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Fibulin-5 protects the extracellular matrix of chondrocytes by inhibiting the Wnt/β-catenin signaling pathway and relieves osteoarthritis

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Abstract. – OBJECTIVE: Osteoarthritis (OA) is a common disease in the elderly and seriously affects the quality of life of patients. The purpose of this study was to explore the protective effect of Fibulin-5 on articular chondrocytes and its mechanism of action.

PATIENTS AND METHODS: Articular cartilage tissues from patients with OA and normal people were selected and tested for differences in Fibulin-5 expression. In addition, human chondrocytes were cultured, and the effects of Fibulin-5 on the extracellular matrix (ECM) of chondrocytes and the level of inflammation were examined by means of cell transfection and cytokine intervention. SKL2001, an agonist of the Wnt/β-catenin signaling pathway, was used to validate the mechanism of action of Fibulin-5 to protect chondrocytes.

RESULTS: Fibulin-5 was lowly expressed in the cartilage tissue of patients with OA. Overexpression of Fibulin-5 significantly increased the expressions of ECM collagen II and aggrecan in chondrocytes, while decreasing the expressions of MMP-3 and MMP-13. In addition, Fibulin-5 reduced IL-1β-induced inflammation of chondrocytes, as well as expressions of IL-6, IL-8, and TNF-α. Overexpression of Fibulin-5 also reduced the activity of Wnt/β-catenin signaling pathway, and activation of Wnt/β-catenin signaling pathway attenuated the protective effects of Fibulin-5 on the ECM of chondrocytes.

CONCLUSIONS: Fibulin-5 can protect the ECM of chondrocytes and reduce the inflammatory response of chondrocytes by inhibiting the Wnt/β-catenin signaling pathway.

Key Words:
Fibulin-5, OA, Chondrocytes, ECM, Wnt/β-catenin signaling pathway.

Introduction

Osteoarthritis (OA), also known as degenerative arthritis, is a chronic and deformed joint disease that is more common in the elderly and involves multiple small joints of the hand and weight-bearing joints[1]. Its clinical features are mainly joint pain, deformation, and functional limitation. The incidence of OA is quite high worldwide. 25% of people over the age of 60 have varying degrees of bone and joint disease, such as pain and limited function. Among them, the most common is knee arthritis[2]. At present, the destruction of articular cartilage is considered to be the main pathogenesis of arthritis. Articular cartilage tissue is mainly composed of chondrocytes and its secreted extracellular matrix (ECM)[3]. In a healthy population, the synthesis and decomposition of the cartilage matrix is in equilibrium. The production of OA is due to the disorder of this balance, and the reduction of cartilage matrix synthesis and the increase of decomposition[4]. In addition, the increased activity of matrix metalloproteinases (MMPs) in articular cartilage also increases the loss of cartilage matrix proteoglycans and collagen, leading to cartilage destruction and dissolution[5].

A variety of signal transduction pathways are involved in the pathogenesis of OA. Among them, the Wnt/β-catenin signaling pathway plays a crucial role[6]. By artificially culturing the chondrocytes of healthy rabbits, Spater et al[7] found that Wnt9α can activate the Wnt/β-catenin signaling pathway and inhibit the proliferation of cartilage.

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Dong et al[8] found that overexpression of Wnt8 can also increase the content of collagen X and RUNX2, leading to hypertrophy of chondrocytes. In the embryonic period, β-catenin is important for the formation of bone joints, the development, and differentiation of postnatal joints. After activation of β-catenin, the expressions of inflammatory factors such as TNF-α, IL-1β, IL-6, and PGE-2 were up-regulated, suggesting an increase in inflammatory response.

The Fibulin protein family is a class of ECM that is distributed throughout the tissues and organs and have important cellular functions[9]. The Fibulin family assists in the synthesis of ECM complexes and participates in the synthesis of elastic fibers and basement membranes. Fibulin-5 is a glycoprotein composed of 448 amino acids that plays an important role in regulating the balance of the extracellular environment[10]. In addition to the stabilizing effect on ECM, Fibulin-5 has an inhibitory effect on Wnt/β-catenin signaling pathway. Chen et al[11] found that Fibulin-5 can suppress the proliferation and metastasis of lung cancer cells by inhibiting Wnt/β-catenin signaling pathway in lung cancer cells. However, the role of Fibulin-5 in normal chondrocytes has not been studied.

Therefore, it was hypothesized that Fibulin-5 can promote the synthesis of ECM of chondrocytes and inhibit its degradation, and it may play a role in the prevention and treatment of OA by inhibiting the Wnt/β-catenin signaling pathway. This study can provide new directions and targets for the treatment of OA in the clinic.

Patients and Methods

Patient Tissue Samples

The knee cartilage tissue of patients with OA and normal people was extracted to detect changes in related indicators. The knee cartilage tissue of patients with severe OA requiring knee replacement surgery was taken out as a degenerative group. The knee cartilage tissue of the patient who had a lower limb amputation because of trauma or tumor was taken out as control group, and it was confirmed by imaging diagnosis that there was no evident OA. Articular cartilage tissue taken during surgery was immediately placed in liquid nitrogen for further experiment. This study was approved by the Ethics Committee of Third Hospital of Shijiazhuang. Ten articular cartilage specimens from patients with OA and ten articular cartilage specimens from patients without OA were collected. All patients provided written informed consent. This study was conducted in accordance with the Declaration of Helsinki.

