

SD0006 promotes nucleus pulposus cell proliferation *via* the p38MAPK/HDAC4 pathway

Y.-D. WU, Z.-G. GUO, W.-J. DENG, J.-G. WANG

Department of Orthopaedics, Rizhao Hospital of Traditional Chinese Medicine, Rizhao, China

Yadong Wu and Zhenguang Guo contributed equally to this work

Abstract. – **OBJECTIVE:** p38 mitogen-activated protein kinase (p38MAPK) is a critical negative regulator for nucleus pulposus (NP) cell metabolism contributing to intervertebral disc degeneration (IDD). Histone deacetylase 4 (HDAC4) has the ability to mediate cell proliferation and is affected by p38MAPK. It is unclear whether the p38 MAPK inhibitor SD0006 (SD) can regulate IDD. Our study aims to explore the effect of SD in the progression of IDD, as well as its potential mechanism.

PATIENTS AND METHODS: NP cells isolating from mild degenerated NP tissues were cultured, and IL-1 β or Asiatic acid (AA) was used to activate p38MAPK and accelerate the NP cell degradation. Then, SD was used to reject the p38MAPK activation. After that, the levels of phosphorylated p38MAPK (p-p38), HDAC4, proliferating cell nuclear antigen (PCNA), inflammatory factor, proliferative cell rate, and cell cycle were determined by Western blot, RT-PCR, flow cytometry, and immunofluorescence, respectively.

RESULTS: The results showed that the activation of p38MAPK by IL-1 β and AA decreased the HDAC4 expression, affected the collagen-II expression, upregulated the TNF- α , IL-6, MMP-3, and ADAMTS mRNA levels, and prevent the NP cell proliferation by mediating cell cycles. However, the application of SD alleviated the negative effect of p-p38 by upregulating HDAC4, anti-inflammation, and promoting cell proliferation, while the blocking of HDAC4 expression partly abolished the effect of SD.

CONCLUSIONS: SD can prevent NP cell degeneration by promoting cell proliferation and suppressing inflammation via p38MAPK/HDAC4 pathway.

Key Words:

Nucleus pulposus cells, p38MAPK, HDAC4, Proliferation, SD0006.

Introduction

Intervertebral disc degeneration (IDD) is the core of spinal degeneration and is one of the important

factors of low back pain and lumbar disc herniation in adults¹. At present, the short-term effect of surgical treatment of lumbar disc herniation is good, but the long-term impact is not very satisfactory². As time goes by, the cure rate of surgical procedure has a tendency to decline. IDD is mainly manifested as the reduction of nucleus pulposus (NP) cells and changes in the extracellular matrix (ECM). When the disc gets aging or the nutrient supply is impaired, the activity of NP cells is decreased with a decreased proliferation and increased apoptosis, which is the pathological basis of IDD³.

Mitogen-activated protein kinase (MAPK) is present in most eukaryotic cells and plays a role in intracellular and extracellular information transmission⁴. The p38MAPK is one of the four MAPK pathways that have been found in mammals⁵, and it has such functions as mediating inflammation, proliferation, differentiation, apoptosis, and cytokine synthesis of cell metabolism⁶. After activated by phosphorylation, p38 affects the production of inflammatory factors such as IL-1 and TNF- α , inhibits cell proliferation, participates in the regulation of apoptosis⁷. Phosphorylated p38MAPK substrates include activated transcription factor 2 (ATF2), myocyte enhancer factor 2c (MEF2C) and C/EBP homolog 10 (CHOP10). p38MAPK also affects histone deacetylase 4 (HDAC4) expression, which regulates transcriptional levels in cell proliferation by regulating cycle progression⁸⁻¹¹.

Activated p38MAPK accumulates inflammatory mediators in IDD, significantly increasing the content of inflammatory factors and participating in the regulation of macrophage function¹². The content of p38MAPK is positively related to the increase of degenerative degree¹³. However, the usage of p38 blocker improves the extent of disc degeneration¹⁴. Studer et al¹⁵ reported that p38MAPK inhibition balances the anabolic and catabolic of NP cells, which slows the course of

IDD. Pang et al¹⁶ found that the suppression of the p38MAPK pathway prevents high-magnitude compression induced NP cell senescence. Zhu et al¹⁷ elucidated that SB203580 could inhibit p38 phosphorylation and decrease NP cell apoptosis in rats with IDD. However, little research on the mechanism of p38MAPK inhibition is about NP cell proliferation in IDD. Therefore, this study aims to explore the efficiency of a novel p38MAPK inhibitor named SD0006 (SD) in the development of IDD, mainly focusing on the effect on HDAC4 and the proliferation of NP cells.

