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RBMS3 delays disc degeneration by inhibiting Wnt/β-catenin signaling pathway

J.-J. WANG[1], X.-Y. LIU[2], W. DU[1], J.-Q. LIU[1], B. SUN[1], Y.-P. ZHENG[3]

[1]Department of Spine, Yantai Shan Hospital, Yantai, Shandong Province, China
[2]Department of Laboratory, Yuhuangding Hospital, Yantai, Shandong Province, China
[3]Department of Orthopedic Surgery, Qilu Hospital of Shandong University, Qingdao, Shandong Province, China

Jingjie Wang and *Xiaoyan Liu* contributed equally to this work

Abstract. – OBJECTIVE: To study the effect of RBMS3 on nucleus pulposus cells and its effect on the Wnt/β-catenin signaling pathway.

PATIENTS AND METHODS: We measured the expression of RBMS3 in human nucleus pulposus tissues with different degrees of degeneration. Recombinant human IL-1β is used to stimulate the degeneration of human nucleus pulposus cells. We used Wnt/β-catenin signaling pathway inhibitors and cell transfection to study the effect of RBMS3 on nucleus pulposus cells and its mechanism.

RESULTS: RBMS3 was less expressed in the nucleus pulposus tissue of people with higher degeneration degree. IL-1β reduced the expression of RBMS3 in nucleus pulposus cells. Overexpression of RBMS3 can promote the proliferation of nucleus pulposus cells and reduce the apoptosis and inflammation of cells. In addition, RBMS3 can reduce the expression of β-catenin and c-myc in nucleus pulposus cells, and inhibit the activity of the Wnt/β-catenin signaling pathway.

CONCLUSIONS: RBMS3 inhibits the Wnt/β-catenin signaling pathway, improves the proliferation ability of nucleus pulposus cells, inhibits their apoptosis and inflammation, and thus delays the degeneration of the intervertebral disc.

Key Words:
RBMS3, Disc degeneration, Wnt/β-catenin signaling pathway.

Introduction

Chronic low back pain is still a high-incidence and intractable orthopedic disease worldwide. Also, it is one of the most serious diseases associated with chronic aging in the world[1]. There are about 600 million patients with low back pain worldwide and 70%-85% of adults will experience different degrees of low back pain in their lifetime, which will have a great impact on patients' life, national medical expenses, and social productivity[2]. It is estimated that the direct and indirect cost of low back pain in the United States exceeds $100 billion annually. Clinically, data based on histopathology and imaging suggest that the majority of low back pain begins with degenerative changes in the nucleus pulposus of the disc[3]. Therefore, it is one of the important directions of medical research to explore its effective treatment.

A disc is a soft connective tissue that connects adjacent vertebral bodies of the spine. It is a complex tissue composed of the nucleus pulposus, annulus fibrosus, and cartilaginous endplate. It has the function of transferring and cushioning spinal stress caused by weight and muscle contraction[4]. Nucleus pulposus is composed of collagen II, proteoglycan, and nucleus pulposus cells. Actually, it is mainly used to resist the longitudinal pressure. Annulus fibrosus is rich in crisscrossed collagen I and annulus fibrosus cells, and its main function is to cushion the lateral extension movement[5]. In the process of disc nucleus pulposus degeneration, the structure and biochemical components of the nucleus pulposus change. The decrease of proteoglycan and water content affects the bearing capacity of the disc[6]. Therefore, the intervertebral disc cannot play the original function as a cushion between the vertebral bodies, causing varying degrees of low back pain and nerve stimulation symptoms.

Corresponding Authors: Xiaoyan Liu, MD; e-mail: xiaoyanliu287@126.com
Yanping Zheng, MD; e-mail: w74887@126.com

The Wnt/β-catenin signaling pathway is an important branch of the Wnt signaling pathway, which is mediated by β-catenin protein and has an important regulatory system for cell proliferation, metabolism, growth, and development[7]. Reports[8] have shown that the Wnt pathway can induce the synthesis of many growth factor proteins such as MMPs, BMPs, TGF-β, and insulin growth factor in intervertebral disc cells to induce the synthesis of the matrix in the nucleus pulposus tissue and affect the biological activity of other cells, thereby accelerating the degeneration process. Therefore, the abnormal activation of the Wnt/β-catenin signaling pathway can cause or accelerate intervertebral disc degeneration. Studies[9] have shown that RBMS3 has an inhibitory effect on the Wnt/β-catenin signaling pathway. However, the role of RBMS3 in disc degeneration has not been explored. Therefore, this work used human nucleus pulposus cells to study the effect of RBMS3 on disc and its mechanism. We hope to find a new target for the treatment of disc degeneration by studying RBMS3 to provide a theoretical basis for clinical treatment of disc degeneration.