Cells Culture and Drug Treatment

Human chondrocytes were purchased from Shanghai Guyan Industrial Co., Ltd., cultured using Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum and 1% penicillin plus streptomycin (Gibco, Grand island, NY, USA) and placed the cells in an incubator at 37°C and 5% CO_2. After the cell growth density reached 50-60%, the cells were transfected or stimulated with drugs. All cell experiments were performed in a clean bench. After that, SKL2001 (Selleck, China), an agonist of the Wnt/β-catenin signaling pathway, was used to activate the Wnt/β-catenin signaling pathway in chondrocytes. Finally, recombinant human IL-1β (Lianke, China) was used to stimulate degeneration of chondrocytes.

Lentiviral Transfection

Lentivirus Lenti-NC or Lenti-Filbulin-5 was constructed at Shanghai Jima Bio and were then transfected in chondrocytes using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA). Lenti-Filbulin-5 was used to increase the expression of Fibulin-5 in chondrocytes, while Lenti-NC was used as negative control.

Western Blot Analysis

Protein lysates (NCM Biotech, China) were used to lyse articular cartilage or chondrocytes, and cell debris was removed by centrifugation. Then, the bicinchoninic acid assay (BCA) kit (Yi Fei Xue Biotechnology, Nanjing, China) was used to detect protein concentration. After that, an appropriate amount of protein was added to the electrophoresis gel for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the protein was transferred to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking the non-specific antigen with 5% skim milk, primary antibodies (collagen II 1:3000, Rabbit, Abcam, Cambridge, MA, USA; aggrecan, 1:3000, Rabbit, Abcam; MMP3, 1:2000, Rabbit, Abcam; MMP13, 1:1000, Rabbit, Abcam; IL-6, 1:3000, Rabbit, Abcam, IL-8, 1:2000, Rabbit, Abcam; TNF-α, 1:1000, Rabbit, Abcam; β-catenin, 1:5000, Rabbit, Abcam; c-myc, 1:5000, Rabbit, Abcam; β-actin, 1:3000, Rabbit,

Abcam) were used to incubate the polyvinylidene fluoride (PVDF) membrane overnight at 4°C. The next day, after washing the PVDF membrane, a secondary antibody (Goat anti-rabbit, 1:3000, Abcam) was used to incubate the PVDF membrane for 2 h at room temperature. Finally, chemiluminescence was applied to detect protein expression.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from articular cartilage or chondrocytes using TRIzol (Invitrogen, Carlsbad, CA, USA). The SuperScript IV kit (Invitrogen, Carlsbad, CA, USA) was used to reversely transcribe mRNA to cDNA. Then, the SYBR Real-Time PCR kit (Invitrogen, Carlsbad, CA, USA) was used to amplify cDNA. With GAPDH as endogenous control, the $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression level. The primer sequences of mRNA are shown in Table I.

Enzyme Linked Immunosorbent Assay (ELISA)

Six-well plates were used to culture human chondrocytes. After the cells were treated, the cell supernatant was collected, and the cellular impurities were removed by centrifugation. Finally, the enzyme-linked immunosorbent assay (ELISA) kit (Lianke, China) was used to detect the expression levels of MMP3, MMP13, IL-6, and TNF-α.

Immunocytofluorescence Staining

Cell slides were placed in 24-well plates and chondrocytes were cultured therein. After the cells were treated, the 24-well plates were taken out and the medium was discarded. 4% paraformaldehyde was used to fix chondrocytes. Then, the chondrocytes were immersed in 0.5% Triton-PBS for 15 min, and 10% goat serum was used to block non-specific antigens. Thereafter, the cells were incubated with primary antibodies (collagen II, 1:500, rabbit, Abcam, TNF-α, 1:500, rabbit, Abcam, β-catenin, 1:500, rabbit, Abcam) at 4°C overnight. The next day, after washing the cells, the cells were incubated for 1 h at room temperature using a fluorescent secondary antibody (Goat anti-rabbit-FITC, 1:500, Abcam). The slides were then removed and fixed to the glass slides using a mounting medium containing 4',6-diamidino-2-phenylindole (DAPI). Finally, the staining results were observed and recorded using a fluorescence microscope.

Statistical Analysis

SPSS 18.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data from this study and use GraphPad Software (La Jolla, CA, USA) to create and analyze charts. For the measurement data in the results of this study, the mean ± standard deviation was used to represent. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Signif-

Table I. RT-PCR primer sequences.

