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HBP1 deficiency protects against stress-induced premature senescence of nucleus pulposus

W. LIU[1], W.-C. LI[2], X.-F. PAN[3], H. SHA[1], W.-Q. GONG[1], M. YAN[1]

[1]Department of Spine Surgery, The First Hospital of Jilin University, Changchun, China
[2]Department of Neurotrauma, The First Hospital of Jilin University, Changchun, China
[3]Department of Obstetrics, The First Hospital of Jilin University, Changchun, China

Abstract. – OBJECTIVE: Senescence of nucleus pulposus (NP) cells is involved in the pathological process of intervertebral disc degeneration (IVDD). HMG-box transcription factor 1 (HBP1) is a transcriptional inhibitor that prevents proliferation and regulates premature senescence of cells. The aim of this study was to confirm whether HBP1 deficiency could protect stress-induced NP cells premature senescence.

PATIENTS AND METHODS: Firstly, HBP1 protein level in human degenerated intervertebral disc tissues was detected. Then, NP cells were isolated from disc samples and transfected with plasmid to upregulate HBP1expression. H2O2 and interleukin-1β (IL-1β) were used to induce NP cells premature senescence in a different manner. Thereafter, cell viability, proliferation, and apoptosis were measured, and the protein expressions of collagen II, HBP1, and p16, were determined by Western blot or immunofluorescence. Finally, the mRNA levels of aggrecan, collagen I, IL-6, Transforming Growth Factor-α (TNF-α), and matrix metalloproteinase-3 (MMP-3) were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RESULTS: The data indicated that HBP1 was upregulated in degenerated NP tissues. HBP1 gene overexpression increased p16 expression, affected NP cell proliferation, and caused cell apoptosis. In addition, HBP1 also decreased the collagen II and aggrecan expressions but increased collagen I, IL-6, TNF-α, and MMP-3 levels. Moreover, the silencing of HBP1 markedly reversed the H2O2 and IL-1β induced NP cell senescence by reducing p16 expression, apoptotic cell population, and inflammatory response and by promoting cell proliferation.

CONCLUSIONS: In summary, HBP1 accumulation contributes to the senescence of NP cells, and HBP1 deficiency protects stress-induced NP cells premature senescence.

Key Words:
HBP1, Nucleus pulposus cells, Stress-induced premature senescence, p16.

Introduction

The nucleus pulposus (NP) is the main component of the intervertebral disc, and it is a jelly-like structure that can cushion the body's own tension and resist external pressure[1]. Pain in the neck and lower back caused by intervertebral disc degeneration (IVDD) has been one of the most common symptoms in orthopedics. Among many factors that cause the IVDD, such as heredity, inflammatory factors, apoptosis, oxidative stress, and aging, the senescence of NP cells has an important position[2]. Cellular senescence is a process by which normal cells respond to various stressors, and it can be divided into replicative senescence (RS) and stress-induced premature senescence (SIPS)[3]. As the number of cell divisions increases, the shortening of telomeres causes cell RS. However, SIPS is a stagnation of cell division induced by a variety of stressors. The vast majority of cellular senescence in the body is mediated through the p53/p21 signaling pathway or p16 signaling pathway. The telomere-deficient p53/p21 pathway plays a major role in RS, while the p16 pathway mainly mediates SIPS[4].

Under the stress of nutrient deficiency and harmful factors, the cells appear to be aging independently of the shortening of telomere length, called SIPS. At SIPS, the accumulation of the p16 gene causes the cells to remain in the G1 phase, maintaining the cells in an irreversible growth arrest state and resulting in inhibition of cell proliferation[4]. The regulation of p16 is complicated and involves the interaction of many transcription factors, including HMG-box transcription factor 1 (HBP1). Wang et al[5] have revealed that HBP1 activates p16 expression *via* binding to p16 promoter. Li et al[6] reported that HBP1 upregulates p16 expression in Ras-induced premature senescence.