Patients and Methods

Patient Tissue Samples

All of the human disc tissue came from patients who had intervertebral disc resection due to lumbar disc herniation. All patients were diagnosed with intervertebral disc herniation by imaging. We divided patients into two categories according to the Pfirrmann scoring system. Disc degeneration was less severe in patients with Grade I and II and more severe in patients with Grade III and IV. We divided patients into two groups according to the severity of the disease. Then, we extracted the protein and RNA from the disc tissue to test the indicators. This investigation was approved by the Ethics Committee of the Yantai Shan Hospital. All patients provided written informed consent. This research was conducted in accordance with the Declaration of Helsinki.

Cells Culture and Drug Treatment

Human primary nucleus pulposus cells were purchased from the Shanghai Saibaikang Biotechnology company. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA)/F12 medium containing 10% serum and 1% streptomycin plus penicillin. The cells were cultured in an incubator at 5%CO_2 and 37° C. We stimulated the degeneration of nucleus pulposus cells with recombinant human IL-1β and conducted the next research by lentivirus.

Western Blot Analysis

Nucleus pulposus cells were subcultured in six-well plates. After we treated cells in different ways, cell proteins were extracted, and protein concentration was determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After transferring the protein to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), we sealed it with 5% skim milk. Then, the PVDF membrane was immersed in primary antibody [collagen II, Abcam (Cambridge, MA, USA); aggrecan, Abcam (Cambridge, MA, USA); RBMS3, Abcam, (Cambridge, MA, USA); caspase3, Abcam (Cambridge, MA, USA); caspase9, Abcam (Cambridge, MA, USA); β-catenin, Abcam (Cambridge, MA, USA); c-myc, Abcam (Cambridge, MA, USA); β-actin, Abcam (Cambridge, MA, USA)] and incubated overnight at 4° C. On the second day, after the PVDF membrane was washed by Phosphate-Buffered Saline and Tween (PBST), the PVDF membrane was incubated at room temperature for 2 h with secondary antibody. After images were collected by the imaging system, we analyzed the gray value of the bands. The relative expression of the target protein was shown by the ratio of the gray value of the target protein and the internal reference band.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Nucleus pulposus cells were subcultured in six-well plates. After we treated the cells in different ways, we extracted the RNA from the cells and measured the RNA concentration using a spectrophotometer. First, we reversed RNA into cDNA using PrimeScript™ RT Master Mix (Applied Biosystems; Foster City, CA, USA). Next, RT-PCR was performed to quantify RBMS3, collagen II, aggrecan, c-myc, β-catenin, and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA expression levels. GAP-

DH was used for normalization. The primers used for RT-PCR are shown in Table I. RT-PCR was conducted using SYBR Green Master Mix (Applied Biosystems; Foster City, CA, USA). The relative mRNA expression levels were calculated by the $2^{-\Delta\Delta CT}$ methods.

Immunocytofluorescence (IF) Staining

Nucleus pulposus cells are subcultured in 24-well plates. After treating the cells in different ways, we took out the 24-well plates and discarded the medium. The cells were fixed by paraformaldehyde. After washing the cells with phosphate-buffered saline (PBS), we immersed them in 1% Triton for 15 min. Then, we used 10% goat serum to seal the cells for 30 min. The cells were immersed in the primary antibody at 4° C overnight. The next day, after washing the cells with PBS, we immersed them in a fluorescently labeled secondary antibody for 2 h. After washing the cells, we stained the nuclei with DABI. Finally, the cells were visualized using a fluorescence (Carl Zeiss MicroImaging, Jena, Germany).

Cell Transfection

Before transfection, the nucleus pulposus cells at logarithmic growth stage were subcultured into six-well plates and cultured in 5% CO_2 and 37° C constant temperature incubators until the cell density was 60%-70%. NP cells were transfected by Lentivirus-NC, Lentivirus-Rab7, or Lentivirus-Rab7-siRNA (Invitrogen, Carlsbad, CA, USA). RBMS3 was downregulated *via* transfection of siRNA-RBMS3 and was upregulated *via* the transfection of Lenti-RBMS3. Transfection efficacies were measured *via* Western blot and RT-PCR. The siRNA-RBMS3 was designed as siRNA-RBMS3-sense: ACUGACGACUGACGUA and siRNA-RBMS3-antisense: UCGGUAAUGCGAUGCA.

