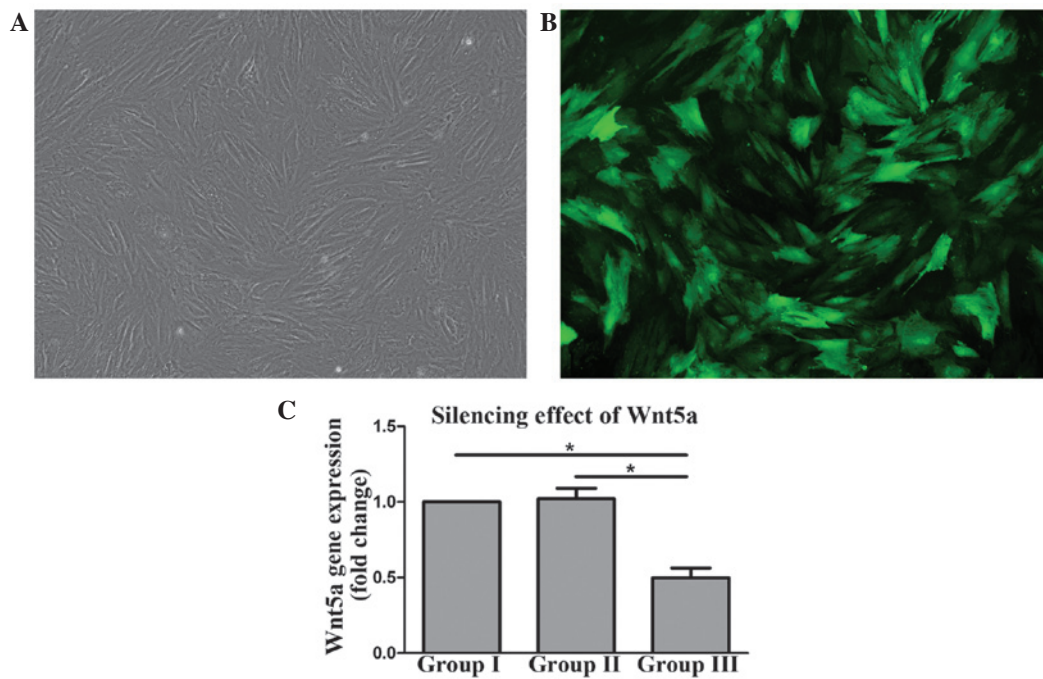


Figure 4. Determining the efficiency of LV-Wnt5a-RNAi transfection. (A) The OA-like chondrocytes observed under the microscope following transfection with LV-Wnt5a-RNAi. Magnification, x100. (B) The OA-like chondrocytes observed under fluorescence microscopy following transfection with LV-Wnt5a-RNAi. The green fluorescence illustrates that the OA-like chondrocytes were successfully transfected by LV-Wnt5a-RNAi with the multiplicity of infection = 50 (magnification, x200). (C) Quantification of the efficiency of Wnt5a mRNA silencing. The expression of Wnt5a mRNA in group III was significantly lower compared with that in groups I and II, which illustrates that the Wnt5a mRNA in group III could be successfully silenced by LV-Wnt5a-RNAi; * $P < 0.05$. OA, osteoarthritis; LV-Wnt5a-RNAi; lentivirus vector-mediated Wnt5a-specific siRNA.