Corresponding Author: Ming Yan, MD; e-mail: 5296827@qq.com

Chen et al[7] found that HBP1 mediates preadipocytes senescence and apoptosis targeting p16 and p53. Additionally, there is no sign of premature senescence in the cells when the HBP1 gene is silent[8], which is sufficient to prove that HBP1 may play an important role in the premature senescence of cells. The underlying reason may be that the silence of the HBP1 gene can block the expression of p16. Besides, HBP1 is also involved in the regulation of the Wnt and p38 mitogen-activated protein kinase (MAPK) pathway, the inhibition of c-Myc gene transcription, thereby blocking cell proliferation[9,10]. However, whether HBP1 takes part in the progression of SIPS in NP cells remains unknown.

Stimulation of H_2O_2 and imbalance of inflammatory response can induce premature senescence[11-13]. Therefore, H_2O_2 and interleukin-1β (IL-1β) were used as inducers to establish an NP cell SIPS model in the experiments of this study. Besides, plasmid and siRNA targeting HBP1 were used to overexpress and silence HBP1 gene expression to explore its function on the NP cell senescent phenotype. The results of this study indicate that HBP1 can be used as a target to prevent NP cell senescence, providing a new idea for the mechanism of IVDD.

Patients and Methods

Intervertebral Disc Sample Collection

A total of 12 intervertebral disc samples of different degenerative degrees were donated from patients (7 males and 5 females, every age, 49, range from 39 to 51) undergoing disc herniation surgery in our hospital. Then, all the samples were divided into 3 groups base on the Pfirrmann score[14] according to the magnetic resonance imaging (MRI) taken before surgery. This investigation was approved by the Ethics Committee of The First Hospital of Jilin University. The signed written informed consents were obtained from all participants before the study.

NP Cells Isolation and Cell Treatment

NP cells were only isolated from the mild degenerated tissues of Pfirrmann Grade III. Disc tissues fragments were digested with 0.2 % type II collagenase at 37°C overnight. The next day, the digested solution was filtrated with a 100 μm pore size cell strainer and the NP cells remain after centrifuge. Next, NP cells were seeded with Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), penicillin 100 UI and streptomycin (100 μg/mL) on six-well plates at 1×10^5 cells. Finally, the stress-induced premature NP cell senescence model was established by incubation with IL-1β (10 ng/mL) or H_2O_2 (1 mM) for 24 hours.

Western Blot (WB)

NP cells were lysed with radioimmunoprecipitation assay (RIPA) and extraction buffer (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Protein was adjusted to equally sing the bicinchoninic acid (BCA) assay kit (Beyotime, Shanghai, China), separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After 5% milk blocking for 1 hour, the membranes were incubated with primary antibodies, collagen II (ab34712), p16 (ab51243), HBP1 (ab83402) and β-actin (ab179467), overnight at 4°C. After washing and incubation with secondary antibody at room temperature for 2 h, the membranes were exposed in the enhanced chemiluminescence (ECL) substrate (Thermo Fisher Scientific, Waltham, MA, USA). All the antibodies were purchased from Abcam (Cambridge, MA, USA.

Cell Viability

Cell viability was determined by Cell Counting Kit-8 (CCK-8; Beyotime, Shanghai, China) according to the manufacturer's protocol. NP cells were seeded in 96-well culture plates at a density of 5000 cells/well and incubated with 10 μL CCK-8 solution at 37°C in the dark for 2 h. Finally, absorbance detection was assessed at 450 nm using a spectrophotometer.

Flow Cytometry

NP cells were harvested and prepared in cold phosphate-buffered saline (PBS), and the proliferation and apoptosis were determined by flow cytometry. Proliferative positive cells were measured using the 5-Ethynyl-2'-deoxyuridine (EdU) assay kit (Keygen, Nanjing, China) according to the manufacturer's protocol. Then, apoptosis was detected by Annexin VFITC/PI assay kit (Keygen, Nanjing, China) according to the manufacturer's protocol. The total apoptotic cells contained both early and late apoptotic NP cells.