Cell Counting Kit (CCK-8) Assay

Nucleus pulposus cells were subcultured in a 96-well plate. When the cell density was 60-70%, we treated the nucleus pulposus cells in different ways. Then, we added 10 μl CCK8 to each well. The cells were incubated in an incubator for 2 h and the absorbance of each well at 450nm was detected by a microplate reader.

Enzyme-Linked Immunosorbent Assay (ELISA)

Nucleus pulposus cells were subcultured in a 6-well plate. When the cell density was 60%-70%, we treated the nucleus pulposus cells in different ways. Then, the supernatant was taken, and the contents of IL-1β and TNF-α in the supernatant were detected by ELISA.

Statistical Analysis

SPSS 22.0 (SPSS IBM, Armonk, NY USA) statistical software is used to analyze the data. For measurement data, we use the mean ± standard deviation. Comparison between multiple groups was made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). The difference was statistically significant with $p<0.05$. All the results were repeated three times.

Table I. RT-PCR primers.

Name	Sense/anti-sense	Sequence (5′-3′)
Collagen II	Sense	GGGAATGTCCTCTGCGATGAC
	Anti-sense	GAAGGGGATCTCGGGGTTG
Aggrecan	Sense	GGTGAACCAGTTGTGTTGTC
	Anti-sense	CCGTCCTTTCCAGCAGTC
RBMS3	Sense	GGTAGCATCTCTCAAGGCAAAT
	Anti-sense	CATGTCCAAAGGGTTTCAGCA
Caspase3	Sense	CAGAATCATAAGCCCCTGGA
	Anti-sense	TCTGCGAGTCAGGCATTTG
Caspase9	Sense	TTCTTGAGCAACACCCTC
	Anti-sense	CGCATACACTGTCTACCT
β-catenin	Sense	GAGTGCTGAAGGTGCTATCTGTCTG
	Anti-sense	TTCTGAACAAGACGTTGACTTGGA
c-myc	Sense	TGCTGCCAAGAGGGTCAAGT
	Anti-sense	TCAGCCAAGGTTGTG
GAPDH	Sense	ACAACTTTGGTATCGTGGAAGG
	Anti-sense	GCCATCACGCCACAGTTTC

Results

Expression of RBMS3 in Human NP Tissues with Different Pfirrmann Grades

To determine whether the expression of RBMS3 is related to the degree of disc degeneration, we examined the expression levels of RBMS3 in different degrees of disc degeneration. Results of Western blot showed that the disc tissues with a higher degree of degeneration had lower expression of collagen II and lower expression of RBMS3 than those in the low degeneration group (Figure 1A). The results of RT-PCR were similar to those of Western blot (Figure 1B, 1C). This suggests that RBMS3 expression is negatively correlated with the degree of disc degeneration.

Expression of RBMS3 Decreased in NP Cells Stimulated by IL-1β

We stimulated the degeneration of nucleus pulposus cells with different concentrations of IL-1β (10 ng/ml, 50 ng/ml) to study its effect on RBMS3 expression. Results of Western blot showed that after the stimulation of IL-1β on nucleus pulposus cells, the expression of collagen II was significantly reduced and presented a dose-dependent pattern (Figure 2A). This suggests that IL-1β successfully stimulates degeneration of nucleus pulposus cells. The expression of RBMS3 in control group was higher than that in IL-1β group (Figure 2A). Wnt/β-catenin signaling pathway-related proteins, β-catenin, and c-myc have opposite results to RBMS3 (Figure 2A). The results of RT-PCR were similar to those of Western blot (Figure 2B-2E). Results of cell immunofluorescence showed that RBMS3 expression was significantly reduced in IL-1β group (Figure 2F).

Overexpression of RBMS3 Delayed the Degeneration of NPCs

We transfected nucleus pulposus cells with Lenti-RBMS3 to up-regulate the expression of RBMS3 in nucleus pulposus cells. Results of Western blot (Figure 3A) and RT-PCR (Figure 3B) showed that after nucleus pulposus cells were transfected with Lenti-RBMS3, the expression of RBMS3 was significantly increased. Results of Western blot showed that the expressions of collagen II and aggrecan in Lentity-RBMS3 group were higher than those in control group, while those in IL-1β+Lenti-RBMS3 group were higher than those in IL-1β group (Figure 3C). The results of RT-PCR were similar to those of Western blot (Figure 3D-3G). Cell Counting Kit-8 (CCK8)

