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Apelin-13/APJ system delays intervertebral disc degeneration by activating the PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: To study the effect of Apelin-13/APJ system on intervertebral disc degeneration and its mechanism.

PATIENTS AND METHODS: This study detected the expression of APJ in human intervertebral disc tissue with varying degrees of degeneration. IL-1β is used to stimulate the degeneration of nucleus pulposus cells. We used recombinant human Apelin-13 and Ala13 to activate and inhibit the APJ receptor, respectively. The inhibitor LY294002 was used to inhibit the PI3K/AKT signaling pathway. We studied the effects of Apelin-13/APJ system on nucleus pulposus cells and its mechanism by Western blot, RT-PCR, and so on.

RESULTS: APJ is lowly expressed in the nucleus pulposus of patients with a high degree of degeneration. IL-1β stimulates the nucleus pulposus cells and reduces the expression of APJ in nucleus pulposus cells. Recombinant human Apelin-13 reduces the degradation of nucleus pulposus extracellular matrix, promotes proliferation, and reduces the levels of apoptosis and inflammation. In addition, the Apelin-13/APJ system increases the expression of PI3K and AKT and activates the PI3K/AKT signaling pathway.

CONCLUSIONS: Apelin-13/APJ system activates PI3K/AKT signaling pathway activity, reduces the degradation of nucleus pulposus extracellular matrix, promotes proliferation, and reduces the level of apoptosis and inflammation, thus delaying the degeneration of the intervertebral disc.

Key Words:

Apelin-13/APJ, Intervertebral disc degeneration, PI3K/AKT signaling pathway, Proliferation, Apoptosis, Inflammation.

Introduction

Intervertebral disc degeneration is the most common cause of low back pain, which brings great economic burden to patients and society[1]. The US National Health Data shows that about 5 million people are diagnosed with low back pain every year in the outpatient department, and nearly 40% of patients with low back pain are caused by lumbar disc herniation[2]. Intervertebral disc degeneration is the pathological basis of lumbar disc herniation[3]. The main features of the intervertebral disc degeneration include a decrease in the number of nucleus pulposus cells, a decrease in extracellular matrices such as glycoproteins and proteoglycans, and an increase in the expression of various inflammatory factors in the intervertebral disc[4]. In the early stage of intervertebral disc degeneration, the proliferation of nucleus pulposus cells in the intervertebral disc tissue can be increased[5]. As the local microenvironment of the intervertebral disc changes, the apoptosis of nucleus pulposus cells increases, and local inflammatory factors accumulate. As the degree of intervertebral disc degeneration increased, the number of nucleus pulposus cells in the intervertebral disc decreased, cartilage-like cells formed, and the extracellular matrix of the intervertebral disc began to degrade. The content of collagen II in the nucleus pulposus decreased, and the proportion of collagen I began to increase[6]. The content of various matrix-degrading enzymes in the intervertebral disc microenvironment increases, such as matrix metalloproteinases[6].

PI3K/AKT signaling pathway plays an important role in the process of intervertebral disc degeneration[7]. Phosphatidylinositol 3-kinase (PI3K) is a major downstream effector of receptor tyrosine kinases and G-protein coupled receptors. The key proximal node downstream of the receptor tyrosine kinase and PI3K complex is serine-threonine protein kinase (Akt). The PI3K/Akt pathway plays an important regulatory role in cell proliferation, anti-apoptosis, glucose metabolism, and protein synthesis[8]. Ren

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et al[9] introduced the exogenous SOX9 gene into bovine intervertebral disc cells cultured *in vitro*, and found that its annulus fibrosus and nucleus pulposus cells can express the morphological structure of chondrocyte-like cells in a short time, suggesting that SOX9 gene can effectively delay the intervertebral disc tissue degeneration. The activation of PI3K/Akt signaling pathway in nucleus pulposus cells can effectively regulate the expression of SOX9 factor[10]. When the inhibitor of the PI3K/Akt signaling pathway is added, the expression and activity of SOX9 factor are also reduced. It is suggested that the expression of SOX9 factor in intervertebral disc cells mainly depends on the regulation of the PI3K/Akt pathway.

Apelin and its receptor APJ are widely distributed in the body. Apelin has a C-terminal which is rich in arginine and lysine residues, which are the cleavage sites of the proteolytic enzymes during reverse transcription and can be decomposed into Apelin-36, Apelin-13, and Apelin-12[11]. Apelin fragments of different lengths have the different binding ability with APJ and the physiological effects played *in vitro* and *in vivo* are also different[12]. Apelin-13 can affect cell proliferation and apoptosis by activating the PI3/AKT signaling pathway[13]. However, the role of Apelin-13 in intervertebral disc tissue is unclear.