Immunofluorescence (IF)

NP cells were washed with cold PBS and then fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 15 min and blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. NP cells were following incubated with the first antibody against collagen II (ab34712, Abcam, Cambridge, MA, USA), p16 (ab51243, Abcam, Cambridge, MA, USA), HBP1 (SC-376831, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Then, NP cells were incubated with a second antibody and 4',6-diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China) in the dark for 1 h. Finally, the fluorescence was visualized using a fluorescence microscope.

Quantitative Real Time-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from NP cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and then, reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript Master (Invitrogen, Carlsbad, CA, USA). After that, qRT-PCR was performed to determine the mRNA levels of collagen I, aggrecan, IL-6, TNF-α, MMP-3, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using SYBR Green Master (Applied Biosystems, Foster City, CA, USA). The primer sequences of the gene used in qRT-PCR were obtained from the Gene Bank (Table I). At last, relative mRNA expressions were calculated using the $2^{-\Delta\Delta Ct}$ method by the normalization of GAPDH.

Transfection

NP cells were seeded in 24-well plates at a density of 1×10^4 cells/well 24 hours before they were transfected with plasmid or siRNA (10 nM) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. HBP1 plasmid was a gift from Nicola Burgess-Brown (Addgene plasmid #39092, http://n2t.net/addgene:39092) and pNIC28-Bsa4 was used as negative control plasmid (null, #26103 BioVector, NTCC, Beijing, China). HBP1-siRNA (# NM_012257) and universal negative control (null, #SIC001) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA). Data were represented as mean ± SD (Standard Deviation). The *t*-test was used for analyzing measurement data. The differences between the two groups were analyzed by using the Student's *t*-test, and the comparison among multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). All the cartograms were generated by GraphPad Prism 6.0 software (La Jolla, CA, USA). $p<0.05$ was considered statistically significant.

Results

HBP1 Increased with the Degeneration of the Intervertebral Disc

To explore the expression of HBP1 in the degenerated intervertebral disc, the disc samples of different degrees of degenerated disc samples from grade (G) 3 to 5 were collected according to the Pfirrmann score. As shown in Figure 1A, the MRI indicated the section for operation and the sample collection. The much more severe degradation the disc was, the lower height and water content the disc had. Thereafter, the expression of collagen II, the most important extracellular matrix (ECM) component secreted by NP cells, in these samples were analyzed, and it was found

Table I. Primer sequences of the genes for RT-PCR.

Gene name	Forward (5'>3')	Reverse (5'>3')
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
MMP-3	AGTCTTCCAATCCTACTGTTGCT	TCCCCGTCACCTCCAATCC
Aggrecan	ACTCTGGGTTTTCGTGACTCT	ACACTCAGCGAGTTGTCATGG
Collagen I	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

qRT-PCR, quantitative Reverse Transcription-Polymerase Chain Reaction.