Name	sense/anti-sense	Sequence (5'-3')
Collagen II	sense	GGGAATGTCCTCTGCGATGAC
	anti-sense	GAAGGGGATCTCGGGGTTG
Aggrecan	sense	GGTGAACCAGTTGTGTTGTC
	anti-sense	CCGTCCTTTCCAGCAGTC
MMP3	sense	ACATGGAGACTTTGTCCCTTTTG
	anti-sense	TTGGCTGAGTGGTAGAGTCCC
MMP13	sense	TACGAGCATCCATCCCGAGACC
	anti-sense	TACGAGCATCCATCCCGAGACC
IL-6	sense	ACTCACCTCTTCAGAACGAATTG
	anti-sense	CCATCTTTGGAAGGTTCAGGTTG
IL-8	sense	AGCCCAGAACACTGGTCTC
	anti-sense	ACTCAGGATTTCAATGGTGCC
TNF-α	sense	CTACCATCACCGCACTGAGAT
	anti-sense	GGTCACTTCACCATAGTGGACA
β-catenin	sense	GAGTGCTGAAGGTGCTATCTGTCTG
	anti-sense	TTCTGAACAAGACGTTGACTTGGA
c-myc	sense	TGCTGCCAAGAGGGTCAAGT
	anti-sense	TCAGCCAAGGTTGTG
GAPDH	sense	ACAACTTTGGTATCGTGGAAGG
	anti-sense	GCCATCACGCCACAGTTTC

icant Difference). All experiments were repeated more than three times. $p<0.05$ was considered to be statistically significant.

Results

Low Expression of Fibulin-5 in Articular Cartilage Tissue of OA Patients

The difference in the expression of Fibulin-5 in articular cartilage tissue of patients with OA compared with normal people was examined. Three cartilage samples were randomly selected from the control group and OA group for western blot analysis. The results of Western blot (Figure 1A) showed that the expressions of collagen II and aggrecan in articular cartilage of patients with OA were significantly lower than those of normal people, indicating that the ECM of articular cartilage is significantly degraded, and articular cartilage degeneration is evident. In addition, the expression level of Fibulin-5 in articular cartilage of patients with OA was also significantly lower than that of the normal people, indicating that the expression of Fibulin-5 is decreased in degenerative articular cartilage. We then extracted RNA from all cartilage samples and performed RT-PCR analysis. The results of RT-PCR (Figure 1B-1D) were similar to those of western blot.

Overexpression of Fibulin-5 Reduced IL-1β-Induced Degradation of ECM of Chondrocytes

The expression of Fibulin-5 in chondrocytes was increased by transfecting Lenti-Fibulin-5. In addition, recombinant human IL-1β (10 ng/ml) was used to stimulate chondrocyte degeneration to construct an OA model at the cellular level. Then, chondrocytes were treated with 10 ng/ml IL-1β and transfected with Lenti-NC and Lenti-Fibulin-5. The results of western blot (Figure 2A) and RT-PCR (Figure 2B-2E) showed that the expressions of collagen II and aggrecan in the IL-1β-stimulated chondrocytes were decreased and the expressions of MMP-3 and MMP-13 were increased, while the overexpression of Fibulin-5 reduced the destruc-