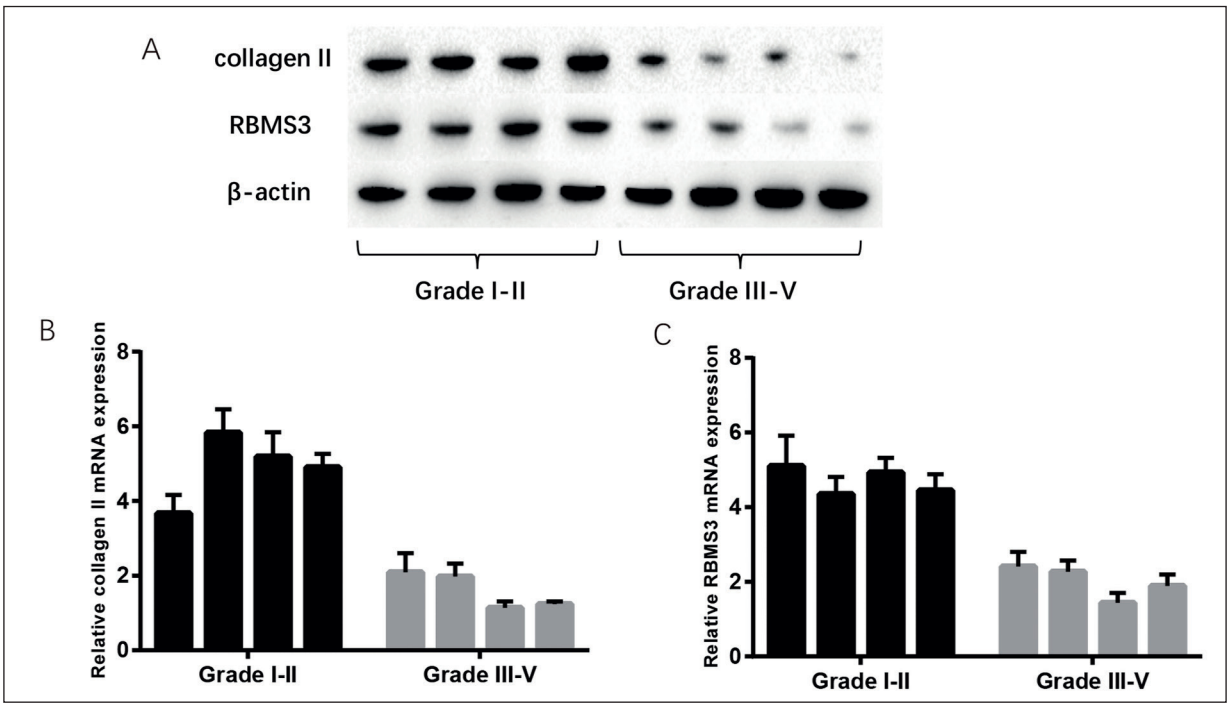

Figure 1. Expression of RBMS3 in the intervertebral disc of people with different degrees of intervertebral disc degeneration. **A,** Results of expression of collagen II and RBMS3 in two groups were determined by Western blot. **B,** Results of expression of collagen II in two groups were determined by RT-PCR. **C,** Results of expression of RBMS3 in two groups were determined by RT-PCR.