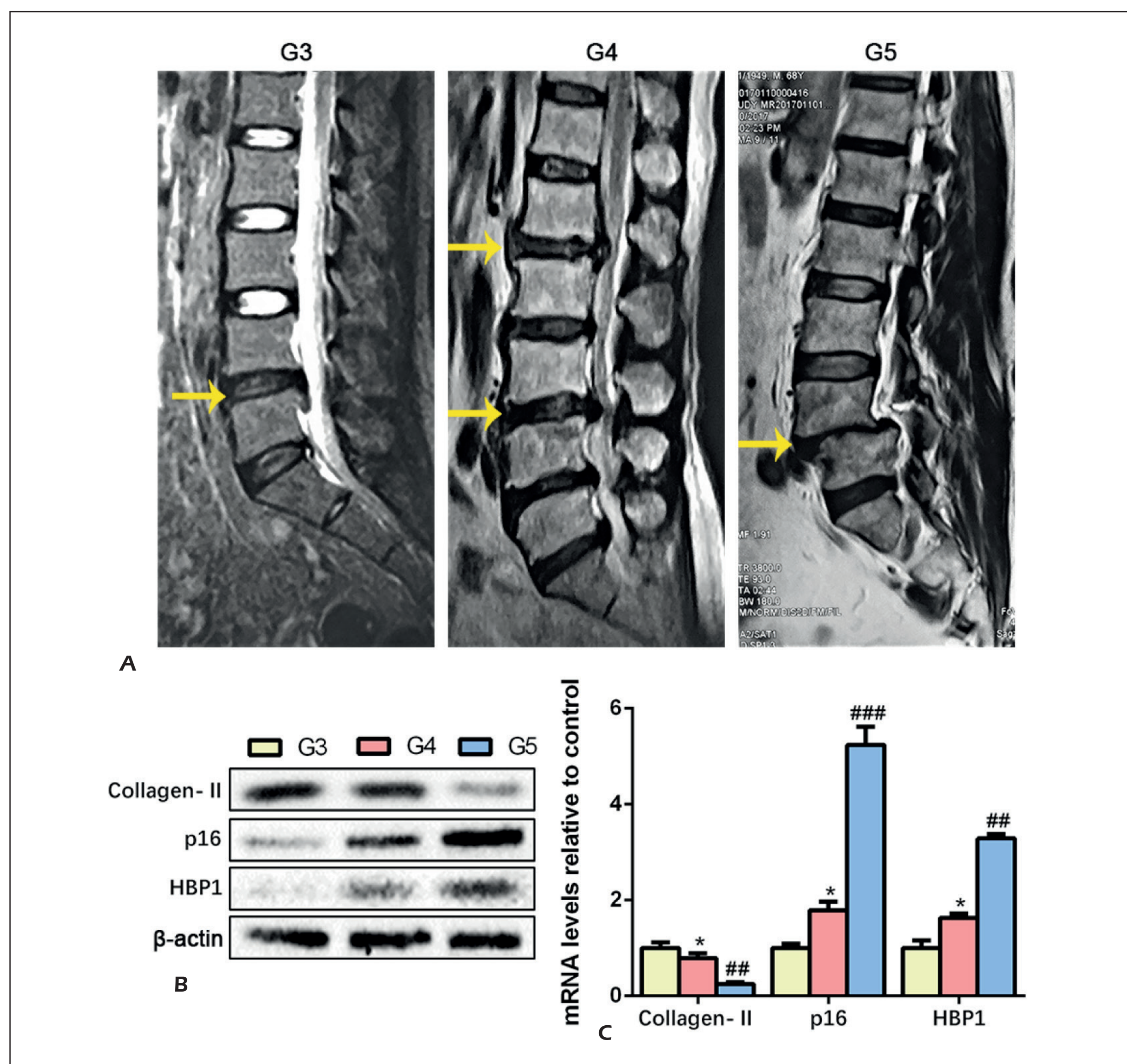

Figure 1. HBP1 protein expression is increased in the degenerated disc. **A**, Representative MRI of the patients from G3 to G4. The yellow arrow indicates the surgical segment **B**, The protein expression levels are determined by WB and **C**, quantification analysis. Data are presented as mean ± SEM of three independent experiments (*$p<0.05$ compared to G3, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to G4).

that the expression of collagen II was decreased with the degeneration of the intervertebral disc. In addition, p16, the senescent marker of cells, was significantly increased resulting from the degeneration getting worse. Additionally, HBP1 was expressed at a low level in mild degenerated discs but substantially increased in the disc of the G5 phase (Figure 1B, 1C). The data suggested that degenerated disc carried a large senescent cell population, and the HBP1 gene might be involved in the progression of IVDD.

HBP1 Overexpression Accelerated NP Cells Senescence In Vitro

Since the HBP1 expression was increased in the meantime with disc degeneration, whether HBP1 overexpression promoted the development of IVDD was explored. Firstly, the HBP1 level in NP cells was upregulated by plasmid transfection. After transfection, the cell viability was not affected statistically compared to the control (Figure 2A). However, the proliferation of NP cells was slightly decreased causing by the HBP1