Patients and Methods

NP Tissues Collection

According to the Pfirrmann classification score based on the Magnetic Resonance Imaging (MRI), eight NP tissues in mild degeneration were collected from the patients with disc herniation undergoing lumbar NP removal surgery in Rizhao Hospital of Traditional Chinese Medicine in January 2019. All specimens taken during surgery were stored in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) and used for NP cell isolation immediately. This investigation was approved by the Ethics Committee of our Hospital and conducted in accordance with the Declaration of Helsinki.

NP Cells Isolation and Treatments

The fragments of NP tissues were digested with 0.2% type II collagenase (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 3 h. The cells were collected by cell sieve and inoculated in 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) in DMEM. All experimental NP cells passaged to the first to third generations were treated as follows: Control group (no treatments), IL-1 β group (treated with 5 ng/mL IL-1 β), SD group (treated with 10-70 nM SD, Selleck, Houston, USA), IL-1 β +SD group (pre-treated with 5 ng/mL IL-1 β and then 70 nM SD), Asiatic acid (AA) group (treated with 10 μ M AA, Abcam, Cambridge, UK), AA+SD group (pre-treated with 10 μ M AA and then 70 nM SD), IL-1 β +SD+Scriptaid group (pre-treated with 5 ng/mL IL-1 β and then co-cultured with 5 μ M Scriptaid and 70 nM SD).

Cell Viability Assay

CCK8 assay was performed to determine NP cell viability. NP Cells were seeded at a density of 1×10^4 cells per well in a 96-well plate and in-

cubated with cell counting kit-8 (CCK-8) reagent (Dojindo, Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions. Finally, the intensity of each well was analyzed at 570 nm.

Flow Cytometry

Flow cytometry was adopted to analyze NP cell cycle distribution and proliferated cell ratio. After treatment, NP cells were harvested by trypsin, fixed with 70% ethanol, and diluted in 1 mL phosphate buffered saline (PBS) containing 1 mg RNase and 50 μ g propidium iodide (PI) or 5-Ethynyl-2'-deoxyuridine (EdU) assay kits (Invitrogen, Carlsbad, CA, USA), respectively. After incubation avoiding light for 30 min, the solution was measured by a flow cytometer.

Western Blot (WB)

After treatments, NP cells were lysed by radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). Total protein was collected, heated and denatured, and the quality of protein was measured with bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). Protein was electrophoretic separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. Then, the membrane was coated by 5% milk and incubated with Phospho-p38MAPK (p-p38, #4511, Cell Signaling Technology, Danvers, MA, USA), HDAC4 (#151641, Cell Signaling Technology, Danvers, MA, USA), and β -actin (#3700, Cell Signaling Technology, Danvers, MA, USA) primary antibodies overnight at 4°C. Subsequently, membranes were incubated with corresponding secondary antibodies for another 1 h. Finally, the membrane was rinsed with enhanced chemiluminescence (ECL) substrate (Beyotime, Shanghai, China) to expose the bands.

Immunofluorescence (IF)

NP cells were seeded at 1×10^4 per well in 12-well plates. After treatments, cells were fixed with 4% paraformaldehyde and then the membranes were permeabilized with 0.1% Triton-X. After blocked with 5% bovine serum albumin (BSA), NP cells were washed and incubated with primary antibodies against collagen II (ab34712, Abcam, Cambridge, MA, USA), and proliferating cell nuclear antigen (PCNA, ab92552, Abcam, Cambridge, MA, USA) overnight. Subsequently, NP cells were incubated with Alexa Fluor488

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 | CCATCTTTGGAAGGTTTCAGGTTG |
| MMP-3 | AGTCTTCCAATCCTACTGTTGCT | TCCCCGTCACCTCCAATCC |
| ADAMTS-5 | GAACATCGACCAACTCTACTCCG | CAATGCCACCGAACCATCT |
| GAPDH | ACAACCTTTGGTATCGTGGAAGG | GCCATCACGCCACAGTTTC |

RT-PCR, quantitative reverse-transcription polymerase chain reaction.

secondary antibody (Invitrogen, Carlsbad, CA, USA) for 1 h in the dark. Finally, the intensity of staining was analyzed using ImageJ software (NIH, Bethesda, MD, USA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

After treatments, total RNA of NP cells was isolated *via* TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using the RT Kit (TaKaRa, Dalian, China). Then, the RNA expressions of TNF- α , IL-6, MMP-3, and ADAMTS-5 were analyzed with SYBR Green Master (TOYOBO, Osaka, Japan). The levels of RNA expression were calculated by the normalization of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) based on the method of $2^{-\Delta\Delta Ct}$. Primer sequences used in RT-PCR are listed in Table I.