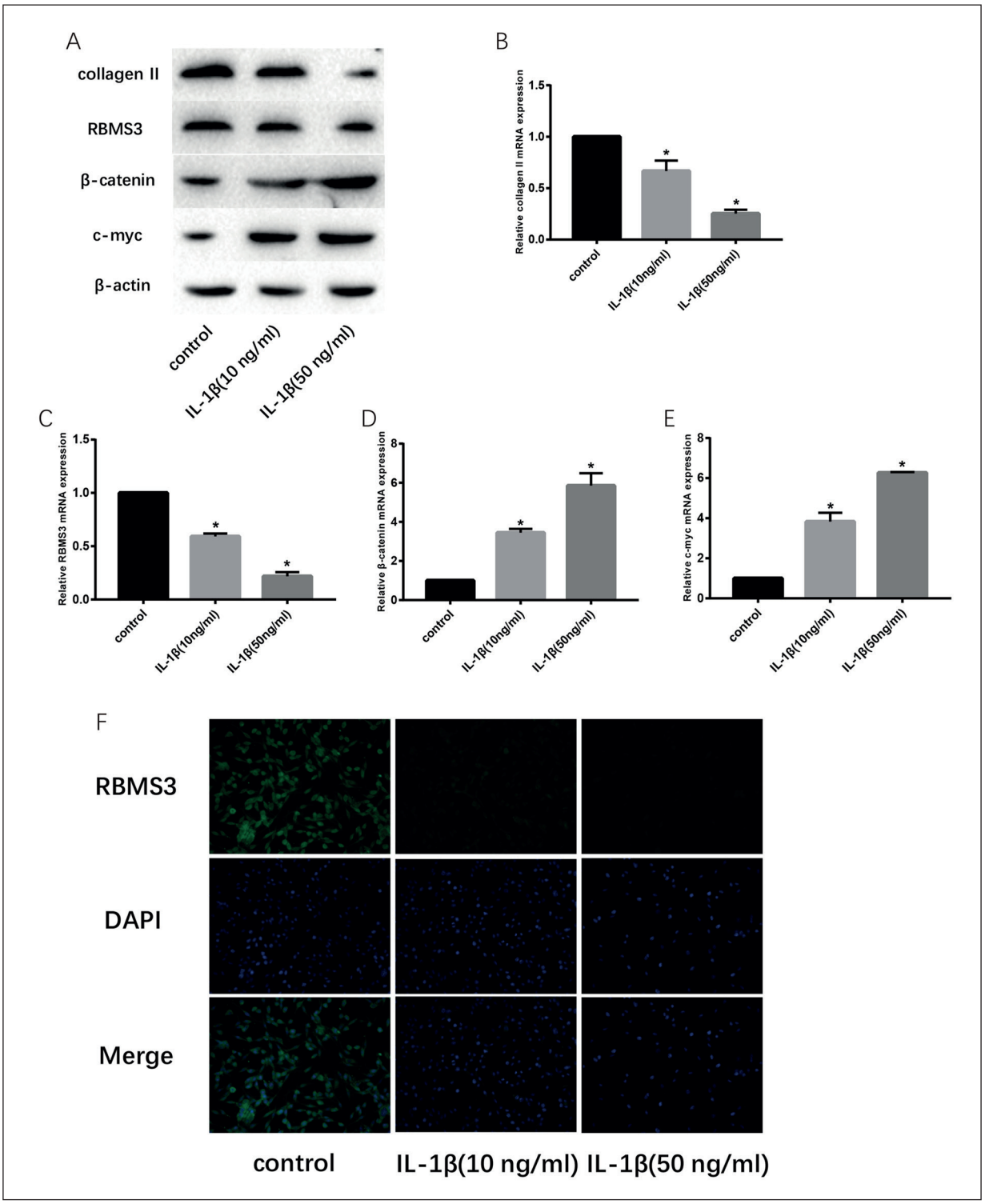

Figure 2. Expression of RBMS3 was reduced in degenerative nucleus pulposus cells. **A,** Expression of collagen II, RBMS3, β-catenin and c-myc in the intervertebral disc of mice was detected by Western blot. **B,** Expression of collagen II in the intervertebral disc of mice was detected by RT-PCR. **C,** Expression of RBMS3 in the intervertebral disc of mice was detected by RT-PCR. **D,** Expression of β-catenin in the intervertebral disc of mice was detected by RT-PCR. **E,** Expression of c-myc in the intervertebral disc of mice was detected by RT-PCR. **F,** Expression of RBMS3 was determined by immunofluorescence (magnification: 40×). ("*" means there is a statistical difference with the control group).