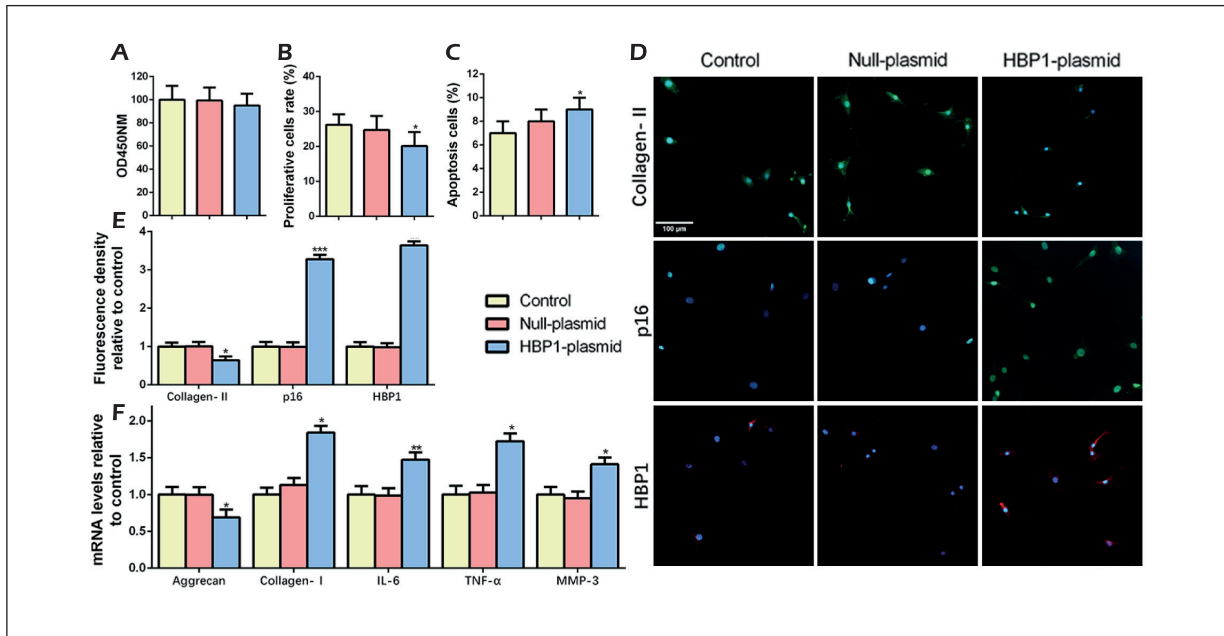

Figure 2. HBP1 overexpression promotes NP cell senescence. NP cells are transfected with plasmid targeting HBP1 or null for 36 hours and the medium is changed. **A**, Cell viability is determined by CCK8 assay. The ratio of **B**, proliferative or **C**, apoptotic NP cells is determined by flow cytometry. **D**, IF staining of collagen-II, p16, HBP1. (magnification: 400×) **E**, Quantification analysis of collagen-II, p16, HBP1. **F**, The mRNA expression levels are assayed by RT-PCR. Data are presented as mean ± SEM of three independent experiments (*p<0.05, **p<0.01, ***p<0.001 compared to the control).

overexpression (Figure 2B), but the apoptosis of NP cells was increased compared to the control (Figure 2C). The upregulation of HBP1 also affected collagen II expression and contributed to the rise of p16 expression (Figure 2D, 2E). Apart from this, the mRNA expression of other members of ECM containing aggrecan and collagen I, the pro-inflammatory factors, such as IL-6 and TNF-α, matrix Metalloproteinases-3 (MMP-3), and the energetic factor of ECM were also measured. It was found that HPB1 rebuilt the ECM components by reducing the aggrecan expression and increasing collagen I expression, indicating that NP cells turned to a fibrosis phenotype[15]. The HPB1 also triggered the IL-6, TNF, and MMP-3 expressions compared to the control (Figure 2F). These results discovered that HBP1 overexpression accelerated the senescent degree of NP cells by affecting the proliferation and ECM statues of NP cells and increasing the apoptosis and the inflammatory response.