Therefore, this study studied the effect of apelin-13 on nucleus pulposus cells and its molecular mechanism using recombinant human Apelin-13 and its inhibitors and PI3/AKT inhibitors by Western blot and other methods.

Patients and Methods

Patient Tissue Samples

All human intervertebral disc nucleus pulposus tissues were obtained from patients with microendoscopic discectomy due to lumbar disc herniation. After the patient's intervertebral disc nucleus tissue was removed during surgery, it was immediately placed in liquid nitrogen for cryopreservation. All patients were diagnosed with lumbar disc herniation by MIRI. We used the Pfirrmann scoring system to grade patients with lumbar disc herniation. The patient's disc degeneration is divided into five grades. The degree of disc degeneration in grade I to V gradually worsens. We divided the intervertebral discs of grade I to II into low degeneration groups, and the intervertebral discs of grade III to V were divided into high degeneration groups. This study was approved by the Ethics Committee of The First Hospital of Jilin University. All patients provided written informed consent. This study was conducted in accordance with the Declaration of Helsinki.

Cells Culture and Drug Treatment

Human primary nucleus pulposus cells were purchased from Shanghai Saibaikang Biotechnology Company. The nucleus pulposus cells were cultured in DMEM/F12 medium containing 10% fetal bovine serum. The culture conditions were 37°C and 5% CO_2. LY294002 (Selleck Chemicals, Houston, TX, USA) is a PI3K inhibitor that is used to inhibit the phosphorylation of AKT. Ala13 is an analog of Apelin-13 and has a structure similar to Apelin-13. It can bind to receptor APJ, but has no responsive biological function, and is therefore used to inhibit the action of Apelin-13.

Western Blot Analysis

After culturing human nucleus pulposus cells for drug stimulation, we used radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) lysate to lyse nucleus pulposus cells. The cell-containing lysate was shaken on ice for 20 minutes. After centrifugation, we extracted the supernatant and determined the protein concentration using a bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). THE Proteins were electrophoresed on a 10% SDS-PAGE gel and then transferred to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking the PVDF membrane with 5% skim milk powder, we added the primary antibody (Collagen II, Abcam, Cambridge, MA, USA, Rabbit, 1:5000; Aggrecan, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; MMP-3, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; MMP-13, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; SOX9, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; PI3K, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; AKT, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; p-AKT, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Proteintech, Chicago, IL, USA, 1:10000) at 4°C overnight. The next day, HRP-conjugated secondary antibody was added and incubated for 2 h at room temperature. Enhanced chemiluminescence (ECL) was used to display the target protein on the exposure machine.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

We cultured the nucleus pulposus cells in six-well plates and treated them with different drugs. Then, we used the TRIzol method to extract the total RNA from nucleus pulposus cells. First, we reversed the mRNA to cDNA using reverse transcriptase. Then, the SYBR Green qPCR kit (Applied Biosystems, Foster City, CA, USA) was used for PCR amplification. The amplification system was 7.0 µl of RNAse Free ddH_2O, 10 µl of 2×SYBR Green solution, 0.5 µl of upstream and downstream primers, and 2 µl of reverse transcription product. The primer sequences of RT-PCR are shown in Table I. Relative mRNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ methods.

Immunocytofluorescence (IF) Staining

We cultured nucleus pulposus cells in 96-well plates and added 5000 nucleus pulposus cells per well. After the nucleus pulposus cells were treated with different drugs, 96-well plates were taken for immunofluorescence staining. After discarding the medium, the cells were washed with Phosphate-Buffered Saline (PBS). The nucleus pulposus cells were then fixed with 4% paraformaldehyde for 30 minutes. After washing the cells, the cells were soaked in 0.1% Triton for 10 minutes. After blocking the cells for 1 hour at room temperature using goat serum, we added the primary antibody dilution at 4°C overnight. The next day, after washing the cells with PBS, we added secondary antibody dilution to each well and incubated for 1 hour in the dark. After washing the cells with PBS, 4',6-diamidino-2-phenylindole (DAPI) was added to each well for 5 minutes in the dark to stain the cell nucleus. After washing away the excess DAPI, we used a laser confocal microscope to observe and record the image.