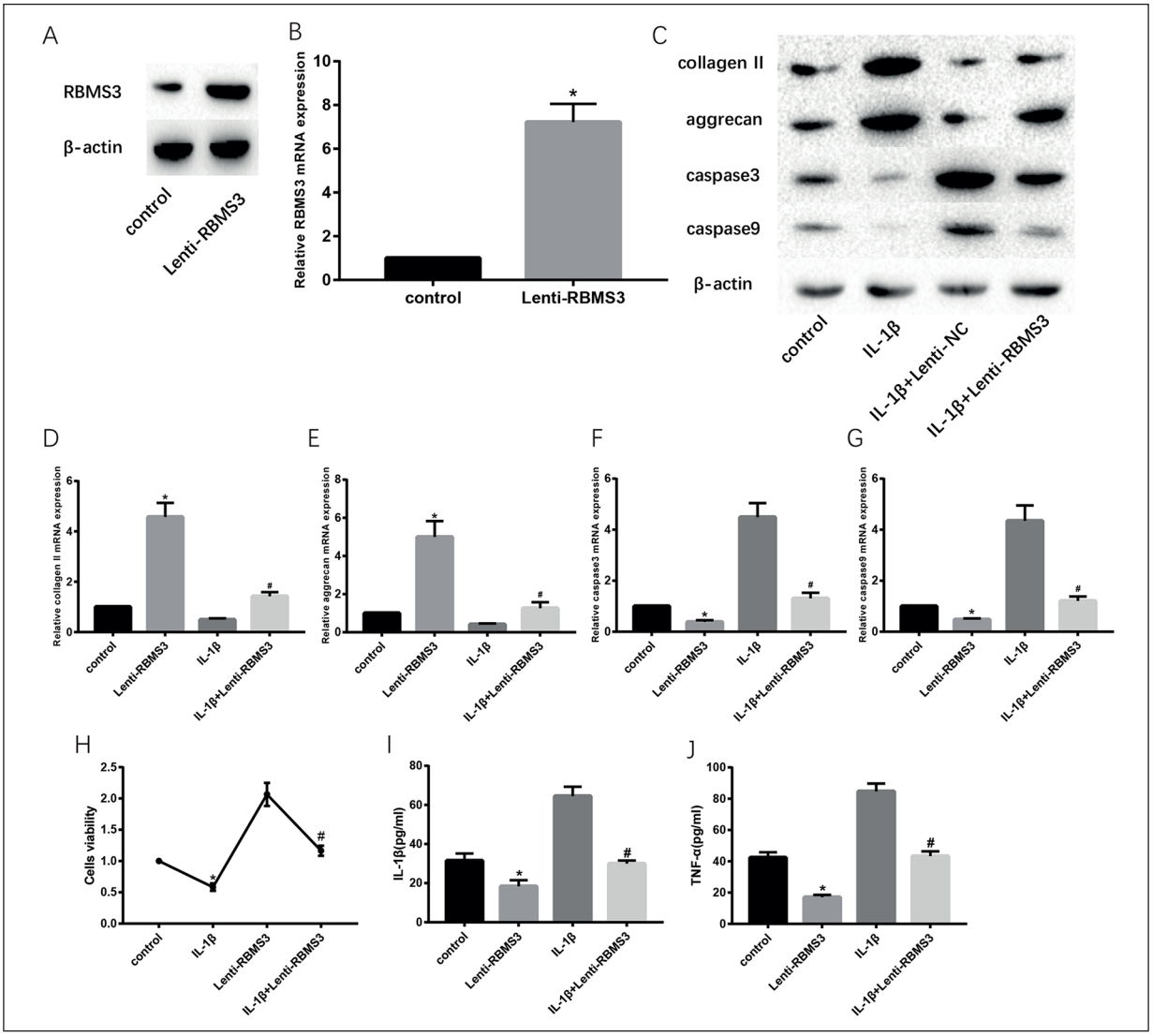

Figure 3. Overexpression of RBMS3 delayed the degeneration of nucleus pulposus cells. **A,** Protein and mRNA expression of RBMS3 in control group and Lenti-RBMS3 group was determined by Western blot. **B,** Protein and mRNA expression of RBMS3 in control group and Lenti-RBMS3 group was determined by RT-PCR. **C,** Results of protein expression of collagen II, aggrecan, caspase3 and caspase9 in four groups were determined by Western blot. **D,** Results of protein expression of collagen II in four groups were determined by RT-PCR. **E,** Results of protein expression of aggrecan in four groups were determined by RT-PCR. **F,** Results of protein expression of caspase3 in four groups were determined by RT-PCR. **G,** Results of protein expression of caspase9 in four groups were determined by RT-PCR. **H,** Proliferation levels of the four groups of cells were determined by CCK8 assay. **I,** Expression of IL-1β was determined by Elisa. **J,** Expression of TNF-α was determined by Elisa. ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β group).

results showed that, after the transfection of Lenti-RBMS3, the proliferation level of nucleus pulposus cells was significantly increased (Figure 3H). Besides, the expression of caspase3 and caspase9 in Lenti-RBMS3 group was lower than those in control group (Figure 3C). ELISA results showed that the overexpression of RBMS3 significantly reduced the expression of IL-1β (Figure 3I) and TNF-α (Figure 3J) in nucleus pulposus cells. These data suggest that the overexpression of RBMS3 significantly improves the degeneration of nucleus pulposus cells.