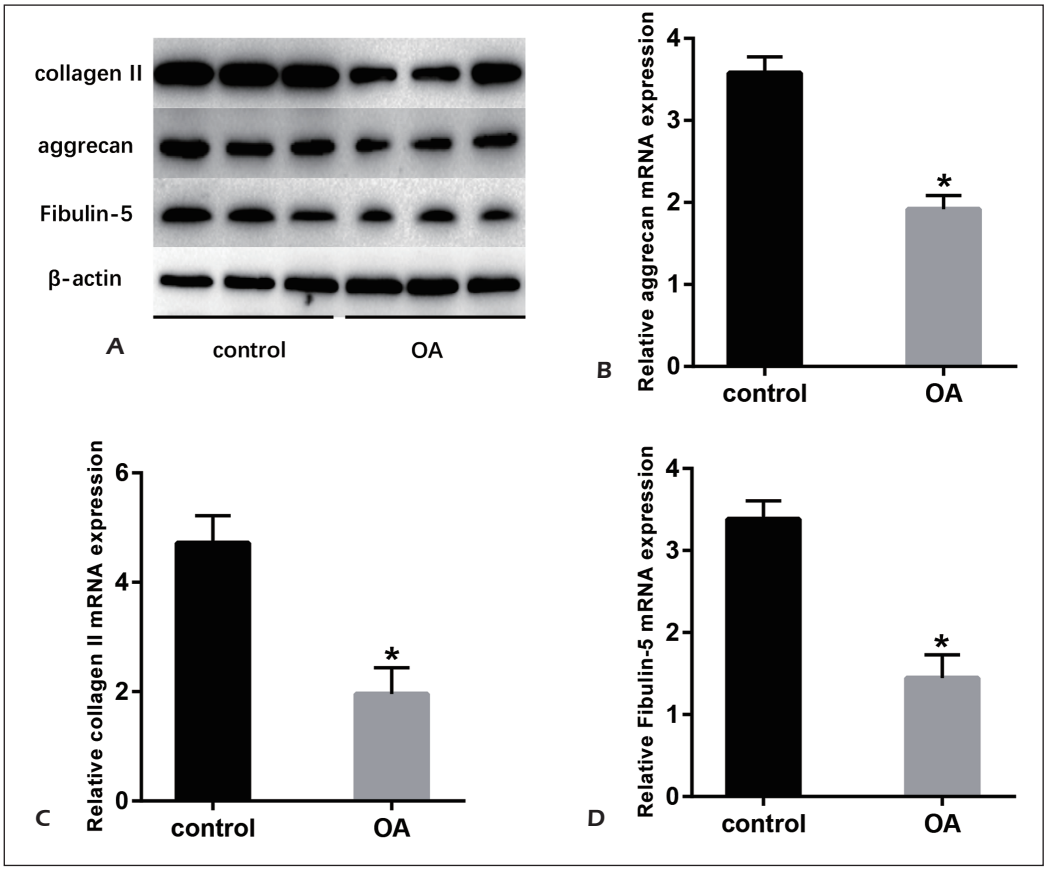

Figure 1. Low expression of Fibulin-5 in articular cartilage tissue of OA patients. **A-D**, Expressions of collagen II, aggrecan and Fibulin-5 in patients with OA and normal people are detected by Western blot (***A***) and RT-PCR (**B-D**).

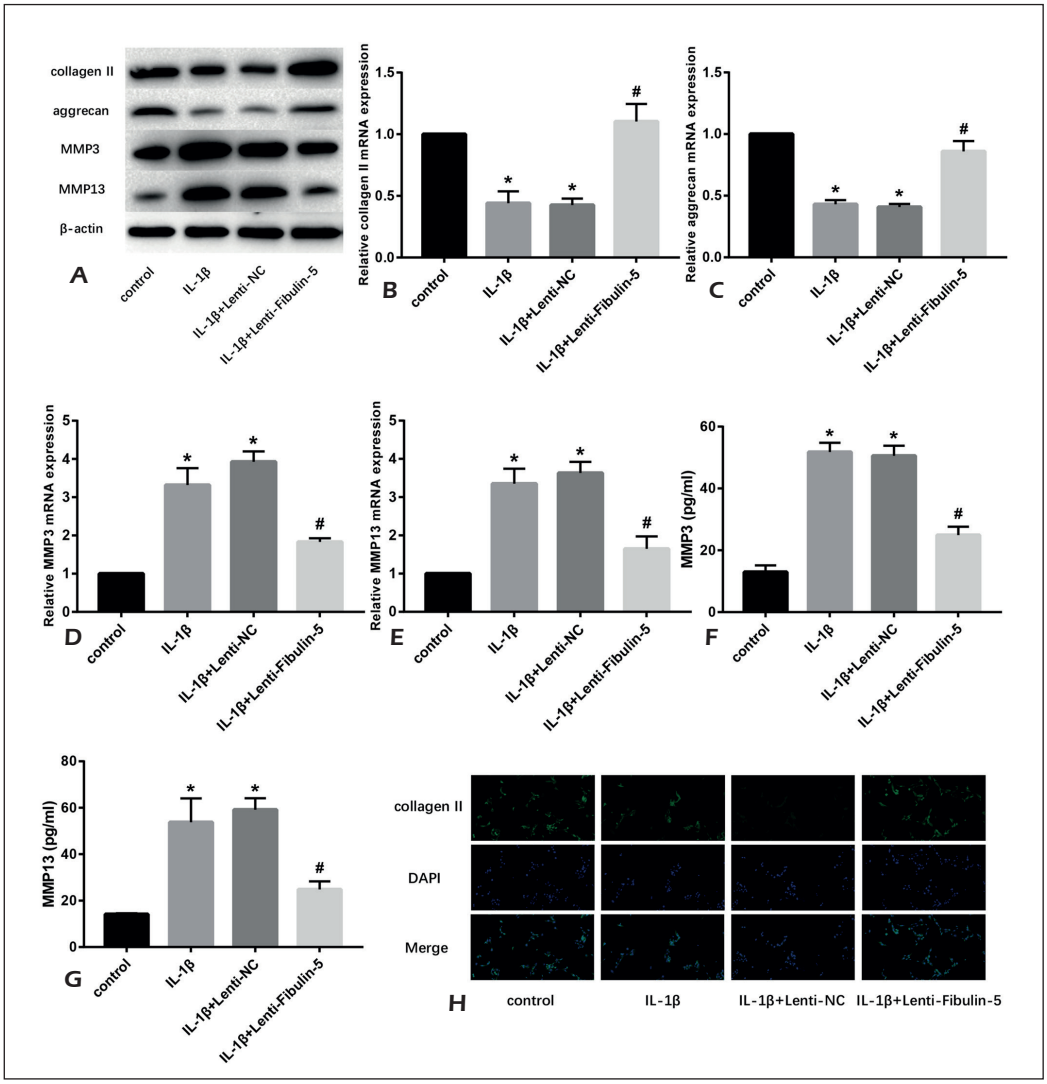

Figure 2. Overexpression of Fibulin-5 reduces IL-1β-induced degradation of ECM of chondrocytes. **A**-**E**, Expressions of collagen II, aggrecan, MMP3 and MMP13 in four groups are determined by Western blot **(A)** and RT-PCR (**B**-**E**). **F**-**G**, ELISA detects the expressions of MMP3 (**F**) and MMP13 (**G**). **H**, IF detects the expression of collagen II (magnification: 40×). ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β+Lenti-NC group).

tion of IL-1β on the ECM of chondrocytes. The results of the ELISA also indicated that Fibulin-5 effectively reduced the expressions of MMP-3 (Figure 2F) and MMP-13 (Figure 2G). The results of IF (Figure 2H) also validated the effect of Fibulin-5 on the ECM collagen II.