Cell Counting Kit (CCK-8) Assay

We cultured nucleus pulposus cells in 96-well plates and added 5000 nucleus pulposus cells per well. After the cells grew to a density of 60% to 70%, the nucleus pulposus cells were treated with different drugs. After 24 hours, 10 µl of CCK8 reagent was added to each well. The 96-well plate was then placed in an incubator at 37°C for 2 hours. Finally, we measured the absorbance of each well using a microplate reader and calculated the cell viability.

Flow Cytometry

We cultured nucleus pulposus cells in six-well plates. After the cells grew to a density of 60%-70%, the nucleus pulposus cells were treated with different drugs. The nucleus pulposus cells in the six-well plates were collected and washed twice with PBS. After centrifugation of the nucleus pulposus cells (1000 rpm, 5 min), the cells were suspended using 500 µl of Binding Buffer. 5 µl of Annexin fluorescein isothiocyanate (V-FITC) and 5 µl of Propidium Iodide (PI) were sequentially added to the centrifuge tube and mixed. Af-

Table I. Primer sequences of RT-PCR.

Name	Sense/anti-sense	Sequence (5′-3′)
Collagen II	Sense	GGGAATGTCCTCTGCGATGAC
	Anti-sense	GAAGGGGATCTCGGGGTTG
Aggrecan	Sense	GGTGAACCAGTTGTGTTGTC
	Anti-sense	CCGTCCTTTCCAGCAGTC
APJ	Sense	CACCTGGTGAAGACGCTGTA
	Anti-sense	TAGGGGATGGATCTCGTG
SOX9	Sense	GTGGGAGCGACAACTTTACC
	Anti-sense	GCGAGCACTTAGCAGAGGC
MMP-3	Sense	ACATGGAGACTTTGTCCCTTTTG
	Anti-sense	TTGGCTGAGTGGTAGAGTCCC
MMP-13	Sense	TACGAGCATCCATCCCGAGACC
	Anti-sense	TACGAGCATCCATCCCGAGACC
PI3K	Sense	GGTGACTGTGTGGGACTTATTGA
	Anti-sense	CTGATGTAGTGTGTGGCTGTTGA
AKT	Sense	CAGGTTCACCCAGTGACAACTCA
	Anti-sense	CACGAGACAGGTGGAAGAAGAGC
GAPDH	Sense	ACAACTTTGGTATCGTGGAAGG
	Anti-sense	GCCATCACGCCACAGTTTC

ter 15 min of reaction at room temperature in the dark, we used flow cytometry to detect apoptosis. The proportion of cell apoptosis is represented by a bar graph.

Enzyme-Immunosorbent Assay (ELISA)

We cultured human nucleus pulposus cells in a six-well plate and treated the cells with different drugs. After the treatment was completed, the cell culture supernatant was aspirated into a centrifuge tube, centrifuged at 1000 × g for 20 minutes to remove cell debris, and collect the supernatant. The concentration of IL-6 and TNF-α in the supernatant was then determined using an ELISA method.

Statistical Analysis

The Statistical Product and Service Solution SPSS 21.0 (IBM Corp., Armonk, NY, USA) was used to analyze the data. For the data of the normal distribution of the measurement data, we used the mean ± standard deviation for analysis. The comparison between multiple groups was done using One-way ANOVA test followed by the post-hoc test (Least Significant Difference). $p < 0.05$ indicates that the difference was statistically significant.

Results

Intervertebral Disc with More Advanced Degeneration Expressed Lower APJ

In order to determine whether the expression of APJ in human intervertebral disc tissue is affected by the degree of degeneration, we selected human nucleus pulposus tissue with different degree of degeneration and detected the expression of APJ. Western blot results showed that the expression levels of collagen II and APJ in the low degeneration group were higher than those in the high degeneration group (Figure 1A). The results of RT-PCR were similar to those of Western blot (Figures 1B and 1C). This indicates that the expression of APJ is gradually reduced as the degree of the degeneration of the disc is aggravated.