Statistical Analysis

Data were calculated by the Statistical Product and Service Solutions (SPSS) 17 software (SPSS Inc., Chicago, IL, USA) and expressed as mean \pm standard deviation (SD). Differences between two groups were analyzed by using the Student's *t*-test. Comparisons among multiple groups were made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p < 0.05$ suggested that the difference was statistically significant.

Results

The Safety and Efficiency of SD Treatment to NP Cell

To determine the optimized concentration of SD in the treatment of NP cells, CCK8 assay was used to test the cell viability and Western blot to test the p38 inhibited efficiency. Com-

pared with the control, there are no statistically significant differences in cell viability under the incubation of SD from 10 nM to 70 nM for 24 hours (Figure 1A). In addition to the 10 nM, SD significantly suppressed the level of phosphorylated p38MAPK up to 70 nM in a dose-dependent manner (Figure 1B, 1C). These data indicate that SD is safe for the viability of NP cells within a

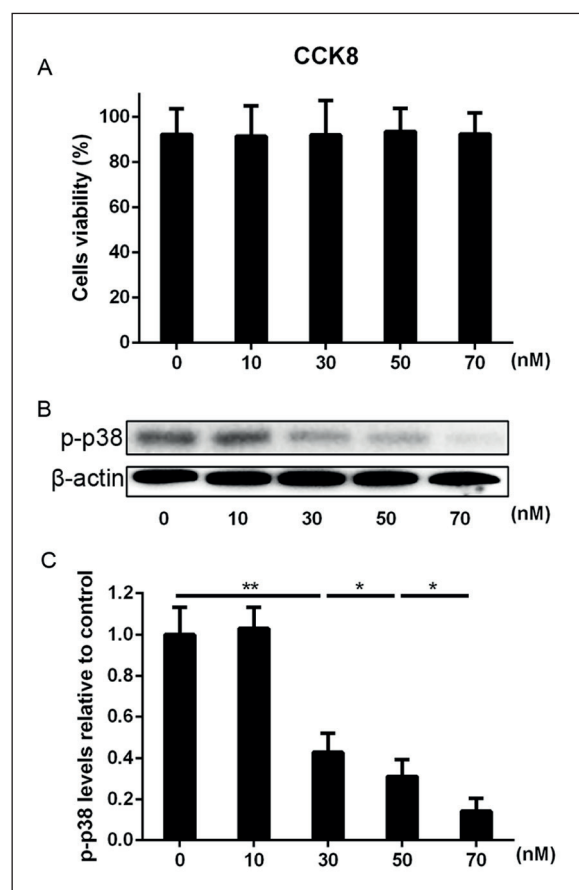


Figure 1. The effect of SD on NP cells. CHs are treated with SD from 0 to 70 nM for 24 h. **A**, Cell viability is measured by CCK8 assay. **B**, **C**, The protein expression level of p-p38 is determined by **(B)** WB and **(C)** quantification analysis. ($n=3$, $*p < 0.05$, $**p < 0.01$).

specific dose, and the 70 nM incubation for 24 h is efficient enough to suppress p38MAPK activity to a great extent.

SD Treatment Protects IL-1 β Treated NP Cell Proliferation In Vitro

As the ability to cause inflammation, IL-1 β is one of the most commonly used inflammatory factors in the NP cell degeneration model¹⁸. As shown

in Figure 2A and 2B, IL-1 β increased the p-p38 expression and decreased the HDAC4 level compared with the control. On the contrary, SD significantly reduced the p-p38 expression and protected the content of HDAC4 compared with the control. Besides, SD also alleviated the IL-1 β induced p-p38 activation and HDAC4 reduction. Collagen II is the main content of ECM in NP, which is only secreted by NP cells, and it decreases in the meantime with