HBP1 Deficiency Alleviated H_2O_2 Induced NP Cells Senescence In Vitro

The accumulation of HBP1 promoted the NP cell's spontaneous senescence *in vitro*. To make it clear the effect of HBP1 deficiency in the H_2O_2 treated NP cells, HBP1 in NP cells was silenced by siRNA transfection. As shown in Figure 3A and 3B, H_2O_2 affected the viability and proliferation of NP cells, but HBP1 silencing partly rejected the harmful influence caused by H_2O_2. H_2O_2 significantly induced cell apoptosis cells. However, the effects can be reduced by the blocking of HBP1 expression (Figure 3C). As a source of reactive oxidative species (ROS), H_2O_2 activated the p16 expression and decreased collagen II, indicating that the cell got senescence and the function of NP cells was damaged. However, the silencing of HBP1 restrained the p16 expression and protected the collagen II level secreted by NP cells (Figure 3D, 3E). Apart from this, HPB1 deficiency balanced the ECM status by maintaining aggrecan level and decreasing collagen I and MMP-3 mRNA expression. Additionally, the mRNA levels of the inflammatory factors IL-6 and TNF-α were also suppressed (Figure 3F). In this part, H_2O_2 significantly induced NP cell's premature senescence depending on an ROS manner, which was inhibited by the deficiency of HBP1 gene expression. The alleviation of premature senescence recalled the proliferative and functional cells population and kept a healthy microenvironment of NP cells.

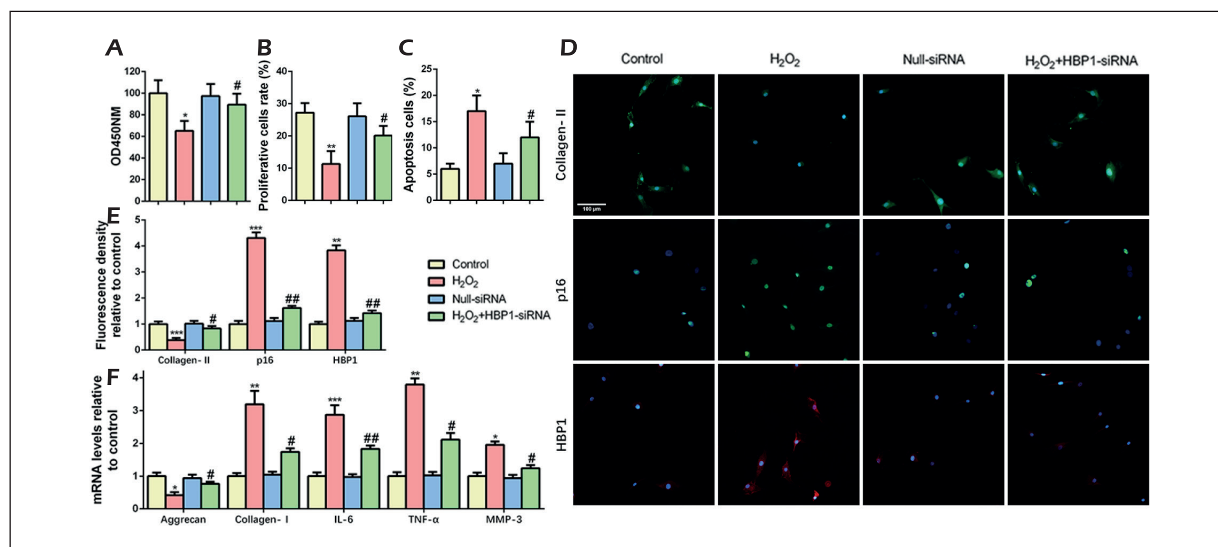

Figure 3. Silencing of HBP1 alleviates H2O2 induced NP cells senescence. CHs with or without siRNA transfection are treated with H2O2 (1 mM) for 24 hours and then the medium is changed. **A**, Cell viability is determined by CCK-8 assay. The ratio of **B**, proliferative or **C**, apoptotic NP cells are determined by flow cytometry. **D**, IF staining of collagen-II, p16, HBP1 (magnification: 400×). **E**, Quantification analysis of collagen-II, p16, HBP1. **F**, The mRNA expression levels are assayed by qRT-PCR. Data are presented as mean ± SEM of three independent experiments (*p<0.05, **p<0.01, ***p<0.001 compared to the control, #p<0.05, ##p<0.01 compared to H2O2 treated group).