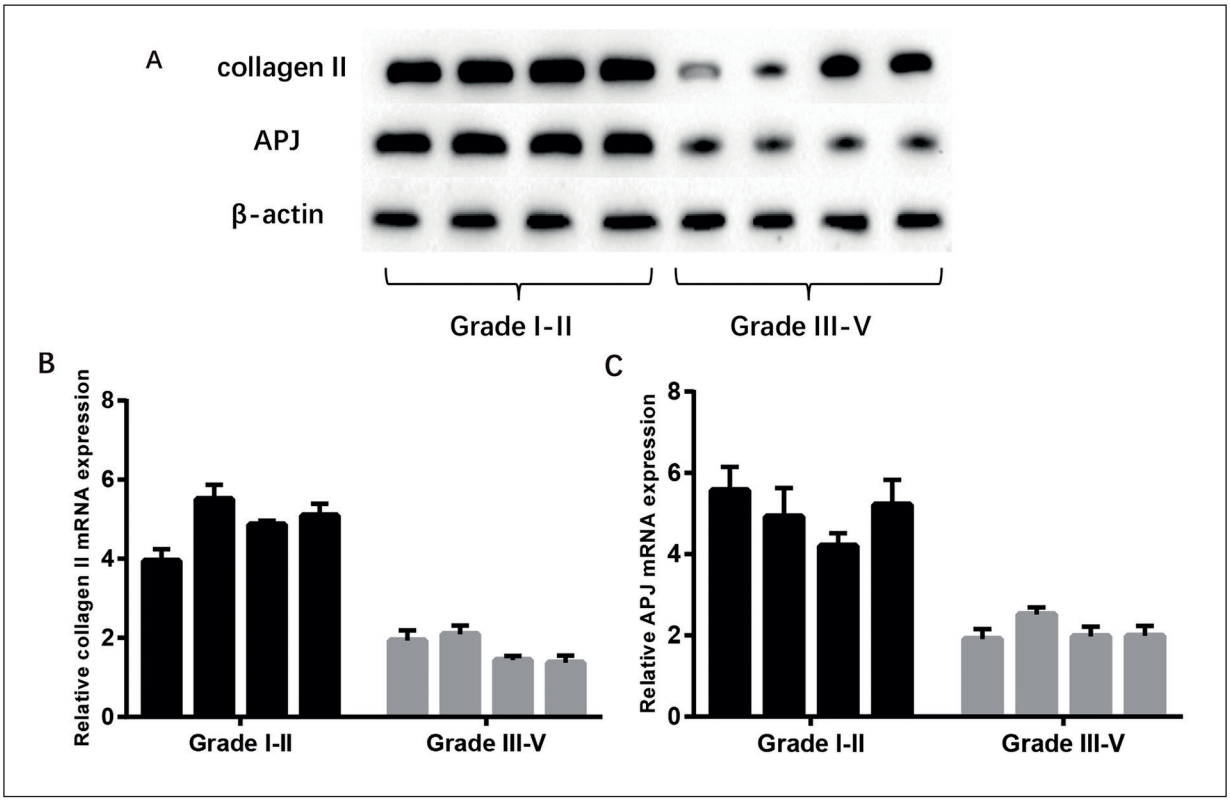

Figure 1. Intervertebral disc with more advanced degeneration expressed lower APJ. **A,** The results of the expression of collagen II and APJ in two groups were determined by Western blot. **B-C,** The results of the expression of collagen II and APJ in two groups were determined by RT-PCR.

The Expression of APJ in IL-1β-Stimulated Nucleus Pulposus Cells was Decreased and the Activity of PI2K/AKT Signaling Pathway was Decreased

We cultured human primary nucleus pulposus cells and stimulated the nucleus pulposus cell degeneration using IL-1β (10 ng/ml, 50 ng/ml). Western blot results showed that the expression of collagen II, APJ, PI3K, and AKT gradually decreased with the increase of IL-1β concentration (Figure 2A). The results of cell immunofluorescence showed that the expression of APJ was decreased after IL-1β stimulated the degeneration of nucleus pulposus cells (Figure 2B). The results of RT-PCR were similar to those of the Western blot (Figures 2C-2F). This indicates that after IL-1β stimulates the degeneration of nucleus cells, the expression of APJ is decreased and the activity of PI3K/AKT signaling pathway is decreased.