Overexpression of Fibulin-5 Reduced IL-1β-Induced Inflammation of Chondrocytes

To determine the effect of Fibulin-5 on inflammation of chondrocytes, the expression levels of inflammatory factors in chondrocytes were examined. The results of Western blot (Figure 3A) and RT-PCR (Figure 3B-3D) showed that IL-1β-induced chondrocytes expressed a large number of inflammatory factors, such as IL-6, IL-8, and TNF-α, while overexpression of Fibulin-5 significantly reduced the expression of inflammatory factors. The results of the ELISA (Figure 3E-3G) were similar to those of the Western blot. The results of IF (Figure 3H) also detected the inhibitory effect of Fibulin-5 on inflammation in chondrocytes.

Fibulin-5 Decreases the Activity of Wnt/β-Catenin Signaling Pathway in Chondrocytes

To examine the effect of Fibulin-5 on Wnt/β-catenin signaling pathway in chondrocytes, changes in the expression of Wnt/β-caten-

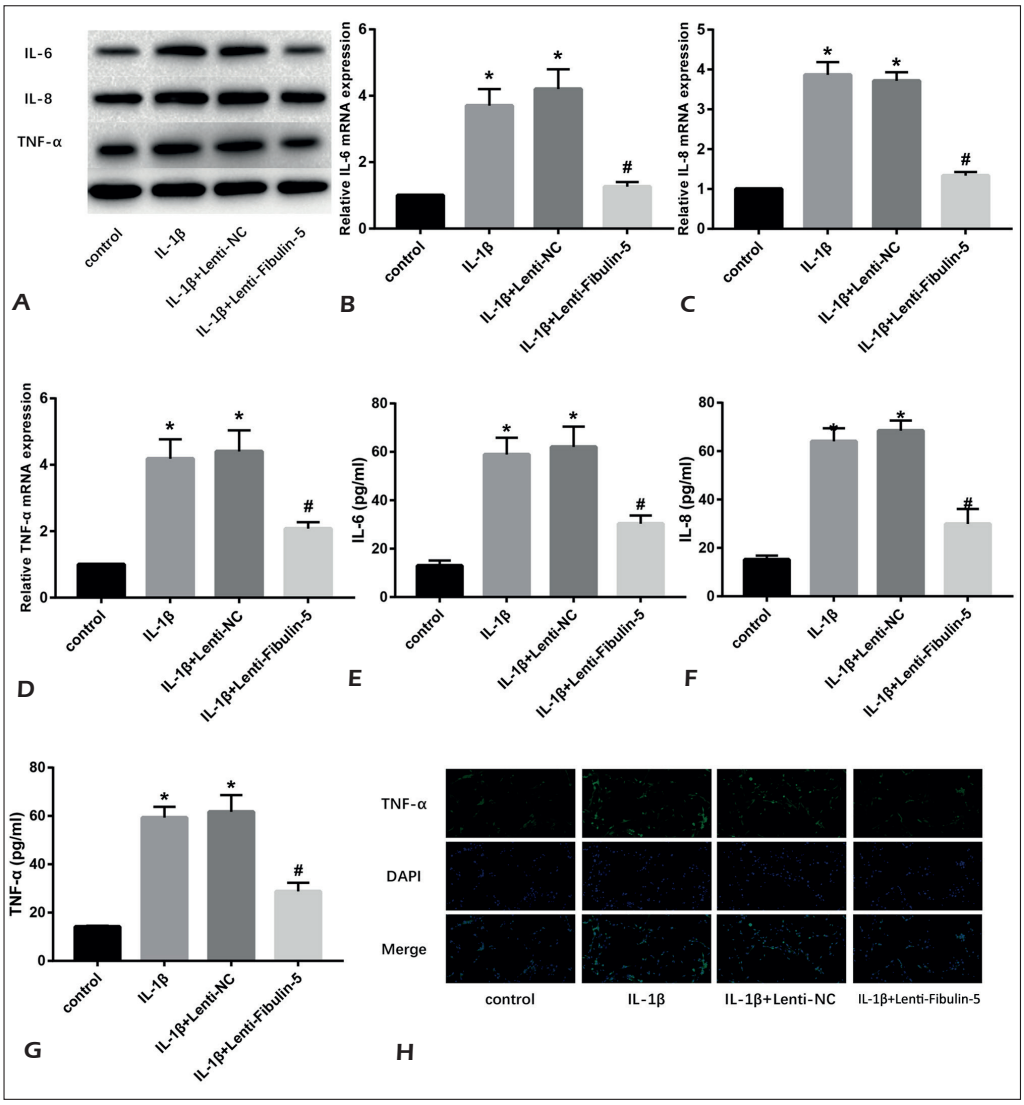

Figure 3. Overexpression of Fibulin-5 reduces IL-1β-induced inflammation of chondrocytes. **A**-**D**, Expressions of IL-6, IL-8 and TNF-α in four groups are determined by Western blot (**A**) and RT-PCR (**B**-**D**). **E**-**G**, ELISA detects the expressions of IL-6 (**E**), IL-8 (**F**) and TNF-α (**G**). **H**, IF detects the expression of TNF-α (magnification: 40×). ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β+Lenti-NC group).