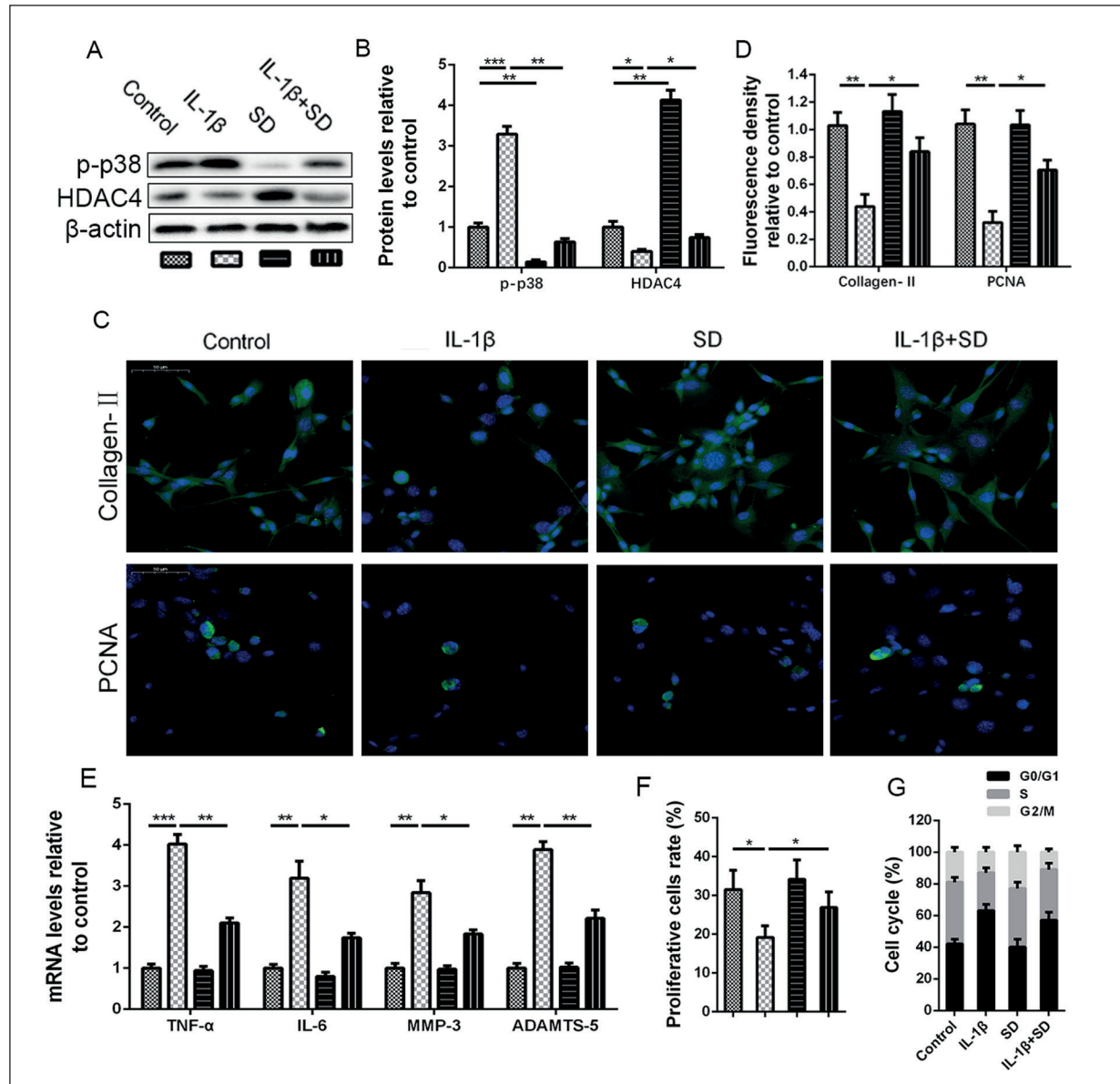


Figure 2. SD protects IL-1 β treated NP cell proliferation. NP cells are treated with 5 ng/mL IL-1 β or 70 nM SD for 24 h. For the co-treated group, NP cells are pre-treated with 5 ng/mL IL-1 β and then cultured with 70 nM SD for another 24 h. A, B, The protein expression levels of p-p38 and HDAC4 are determined by (A) WB and (B) quantification analysis. C, D, The protein expression levels of collagen II and PCNA are determined by (C) IF (magnification: 400 \times) and (D) quantification analysis. E, The mRNA expression levels of TNF- α , IL-6, MMP-3, and ADAMTS-5 are assayed by RT-PCR. F, G, (F) Proliferative cell rate and (G) cell cycle are assayed by flow cytometry. (n=3, * p <0.05, ** p <0.01, *** p <0.001).