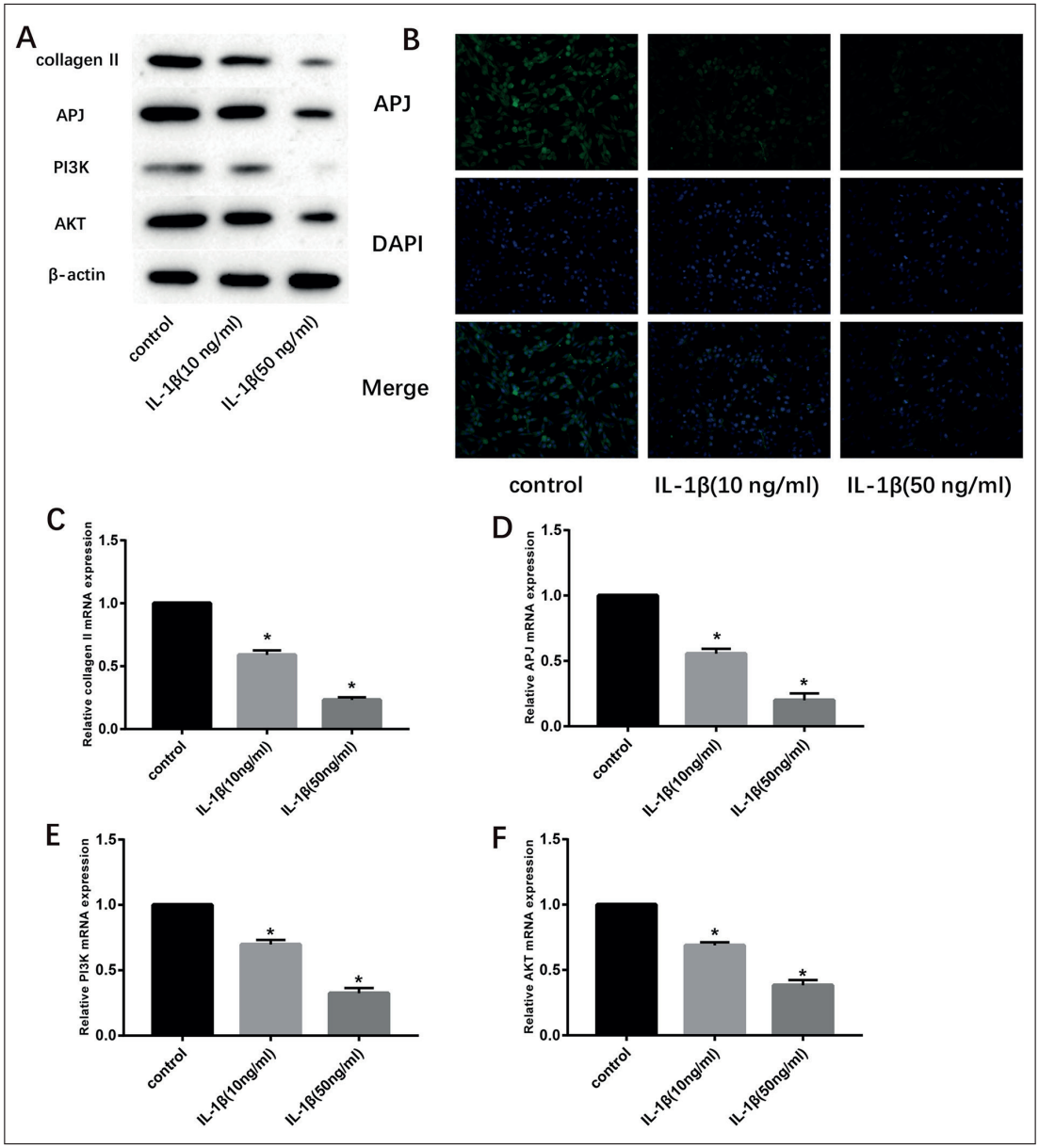

Figure 2. The expression of APJ in IL-1β-stimulated nucleus pulposus cells was decreased and the activity of PI2K/AKT signaling pathway was decreased. **A,** The expression of collagen II, APJ, PI3K, and AKT in the intervertebral disc of mice was detected by Western blot. **B,** The expression of APJ was determined by immunofluorescence (100×). **C-F,** The expression of collagen II, APJ, PI3K, and AKT in the intervertebral disc of mice was detected by RT-PCR. ("*" means that there is a statistical difference with the control group).

Apelin-13 Delays Nucleus Pulposus Cells Degeneration

Recombinant human Apelin-13 (4 μmol/l) was used to activate the APJ receptor of the nucleus pulposus cells. Western blot (Figure 3A) and RT-PCR (Figure 3B-3F) results showed that Apelin-13 increased the expression of collagen II, aggrecan, SOX9, MMP-3, and MMP-13, both at the basal level and at the level of IL-1β stimulation. CCK8 results showed that Apelin-13 increased the proliferation of nucleus pulposus cells (Figure 3G). ELISA results showed that Apelin-13 can effectively reduce the expression of inflammatory factors IL-6 and TNF-α in nucleus pulposus cells (Figures 3H-3I). Flow cytometry results showed that Apelin-13 reduced the level of apoptosis in nucleus pulposus cells (Figure 3J).