in signaling pathway-related molecules were examined. The results of Western blot (Figure 4A) and RT-PCR (Figure 4B, 4C) showed that the expressions of β-catenin and c-myc in IL-1β-stimulated chondrocytes were significantly higher than those in control group, indicating that after IL-1β stimulates chondrocyte degeneration and the activity of the Wnt/β-catenin signaling pathway is increased. However, overexpression of Fibulin-5 reduced the expressions of β-catenin and c-myc. The results of IF (Figure 4D) also indicate that Fibulin-5 significantly decreases the expression of β-catenin.

Activation of Wnt/β-Catenin Signaling Pathway Attenuated the Protective Effect of Fibulin-5 on Chondrocytes

The Wnt/β-catenin signaling pathway agonist SKL2001 was used to activate the Wnt/β-catenin signaling pathway, thereby exploring the mechanism of action of Fibulin-5 to protect chondrocytes. The results of western blot (Figure 5A)

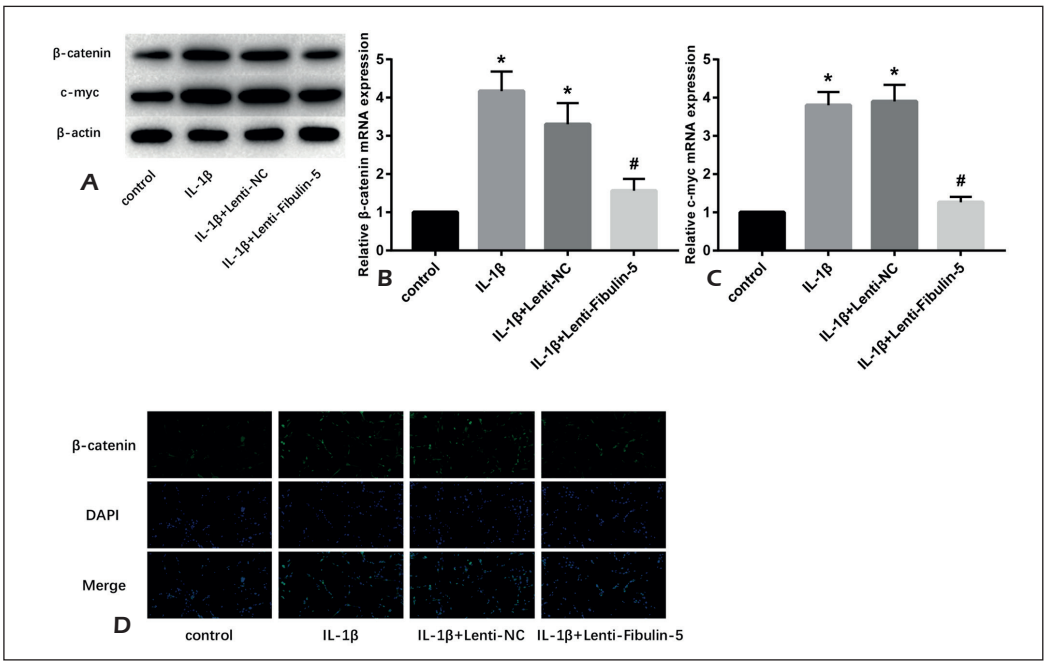

Figure 4. Fibulin-5 decreases the activity of Wnt/β-catenin signaling pathway in chondrocytes. **A-C**, Expression of β-catenin and c-myc in four groups was determined by Western blot (**A**) and RT-PCR (**B-C**,). **D**, IF detects the expression of β-catenin (magnification: 40×). ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β+Lenti-NC group).

and RT-PCR (Figure 5B-5E) showed that after activation of Wnt/β-catenin signaling pathway by SKL2001, the expressions of collagen II and aggrecan in chondrocytes were decreased and the expressions of IL-6 and IL-8 were increased, indicating that SKL2001 attenuates the protective effects of Fibulin-5 on the ECM of chondrocytes.