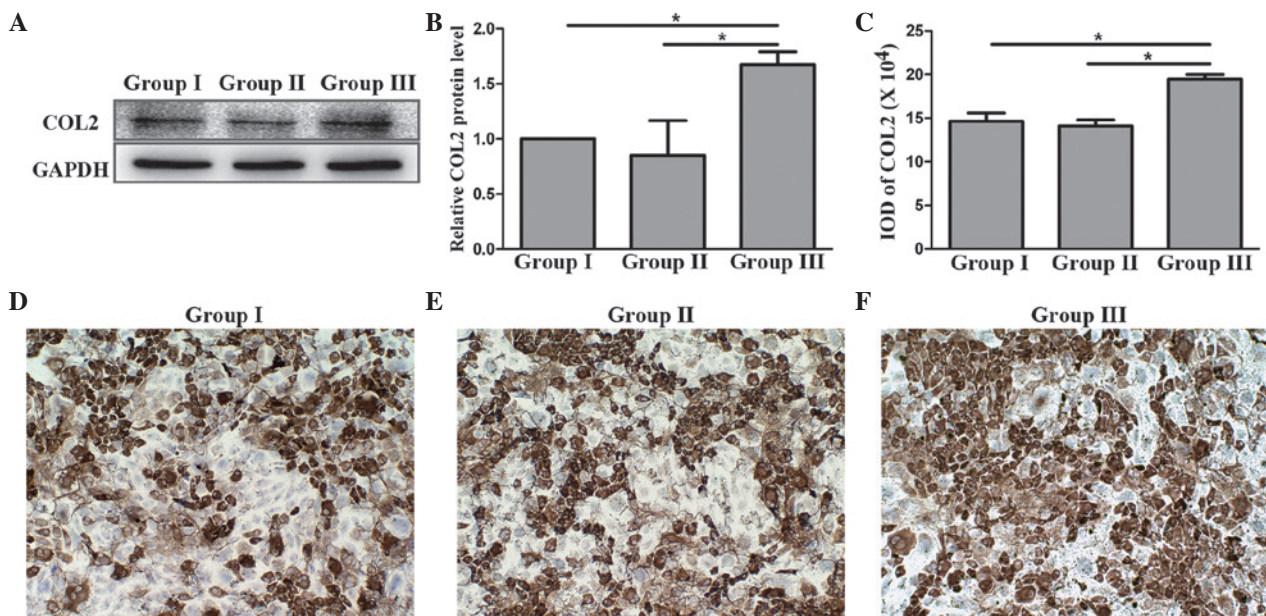


Figure 5. Silencing of Wnt5a mRNA preventing COL2 degradation. (A) Western blotting analysis of COL2 in the three groups. GAPDH was used as the control. (B) Quantitative analysis of western blotting images from three independent experiments, which are normalized to GAPDH; * $P < 0.05$. (C) Integrated option density of immunohistochemical analysis images from three independent experiments; * $P < 0.05$. Representative images of immunohistochemical analysis of COL2 in groups (D) I, (E) II and (F) III (magnification, x100). COL2, collagen type II; IOD, integrated option density.

MMPs result in the degradation and destruction of COL2, thus inducing OA (15,16). That is to say, the Wnt5a protein is the core site for IL-1 β -induced COL2 degradation in OA. Consequently, the silencing of Wnt5a mRNA was chosen as the therapeutic target of Wnt5a-specific siRNA to prevent COL2 degradation in the present study.

The Wnt5a-specific siRNA was packaged in a lentiviral vector to improve the transfection efficiency. Previous studies have reported that the lentiviral vector is an effective siRNA delivery system, which can protect the enclosed siRNA and transport the siRNA to targeted cells (10). In the current study, green fluorescence could be observed in the majority of the

chondrocytes, as shown in Fig. 4B, which indicated that the transfection efficiency of LV-Wnt5a-RNAi was excellent and the MOI used was appropriate.

The Wnt5a mRNA was silenced at least in part by LV-Wnt5a-RNAi, since the expression of Wnt5a mRNA in group III was significantly lower compared with that in groups I and II (Fig. 4C). With the action of LV-Wnt5a-RNAi, the Wnt5a mRNA becomes the component of RNA-induced silencing complexes (28). As a result, the Wnt5a mRNA is silenced and loses its biological activity.

To further explore whether silencing Wnt5a mRNA with LV-Wnt5a-RNAi can prevent COL2 degradation, the synthesis of COL2 was determined in the three groups. As shown in Fig. 5, the content of COL2 in group III was significantly higher compared with that in groups I and II. These results illustrate that the silencing of Wnt5a may prevent the degradation of COL2, the underlying mechanism being the silencing of Wnt5a reducing the synthesis of Wnt5a protein. The decrease of Wnt5a protein may reduce the activation of the JNK signaling pathway, further inducing the downregulation of MMPs (15,16). Consequently, the silencing of Wnt5a may protect COL2 from degradation *in vitro*, which may be a useful method of treating OA. Further animal experiments should be performed in future studies to fully assess the protection of COL2 by the silencing of Wnt5a mRNA.

In conclusion, the present constructed LV-Wnt5a-RNAi, which is siRNA of Wnt-5a packaged into a lentiviral vector. The LV-Wnt5a-RNAi could successfully silence the mRNA of Wnt5a. This silencing of Wnt5a mRNA may prevent the degradation of COL2, which is the key component in cartilage matrix. Therefore, LV-Wnt5a-RNAi may be a useful tool to prevent the progression of OA.

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