NP degeneration. The collagen II level was significantly reduced resulting from IL-1 β stimulation. However, SD partly rejected this side effect caused by IL-1 β . PCNA is a marker of proliferated cells suppressed by IL-1 β , which is also protected by the presence of SD (Figure 2C, 2D). Due to the pro-inflammatory function of p38, SD should affect the inflammatory response of IL-1 β treated NP cells. Then, several inflammatory factors and ECM disruptive factors were tested. As shown in Figure 2E, SD made no difference in NP cells compared with the control. However, it decreased the RNA expressions of TNF- α , IL-6, MMP-3, and ADAMTS-5 in the IL-1 β induced NP cell degeneration. Besides, the SD also promoted the proliferated population of NP cells in the IL-1 β condition (Figure 2F). It is well known that HDAC4 contributes to cell proliferation related to the mediation of the cell cycle. Later, the cell cycle distribution was analyzed by flow cytometry. Compared with the control, IL-1 β delayed more cells in the G0/G1 phase, and SD promoted more cells to go into the G2/M phase. In contrast, SD also promoted more cells into the S phase in the existence of IL-1 β (Figure 2G). Briefly, these data suggest that IL-1 activates the p38 activity but suppresses the HDAC4 expression, accompanied by a higher level of the inflammatory response and a lower proliferative ability resulting in the degeneration of NP cells. The application of SD partly reversed these harmful effects caused by IL-1 β with the increased HDAC4 expression, the suppression of inflammation, and a higher level of proliferation.

SD Treatment Protects AA Treated NP Cell Proliferation In Vitro

To determine the function of SD in the regulation of the p38MAPK/HDAC pathway in NP cells, AA was used as an inducer of p38MAPK phosphorylation. AA, a triterpene, possesses numerous pharmacological activities such as cell cycle arrest and apoptosis through activation of p38MAPK¹⁹. As shown in Figure 3A and 3B, AA promoted the p38 phosphorylation and inhibited HDAC4 expression. However, as the inhibitor of p38, SD rejected the AA-induced p38 activation and the HDAC4 suppression. Meanwhile, AA increased the TNF- α , IL-6, MMP-3, and ADAMTS-5 RNA expressions compared with the control, which reversed by SD (Figure 3C). Due to the activation of p38MAPK, collagen-II expression was significantly decreased after AA treatment compared with the control, and as expected, SD maintained collagen-II content

compared with the AA group (Figure 3D, 3E). As AA accelerated the degeneration of NP cells, whether AA influences the proliferation of NP cells and the effect SD on AA was investigated. Besides, the PCNA expression and proliferative cell rate of these treatments were explored. The results indicated that AA truly affected the proliferation of NP cells with a decreased PCNA level and lower proliferative rate, but SD turned an excellent appearance to oppose the effect of AA protecting the proliferative population of NP cells (Figure 3D-3F). Cell cycle was also measured, and the data showed that AA prevented NP cells going through G1/S checking point resulting in fewer cells stayed in S phase, and SD brought NP cells into S phase, which might be the reason underline SD-promoted cell proliferation under AA treatments (Figure 3G). In this part, it was found that the activation of p38MAPK decreased the expression of HDAC4, and activated the inflammation response, resulting in the reduction of collagen-II expression and the proliferative rate. However, SD could reject the activation of p38MAPK, leading to a protective effect on NP cells.

SD Treatment Promotes NP Cell Proliferation by Protecting HDAC4 Activity

To determine the inhibition of p38MAPK-promoted cell proliferation resulting from the up-regulation of HDAC4, the HDAC4 expression was blocked by its inhibitor named Scriptaid. As mentioned above, IL-1 β suppressed HDAC4 expression and SD protected HDAC4 expression, which was negatively related to the p-p38 level. As a result of Western blot, Scriptaid significantly decreased HDAC4 expression after the protection by SD (Figure 4A and 4B). Furthermore, SD protected the expression of collagen-II decreased by IL-1 β , but Scriptaid destroyed the effect of SD on collagen-II again (Figure 4C, 4D). In addition to this, the suppression of HDAC4 by Scriptaid also upregulated the expressions of TNF- α , IL-6, MMP-3, and ADAMTS-5, which was the composition of NP cells degeneration (Figure 4E). Without the high level of HDAC4, the PCNA positive cells and the proliferative cells are significantly reduced compared with those in SD group (Figure 4C, 4D, 4F). The downregulation of HDAC4 also affected the cell cycle by stagnating more NP cells in the G0/G1 phase, which abolished the function of SD in the antagonist of IL-1 β (Figure 4G). Taken together, SD suppressed p38MAPK activity