Discussion

In the pathogenesis of OA, there are always some activation of cytokines. In recent years, studies on OA-related cytokines have focused on IL-1β and TNF-α[12]. IL-1β stimulates chondrocyte synthesis and secretion of MMPs, inhibits the expression of cartilage collagen II and proteoglycans and degrades cartilage matrix. In addition, IL-1β promotes synovial cell synthesis and releases prostaglandin E2 (PGE2), NO and collagenase, producing a powerful pro-inflammatory effect, causing synovial inflammation and bone resorption. IL-1β can also stimulate the proliferation of human fibroblast-like cells by inducing the synthesis of IL-6 and accelerate the formation of bone around the joint[13]. TNF-α stimulates synovial cells to produce PGE2, accelerates cartilage destruction, selectively inhibits the synthesis of cartilage collagen and proteoglycans and promotes collagen and proteoglycan degradation[14]. In the pathogenesis of OA, TNF-α may promote IL-1β, that is, TNF-α can induce the synthesis of IL-1β, and IL-1β can increase the activity of TNF-α[15]. This study found that overexpression of Fibulin-5 promoted the synthesis of ECM of chondrocytes and increased the expressions of collagen II and aggrecan. In addition, Fibulin-5 also reduced the expressions of MMP-3 and MMP-13, thus reducing the degradation of ECM. Fibulin-5 also plays an important role in anti-inflammatory. Overexpression of Fibulin-5 significantly reduced the expressions of inflammatory factors IL-6, IL-8, and TNF-α in chondrocytes and showed significant anti-inflammatory effects. These results indicate that Fibulin-5 is an important protective factor in OA.

β-catenin is a multifunctional protein with a transcriptional activation in a critical and important position in the Wnt/β-catenin signaling pathway. Under the stimulation of Wnt/β-catenin signaling pathway, the complex formed by APC, GSK-3β, Axin, and other components decomposes, and the function of phosphorylated β-catenin is disabled and cannot be catabolized, resulting in an increase in the accumulation of β-catenin in the cytoplasm. After reaching a certain lev-