Apelin-13/APJ System Delays Intervertebral Disc Degeneration by Activating the PI3K/AKT Signaling Pathway

Ala13 (100 μmol/l) and LY294002 (20 μmol/l) were used to inhibit APJ receptor and PI3K/AKT signaling pathway activities, respectively. Western blot results showed that LY294002 effectively reduced the expression of p-AKT (Figure 4A). Western blot (Figure 4B) and RT-PCR (Figure 4C-4F) results showed that Apelin-13 and Ala13 increase and decrease the expression of collagen II, aggrecan, PI3K, and AKT, respectively. In addition, LY294002 can attenuate the effect of Apelin-13 on nucleus pulposus cells. This suggests that Apelin-13 may attenuate the degeneration of nucleus pulposus cells by activating the PI3K/AKT signaling pathway.

Discussion

Currently, the conservative treatment of degenerative disc disease is mainly medical treatment, such as opioids and some non-steroidal anti-inflammatory drugs, corticosteroid injection, and some physical therapy[14]. However, these measures do not address the underlying causes of degeneration and cannot delay the natural progression of the disease. As the disease progresses, the effects of the conservative treatment gradually deteriorate, and eventually surgery can only be selected[15]. There are many surgical methods. The traditional methods include intervertebral disc fusion and intervertebral bone graft fusion, or the spine endoscopy technique that has gradually emerged in recent years[3]. Surgical treatment can lead to a number of potential complications, including re-protrusion, pseudo-articular formation and adjacent segmental lesions[16]. Therefore, it is particularly important to find new ways to treat degenerative disc disease.

Apelin/APJ is a ligand and receptor widely present in animals. The Apelin/APJ system participates in many basic activities of multiple cells with autocrine or paracrine[17]. In the cardiovascular, Apelin/APJ activates eNOs to dilate blood vessels, repairs the wound vessels through the PI3K/AKT pathway, and promotes neovascularization[18]. In the brain, Apelin/APJ can participate in information transmission as a signaling molecule and regulate the amount of thalamic hormone[19]. At the cellular level, Apelin promotes cell proliferation, maturation, and induces mitochondrial autophagy[20]. This work found that recombinant human Apelin-13 can promote the expression of the extracellular matrix of nucleus pulposus cells, which can effectively reduce the degeneration of intervertebral disc nucleus cells. In addition, the expression of APJ receptors was also significantly reduced in human intervertebral disc tissues with a high degree of degeneration.

The PI3K/AKT signaling pathway is a signaling pathway that plays a key role in the intervertebral disc degeneration[21]. The role of PI3K/AKT signaling pathway in intervertebral disc degeneration is mainly reflected in three aspects[22,23]. First, the PI3K/AKT signaling pathway can regulate the expression of SOX9, promote the synthesis of collagen II, and reduce the degeneration of intervertebral disc. In addition, the PI3K/AKT signaling pathway has an anti-apoptotic effect under hypoxic conditions. Finally, under hypertonic conditions, the PI3K/AKT signaling pathway is activated and reduce the osmotic pressure of the intervertebral disc to form a relatively hypotonic environment, thereby promoting the proliferation of nucleus pulposus cells and inhibiting their apoptosis. This study found that Apelin-13 activates the PI3K/AKT signaling pathway, which effectively increases the proliferation of nucleus pulposus cells and reduces their apoptosis. The level of inflammation of the nucleus pulposus cells is also effectively reduced.

Intervertebral disc degeneration is the result of multiple factors. The intervertebral disc degeneration is accompanied by an increase in apoptosis, cell senescence, oxidative stress and inflammation levels, and a decrease in the proliferative