RBMS3 Delayed Intervertebral Disc Degeneration by Inhibiting Wnt/β-Catenin Signaling Pathway

To demonstrate that RBMS3 inhibits the Wnt/β-catenin signaling pathway, we used the

Wnt/β-catenin signaling inhibitor XAV-939 and used siRNA-RBMS3 to interfere with the expression of RBMS3 in nucleus pulposus cells. Results of Western blot and RT-PCR showed that Lenti-RBMS3 and siRNA-RBMS3 could effectively increase or decrease the expression of RBMS3 in nucleus pulposus cells (Figure 4A, 4B). XAV-939 effectively suppressed the activity of the Wnt/β-catenin signaling pathway (Figure 4C, 4D). Results of Western blot showed that the expressions of collagen II and aggrecan in siRNA-RBMS3 group were significantly reduced, while the expressions of collagen II and aggrecan were increased after the addition of Wnt/β-catenin signaling pathway inhibitor XAV-939. In addition, the expression of β-catenin and c-myc

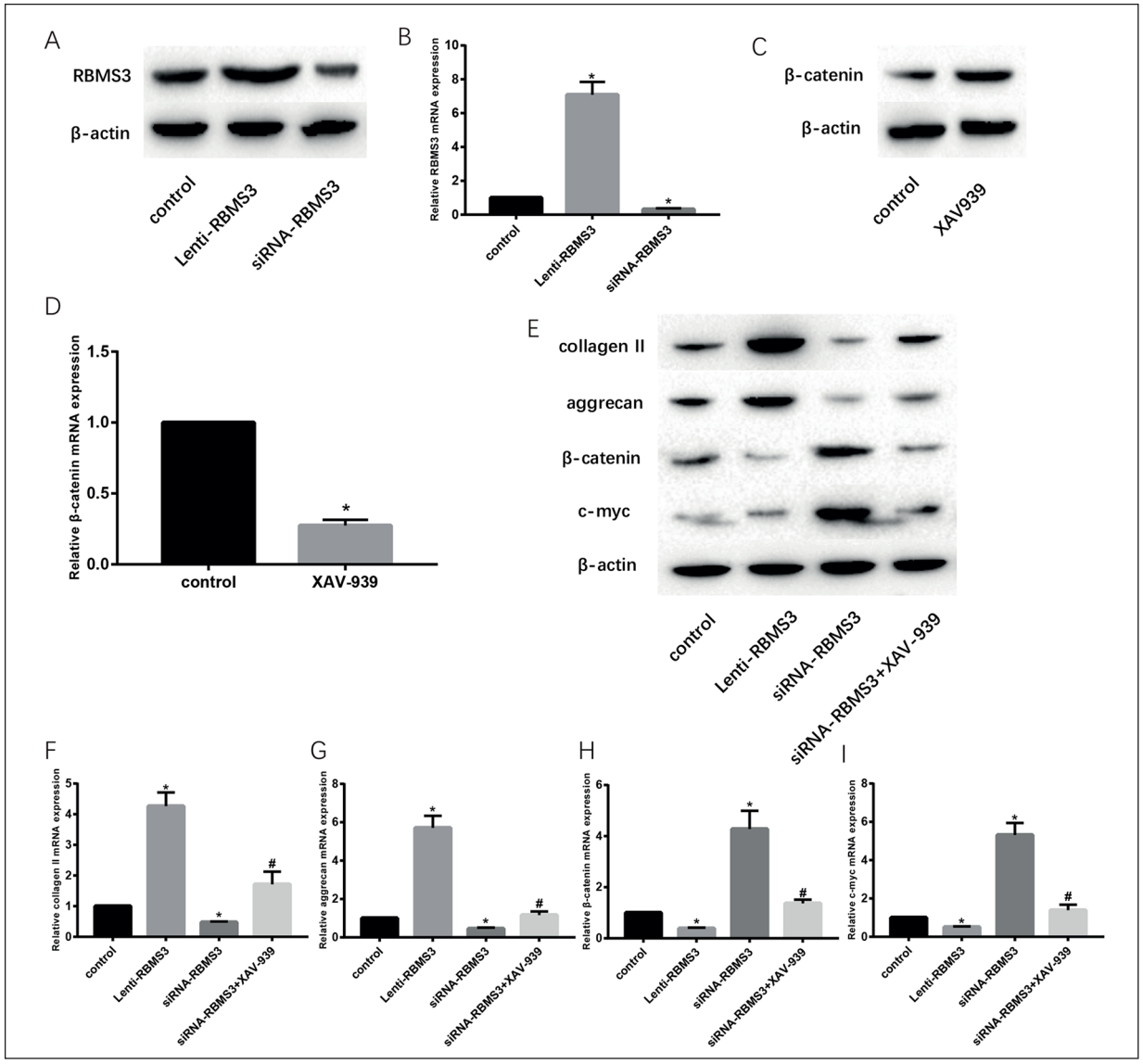

Figure 4. RBMS3 delayed the degeneration of nucleus pulposus cells by inhibiting Wnt/β-catenin signaling pathway. **A,** Protein and mRNA expression of RBMS3 in control group, Lenti-RBMS3 group and siRNA-RBMS3 group was determined by Western blot. **B,** Protein and mRNA expression of RBMS3 in control group, Lenti-RBMS3 group and siRNA-RBMS3 group was determined by RT-PCR. **C,** Protein and mRNA expression of p38 in control group and XAV-939 group was determined by Western blotting. **D,** Protein and mRNA expression of p38 in control group and XAV-939 group was determined by qRT-PCR. **E,** Results of protein expression of collagen II, aggrecan, β-catenin and c-myc in four groups were determined by Western blot. **F,** Results of protein expression of collagen II in four groups were determined by RT-PCR. **G,** Results of protein expression of aggrecan in four groups were determined by RT-PCR. **H,** Results of protein expression of β-catenin in four groups were determined by RT-PCR. **I,** Results of protein expression of c-myc in four groups were determined by RT-PCR. ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the siRNA-RBMS3 group).

decreased or increased after RBMS3 was up-regulated or down-regulated (Figure 4E). The results of RT-PCR were similar to those of Western blot (Figure 4F-4I). These findings suggest that RBMS3 may delay disc degeneration by inhibiting the Wnt/β-catenin signaling pathway.

Discussion