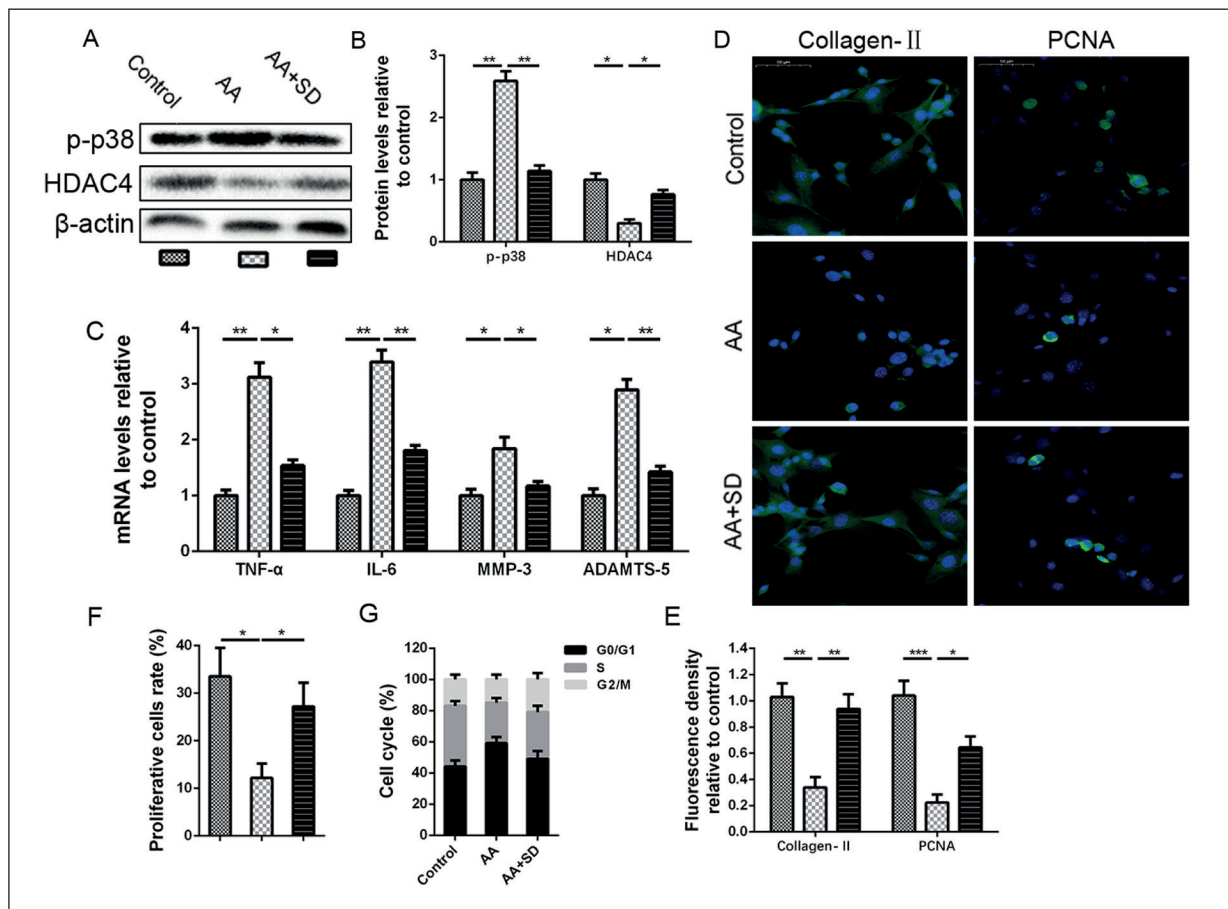


Figure 3. SD protects AA treated NP cell proliferation. NP cells are treated with 10 μ M AA for 24 h with or without 70 nM SD for another 24 h. **A, B**, The protein expression levels of p-p38 and HDAC4 are determined by (A) WB and (B) quantification analysis. **C**, The mRNA expression levels of TNF- α , IL-6, MMP-3, and ADAMTS-5 are assayed by RT-PCR. **D, E**, The protein expression levels of collagen II and PCNA are determined by (D) IF (magnification: 400 \times) and (E) quantification analysis. **F, G**, (F) Proliferative cell rate and (G) cell cycle are explored by flow cytometry. (n=3, * p <0.05, ** p <0.01).

and resulted in the upregulation of HDAC4, which contributed to the stability of NP cells, containing a lower level of inflammation and a higher level of proliferation. However, the blocking of HDAC4 by Scriptaid partly abolished these protective effects of SD in the degeneration of NP cells, indicating that HDAC4 is vital in the development of NP cell degradation, especially in the mediation of cell cycle and the cell proliferation.