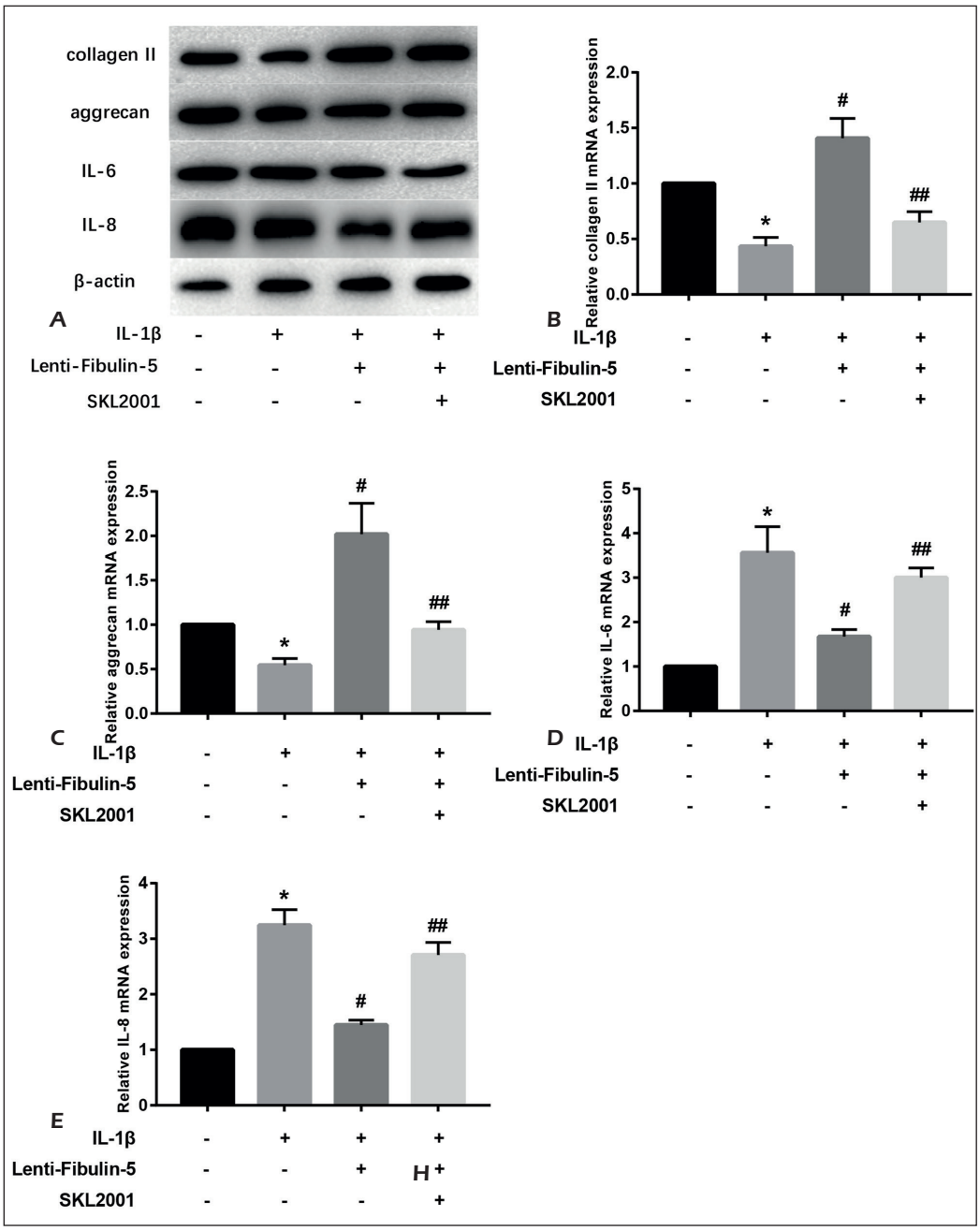

Figure 5. Activation of Wnt/β-catenin signaling pathway attenuates the protective effect of Fibulin-5 on chondrocytes. A-E, Expressions of collagen II, aggrecan, IL-6 and IL-8 in four groups are determined by Western blot (**A**) and RT-PCR (**B**-**E**). ("*" means there is a statistical difference with the control group, "#" means there is a statistical difference with the IL-1β+Lenti-NC group and "##" means there is a statistical difference with the IL-1β+Lenti-Fibulin-5+SKL2001 group).

el, β-catenin moved into the nucleus. When free β-catenin is transferred into the nucleus, it can interact with TCF/LEF, activate TCF transcriptional activity, affect the translation and transcription process of downstream target genes, and thus affect the growth and development of cartilage[16]. A large amount of β-catenin in cells can terminate the differentiation of chondrocytes, which can be used as an important mechanism for the degradation of cartilage tissue in the pathological process of OA[17]. The expression of Fibulin family has a significant correlation with the Wnt/β-catenin signaling pathway. Wang et al[18] studied the effect of Fibulin-4 on endometrial cancer cells

and found that knockdown of Fibulin-4 gene increased the expression of β-catenin, c-myc, and cyclin D1, indicating that Fibulin-4 inhibited Wnt/β-catenin signaling pathway. Ma et al[19] found that overexpression of Fibulin-2 can reduce the expression of β-catenin, cyclin D1, and c-myc in gastric cancer. In addition, Fibulin-3 and Fibulin-5 can inhibit the migration and invasion of lung cancer by inhibiting Wnt/β-catenin signaling pathway[11,20]. Naboulsi et al[21] found that abnormal regulation of Wnt/β-catenin signaling pathway was associated with Fibulin-5 expression in quantitative tissue proteomics analysis of 50 patients with hepatocellular carcinoma and 50 patients with non-hepatocellular carcinoma. However, it is still unclear whether Fibulin-5 has a similar mechanism of action in chondrocytes. This study found that Fibulin-5 has a significant inhibitory effect on Wnt/β-catenin signaling pathway, which may be an important mechanism for Fibulin-5 to exert protective effects on chondrocytes. IL-1β-induced chondrocytes showed a distinct degenerative phenotype and increased activity of the Wnt/β-catenin signaling pathway. Fibulin-5 reduced the expressions of β-catenin and c-myc in chondrocytes, thereby reducing the activity of Wnt/β-catenin signaling pathway.

The carboxy terminus of Fibulin-5 contains six cbGF-like regions that bind to calcium ions, the first of which contains an RGD motif that binds to integrin. The RGD motif binds to the cell surface integrins αγβ3, αγβ5, and α9β1. Fibulin-5 binds to the cell surface by binding to integrin and binds to fibronectin-5 to function as a fixed cell. Integrin also plays an important role in the pathogenesis of OA[22]. In adult chondrocytes, the main integrin is α5β1. α5β1 is a receptor for fibronectin in the ECM of chondrocytes and plays an extremely important role in chondrocyte mechanical signal transduction, cell differentiation, and proliferation[23]. Normal chondrocytes undergo rapid mechanical membrane hyperpolarization after mechanical stimulation, prompting the activation of ion channels. Activation of the α5β1 receptor results in autocrine or paracrine IL-4 secretion, which binds to the IL-4 receptor α subunit on the chondrocyte membrane to form a heterodimer. Heterodimers activate Janus kinase (JAK), such as JAKI, JAK2, JAI3, and Tyk2, activates the corresponding signal transduction and transcriptional activators. The activated transduction factor is transferred to the nucleus, regulates gene transcription, and finally leads to upregulation of proteoglycan mRNA expression and down-regulation of MMP-3 expression, thereby protecting cartilage[24].

To our knowledge, this is the first study to report the protective effect of Fibulin-5 on chondrocyte degeneration, which is believed to provide a new target for the clinical treatment of OA.

Conclusions

Fibulin-5 promotes the synthesis of ECM of chondrocytes and inhibits ECM degradation by reducing the expression of MMPs. In addition, Fibulin-5 reduces the expression of inflammatory factors in chondrocytes, thereby reducing the inflammatory response. Fibulin-5 also inhibits the activity of the Wnt/β-catenin signaling pathway, suggesting that Fibulin-5 may protect against OA by inhibiting the Wnt/β-catenin signaling pathway.

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Conflict of Interests

The authors declare that they have no conflict of interests.

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