HBP1 Deficiency Alleviated IL-1β Induced NP Cells Senescence In Vitro

The HBP1 blocking protected H_2O_2 induced NP cells premature senescence, so the effect of HBP1 deficiency in the stress-induced premature senescence was confirmed by another trigger mode under the presence of IL-1β. Compared to the control, IL-1β decreased the NP cell viability and it was alleviated by HBP1 silencing (Figure 4A). The flow cytometry results suggested that the suppression of HBP1 resulted in more proliferative NP cells and less apoptotic NP cells compared to the IL-1β treated group (Figure 4B, 4C). IL-1β suppressed collagen II expression and upregulated the p16 level compared to the control group. As expected, HBP1 silencing inhibited the activation of p16 expression and protected the collagen II level (Figure 4D, 4E). Finally, the mRNA expressions of aggrecan, collagen I, IL-6, TNF-α, and MMP-3 were also tested, which showed that HPB1 deficiency was helpful to the aggrecan expression but suppressed collagen I, IL-6, TNF-α, and MMP-3 expression increased by IL-1β (Figure 4F). In addition, to protect NP cells premature senescence caused by a ROS-depended manner, the deficiency of HBP1 could prevent redundant inflammatory factor-induced premature senescence with suppression of p16 and a promotion in cell proliferation. It was believed that HBP1 is related to the premature senescence of NP cells regardless of its occurrence.

Discussion

Cell senescence refers to an irreversible state in which normal cells undergo slow growth, and reduced metabolic activity and stagnation after a limited number of cell divisions under the stimulation of physiological conditions or pathological factors[16]. IVD is the largest avascular tissue in the body, and NP relies primarily on the osmotic effects of the endplate to provide nutrients. In this hypoxic and hypertrophic environment, the activity of NP cells is low, which is one of the reasons for the poor self-healing ability of function and the tendency to senescence after disc tissue damage[17,18]. A variety of stress effects can cause cells to senescence prematurely, among which oxidative stress is closely related to DNA damage and cause premature senescence of cells[19]. The level of oxidative stress increases with age. Under physiological conditions, the production of ROS and the elimination of antioxidant enzymes are in equilibrium. If ROS is excessively produced or antioxidant enzyme activity is decreased, the ac-