Discussion

As an inhibitor of p38MAPK, SD significantly decreased the inflammation factors and protected collagen-II expression. The inflammatory factor promoted the degradation of type II collagen and inhibits the synthesis of NP cells, which sup-

ported the development of IDD. P38MAPK was discovered in 1993 by Brewster et al²⁰ in a study on the effects of hypertonic environments on fungi. It is a stress-activated protein kinase that triggers the inflammatory process. Inhibition of p38MAPK activity by pyridine isoxazole derivatives reduces or blocks the production of many inflammatory factors like IL-1 β , IL-6, IL-8, and TNF- α ²¹. Animal experiments have shown that suppression of the p38MAPK pathway reduces the response of NP cells to IL-1, suggesting that silencing the p38MAPK pathway plays a role in inflammation-mediated degeneration of the intervertebral disc²². *In vitro*, inhibition of p38MAPK delays the degeneration of human NP cells, and reduces the production of factors related to inflammation, pain, and intervertebral disc catabolism¹⁵.

HDAC4 is one of the Class IIa HDACs sub-families, playing a vital role in cell growth, repressing the transcriptional action by chromatin condensation and structures regulation²³. HDAC4 has critical roles in cancer development, growth and aggressiveness²⁴. Furthermore, the inhibition of HDAC4 has been regarded as one

of the most promising anti-deacetylase strategies for suppressing the oncogenic roles in glioma cell²⁵, leukemia cell²⁶, breast cancer²⁷ and so on. It has been reported that HDAC4 also takes part in the mediation of the cellular response by protein post-translational modification²⁸. Choi et al²⁹ elucidated that HDAC4 contributes to satellite