Disc degeneration is the result of many factors. Studies[10] have shown that apoptosis and senescence of nucleus pulposus cells were found in abundance in the process of disc degeneration. In addition, disc degeneration is accompanied by a large amount of inflammatory reaction. The proliferation ability of nucleus pulposus also decreased with the increase of degeneration[11]. In our work, we found that the extracellular matrix of nucleus pulposus cells was degraded during the degeneration of nucleus pulposus cells, while the level of apoptosis was increased. CCK-8 assay showed that the proliferation level of nucleus pulposus cells decreased with degeneration. However, overexpression of RBMS3 reversed these effects. These data suggested that RBMS3 may be a protective factor for disc degeneration.

Wnt/β-catenin signaling pathway is activated during disc degeneration and plays an important regulatory role. Compared with patients with lumbar disc herniation, the mRNA and protein expression of β-catenin in the nucleus pulposus of the degenerative disc tissues were increased in patients with lumbar disc trauma at the same time period[12]. Compared with the normal nucleus pulposus cells, the mRNA expressions of β-catenin and matrix metalloproteinase-13 (MMP-13) in rabbit intervertebral disc nucleus pulposus cell degeneration model stimulated by exogenous TNF-α were significantly increased, while the mRNA and protein expression of collagen II was significantly reduced[13]. After the application of the Wnt/β-catenin signaling pathway inhibitor DKK1, the above effect was significantly weakened. Hiyama et al[14] cultured intervertebral discs of beagles with different MRI signals. They found that MRI signal intensity in nucleus pulposus tissue was correlated with mRNA expression of β-catenin and Runx2, indicating that the activation of Wnt/β-catenin signaling pathway in degenerative intervertebral disc tissue could increase the expression of Runx2, thus leading to lumbar disc calcification. The activation of Wnt/ β-catenin signaling pathway can increase the expression of downstream target genes, thus accelerating the degeneration of intervertebral discs[15]. In our research, we found that the expression of Wnt signaling pathway-related molecules increased after IL-1β stimulated the degeneration of nucleus medullary cells, and decreased after the over-expression of RBMS3. This suggested that RBMS3 inhibits the Wnt/β-catenin signaling pathway and thereby delays disc degeneration.

Therefore, RBMS3, as a protective factor in the process of disc degeneration, has a very good clinical application prospect. Therefore, we can take RBMS3 as a drug target to provide a new direction for the clinical treatment of disc degeneration.

Conclusions

The above results showed that RBMS3 reduced the apoptosis and inflammation level of nucleus pulposus cells, promoted the proliferation of nucleus pulposus cells, inhibited the degradation of the extracellular matrix, and delayed the degeneration of the intervertebral disc by inhibiting the Wnt/β-catenin signal pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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