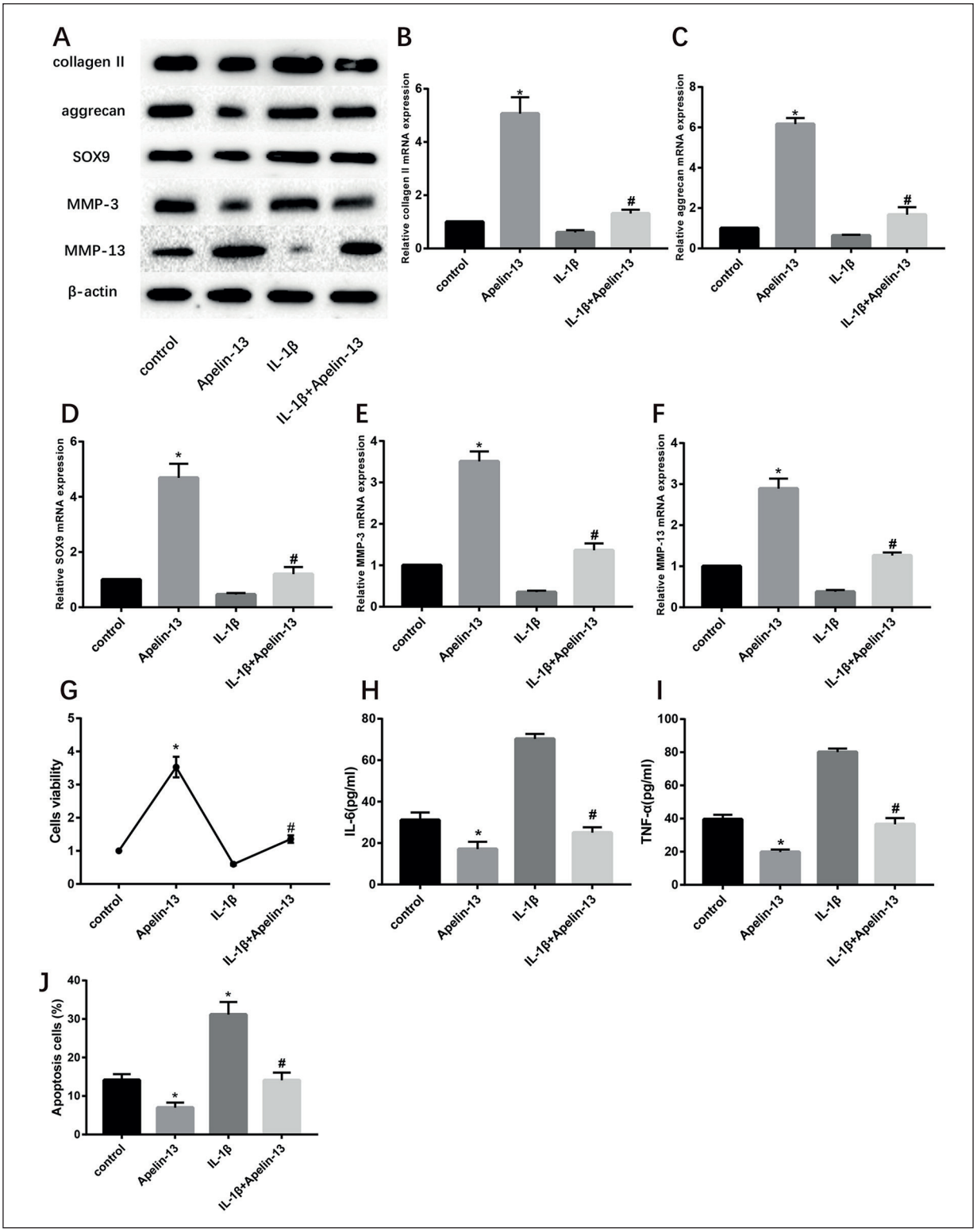

Figure 3. Apelin-13 delays nucleus pulposus cells degeneration. **A,** Results of protein expression of collagen II, aggrecan, SOX9, MMP-3, and MMP-13 in four groups were determined by Western blot. **B-F,** Results of protein expression of collagen II, aggrecan, SOX9, MMP-3, and MMP-13 in four groups were determined by RT-PCR. **G,** The proliferation levels of the four groups of cells were determined by CCK8 assay. **H,** The expression of IL-6 was determined by ELISA. **I,** The expression of TNF-α was determined by ELISA. **J,** The level of apoptosis was detected by flow cytometry. ("*" means that there is a statistical difference with the control group, and "#" means that there is a statistical difference with the IL-1β group).