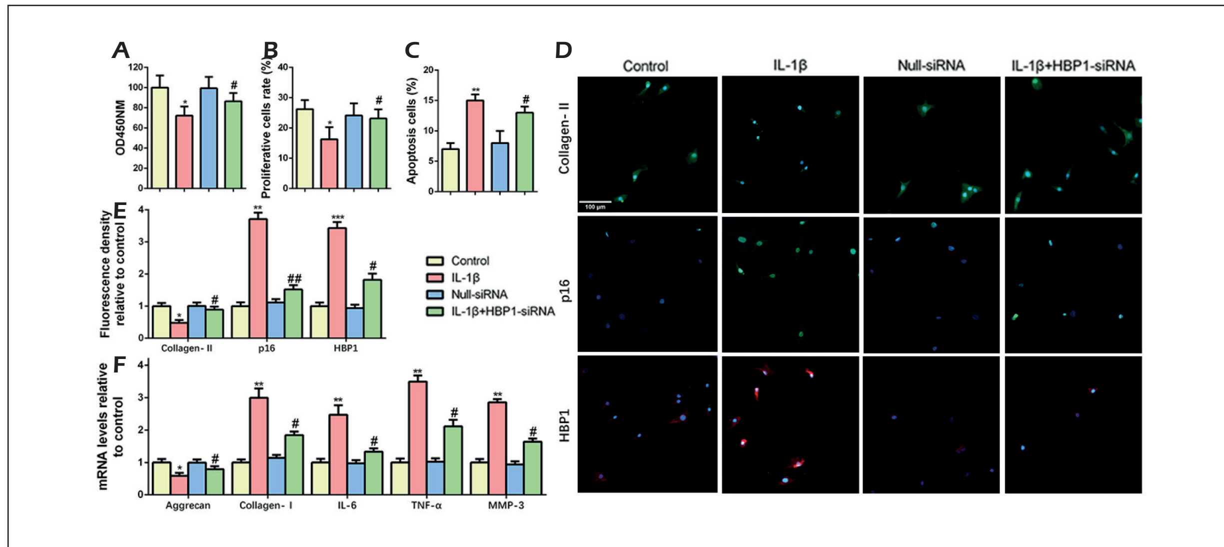

Figure 4. Silencing of HBP1 alleviates IL-1β induced NP cells senescence. CHs with or without siRNA transfection are treated with IL-1β (10 ng/mL) for 24 hours and then the medium is changed. **A**, Cell viability is determined by CCK-8 assay. The ratio of **B**, proliferative or **C**, apoptotic NP cells is determined by flow cytometry. **D**, IF staining of collagen-II, p16, HBP1 (magnification: 400×). **E**, Quantification analysis of collagen-II, p16, HBP1. **F**, The mRNA expression levels are assayed by RT-PCR. Data are presented as mean ± SEM of three independent experiments (*$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to the control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to IL-1β treated group).

cumulation of ROS damages various components in the cell and accelerates the process of cellular senescence[20]. In this study, H_2O_2 was used to induce NP cell senescence by the overplus of ROS. In this situation, it was found that the viability and proliferation of NP cells were decreased, and p16 gene expression was significantly increased. The p16 protein can block the cell cycle at the G1/S detection site failing to complete proliferation, which is a marker of cellular senescence. Upregulation of p16 promotes cell senescence, on the contrary, the removal of p16 delays cell senescence.

HBP1 also can regulate the G1 phase of the cell cycle by interacting with RB to repress cell proliferation. Overexpressed HBP1 gene expression in skeletal muscle cells, adipocytes, erythrocytes, and other highly differentiated cells inhibits cell proliferation and makes cell senescence[21]. HBP1 is a transcriptional repressor targeting some cell proliferation-related genes, and it may become a general inhibitor of cell proliferation. Pan et al[22] found that HBP1 regulates cellular senescence by DNA methylation. Zhang et al[8] reported that HBP1 is involved in Ras-induced cell premature senescence. However, how HBP1 mediates NP cell premature senescence, and whether it is related to the procession of IVDD are not fully understood. The novelty of this study was that HBP1 was noticed to be significantly increased in the severely degenerated NP tissue for the first time, and the overexpression of HBP1 promoted the NP cell premature senescence compared to the control, indicating that HPB1 is an effective target for the treatment of IVDD. Apart from this, the deficiency of HBP1 also made sense to the prevention of ROS mediated premature senescence and protected the ECM stability, which was really important for the intervention in the IVDD caused by redundant ROS.

The inflammatory response and cytokines are also the pathological basis closely to IVDD[23], and the expressions of IL-1β and IL-1RI are increased as the degree of IVD degeneration increases[24]. *In vitro*, IL-1β upregulates MMPs gene expression in NP cells, inhibits the expression of Sox9 and collagen II in IVD cells by nuclear factor kappa B (NF-κB)[25], and triggers inflammatory response and catabolism by p38MARK pathway[26]. Therefore, IL-1β was used as another inducer of NP cell premature senescence. As H_2O_2, IL-1β caused NP cell apoptosis and led to a poor proliferation with the upregulation of p16 expression. However, the silencing of HBP1 surprisingly alleviated the side effects caused by IL-1β. HBP1 downregulation also decreased the IL-6 and TNF-α expressions, which might not be an ability of inflammatory suppression of HBP1 but a signal that HBP1 made

the NP cells in a much healthier statue. The findings in this study indicated that HBP1 could be a potential target in the mediation of cell senescence, proliferation, inflammation, apoptosis, and ECM stability during IVDD.

Conclusions

In short, this research elucidated a novel method to protect NP cells from stress-induced premature senescence by the suppression of HBP1. The deficiency of HBP1 can promote the proliferation of NP cells and keep the stability of ECM, which is greatly meaningful to maintain the viability and function of NP cells.

Conflict of Interests

The authors declare that they have no conflict of interests.

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