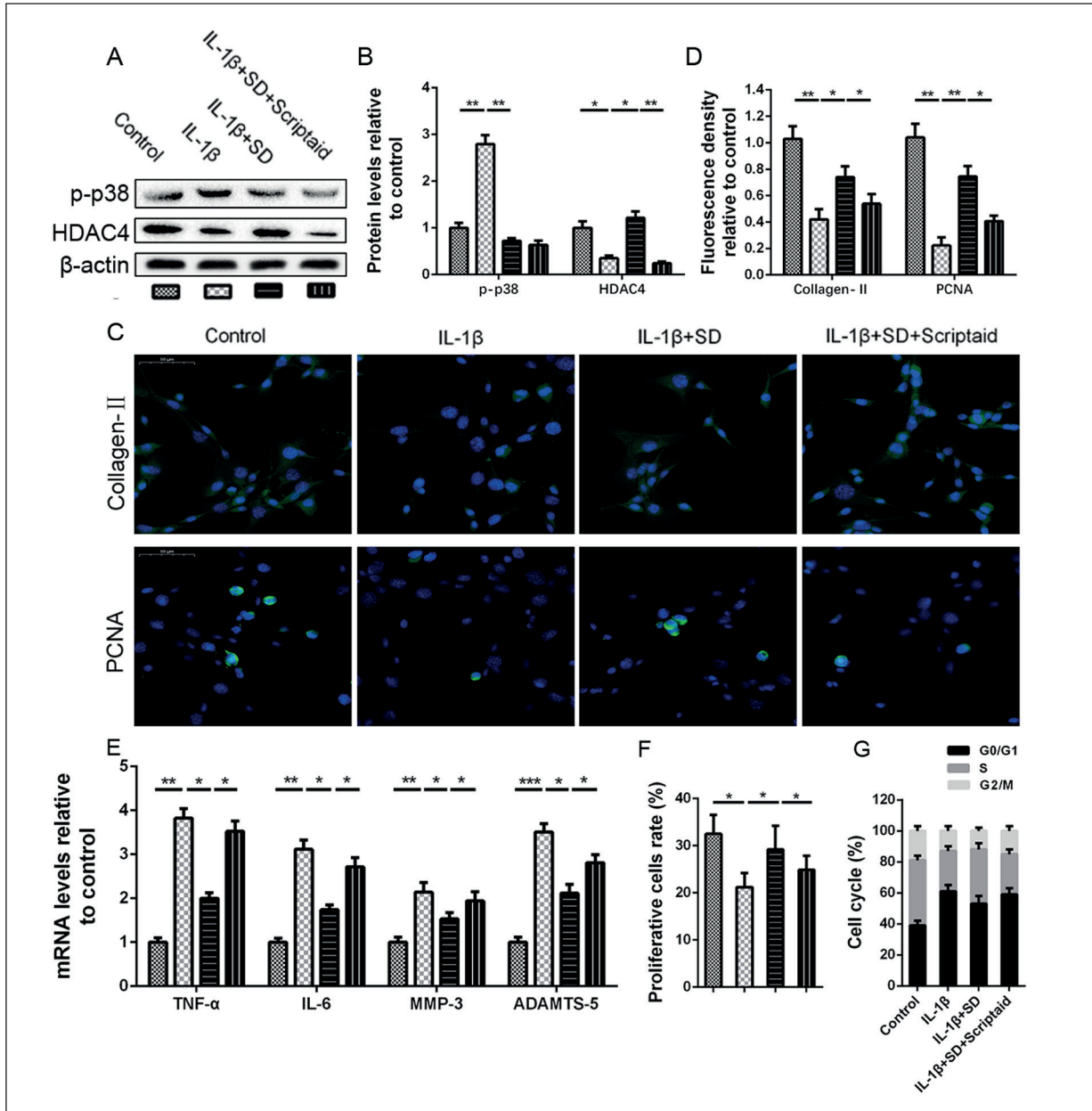


Figure 4. SD promotes NP cell proliferation by protecting HDAC4 activity. NP cells are pre-treated with 5 ng/mL for 24 h, and then treated with 70 nM SD with or without 5 μM Scriptaid for another 24 h. **A, B,** The protein expression levels of p-p38 and HDAC4 are determined by **(A)** WB and **(B)** quantification analysis. **C, D,** The protein expression levels of collagen II and PCNA are determined by **(C)** IF (magnification: 400×) and **(D)** quantification analysis. **E,** The mRNA expression levels of TNF-α, IL-6, MMP-3, and ADAMTS-5 are assayed by RT-PCR. **F, G,** **(F)** Proliferative cell rate and **(G)** cell cycle are quantified by flow cytometry. (n=3, **p*<0.05, ***p*<0.01, ****p*<0.001).

cell activation and muscle regeneration targeting Pax7. Zhang et al³⁰ found that HDAC4 inhibits ER stress-induced apoptosis *via* ATF4 and its transcriptional activity. Liu et al³¹ stated that HDAC4 overexpression promotes Schwann cell proliferation after sciatic nerve crush. The activation of HDAC4 is mediated by many proteins, among which p38MAPK develops the deficiency of HDAC4. Rong et al³² elucidated that p38 inhibited ATF-2 expression resulting in the reduction of HDAC4. Zhou et al³³ found that p38 degrades HDAC4 through caspase activation in chondrocytes. However, little is known about whether p38 affects HDAC4 expression resulting in the inhibition of NP cell proliferation. In this study, the activation of p38MAPK by either IL-1 β or AA environment downregulated HDAC4 expression, and the suppression of p38MAPK by SD recalled the loss of HDAC4 leading to a positive effect of NP cell proliferation. However, the inhibition of HDAC4 by Scriptaid somehow abolished the protection of SD to NP cells, indicating that SD promotes the NP cell proliferation associated with the maintaining of HDAC4.

This study illustrated that the activation of p38MAPK by phosphorylation affected the HDAC4 expression, resulting in a degenerated NP cell phenotype with a decreased collagen-II and proliferation and increased inflammatory response. After inspection, SD is a safe and efficient p38MAPK inhibitor of NP cells, and it can protect the content of HDAC4, contributing to NP cell proliferation. In conclusion, it is believed that SD plays a positive role in the prevention of IDD by promoting NP cell proliferation *via* the p38MAPK/HDAC4 pathway.

Conclusions

Taken together, the above results show that SD can prevent NP cell degeneration by promoting cell proliferation and suppressing inflammation *via* p38MAPK/HDAC4 pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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