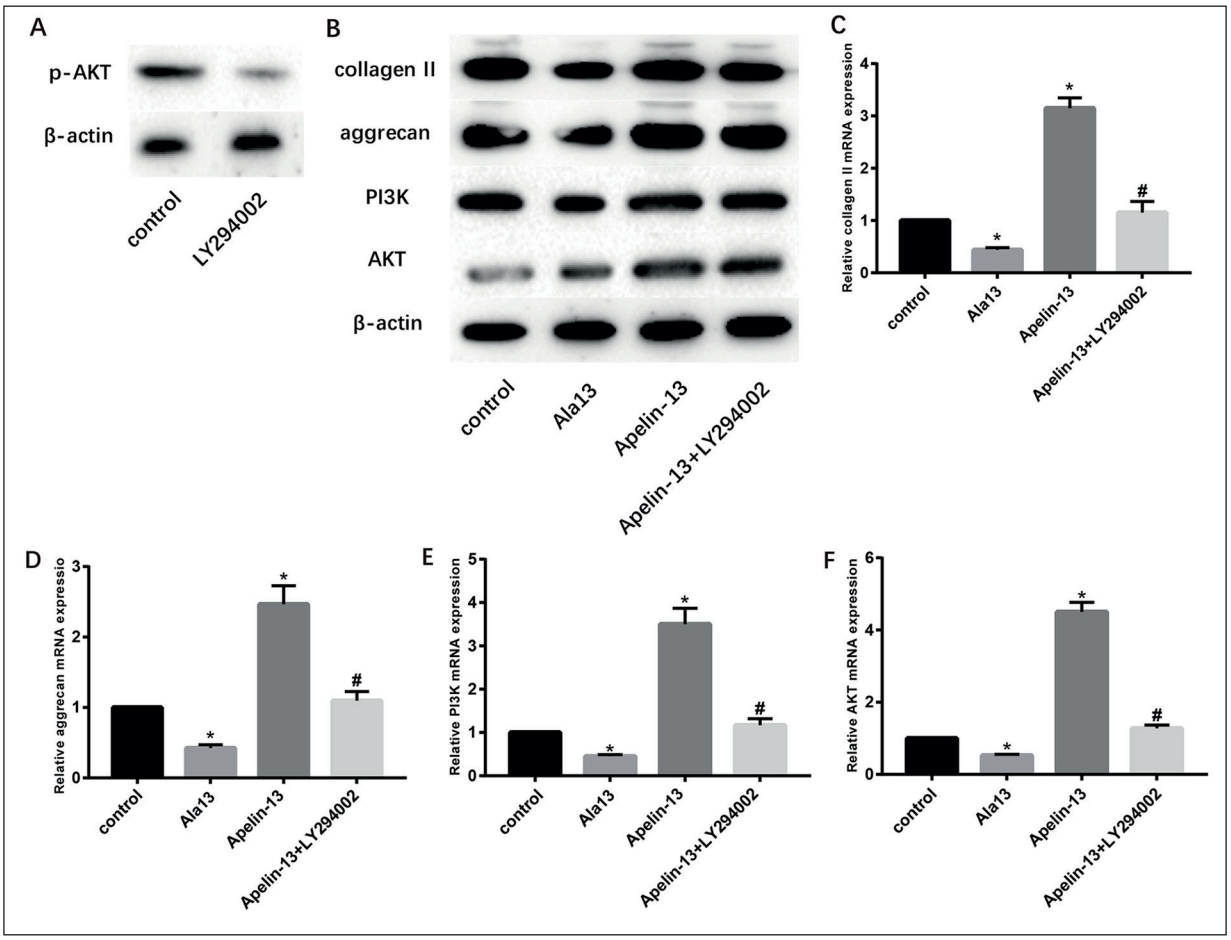

Figure 4. Apelin-13/APJ system delays intervertebral disc degeneration by activating the PI3K/AKT signaling pathway. **A,** The expression of p-AKT in the control group and LY294002 group was determined by Western blot. **B,** The results of expression of collagen II, aggrecan, PI3K, and AKT in four groups were determined by Western blot. **C-F,** The results of expression of collagen II, aggrecan, PI3K, and AKT in four groups were determined by RT-PCR. ("*" means that there is a statistical difference with the control group, and "#" means that there is a statistical difference with the Apelin-13 group).

capacity[24]. Apelin-13 delays the intervertebral disc degeneration by reducing the degradation of extramedullary matrix of nucleus pulposus, promoting the proliferation of nucleus pulposus cells, reducing the level of apoptosis and inflammation. This may provide a new direction for the clinical treatment of the degenerative disc disease.

Conclusions

We demonstrated that apelin-13/APJ system activates PI3K/AKT signaling pathway activity, reduces the degradation of nucleus pulposus extracellular matrix, promotes proliferation, and reduces the level of apoptosis and inflammation, thus delaying degeneration of intervertebral disc.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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