Asperosaponin VI promotes human mesenchymal stem cell differentiation into nucleus pulposus like-cells via up-regulation of ERK1/2 and Smad2/3 signaling pathways.

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Research

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Abstract

Background

At present, the regeneration of nucleus pulposus cells is an effective method to prevent intervertebral disc degeneration (IVDD). In this study, we investigated the role of Asperosaponin VI (ASA VI), isolated from a traditional Chinese medicine (TCM), the root of Dipsacus asper Wall, in promoting human mesenchymal stem cell (HMSC) proliferation and differentiation into nucleus pulposus like-cells and explored its possible mechanism of action.

Methods

First, the effects of ASA VI on HMSC vitality and proliferation were determined by the XTT method and EDU staining. Then, HMSCs were cultured with ASA VI. Real-time PCR, immunocytochemistry and immunofluorescence were used to measure the expression of extracellular matrix (ECM) components in nucleus pulposus cells, such as type II collagen(COL2A1), aggrecan, SOX9, KRT19, PAX1 and glycosaminoglycans (GAGs), and Western blot was used to investigate its potential mechanism.

Results

ASA VI could promote HMSC differentiation into nucleus pulposus like-cells, and its optimum concentration was 1 mg/L. The Western blot assays indicated that the possible mechanisms involved upregulating the expression of P-ERK1/2 and P-Smad2/3.

Conclusions

ASA VI can promote HMSC proliferation and differentiation into nucleus pulposus like-cells, which can be used as a potential treatment for IVDD.

Background

Low back pain (LBP) is a common disease, and approximately 80% of the general population experience it at least once in their lives[1]. IVDD is related to age, load-bearing labor, trauma and heredity and leads to chronic low back pain and economic burden[2]. Although there are many factors that can lead to LBP, IVDD is considered as a major factor[3, 4]. Treatment of IVDD remains a clinical challenge due to conservative treatment, in which only symptoms can be alleviated, while surgical treatment leads to complications and structural damage[5]. Thus, there is no approved program for the treatment of degenerative intervertebral discs; therefore, it is very important to find a treatment for the etiology of IVDD.
The intervertebral disc (IVD) is mainly composed of cartilage tissue, including nucleus pulposus, annulus fibrous and endplates. Some studies have shown that the changes of ECM biosynthesis and the decreases in nucleus pulposus cell function and number are the primary factors of IVDD[6]. Therefore, increasing extracellular matrix and promoting the regeneration of NP cells have become an ideal treatment. Due to poor regeneration of NP cells, cell-based therapy may be a promising treatment for IVDD[7]. At present, increasing amounts of attention have been paid to using the multidirectional differentiation potential of stem cells to promote the regeneration of degenerative IVD[8].

Moreover, a large number of experimental studies have confirmed that stem cell applications are effective treatments with great clinical application potential[9, 10]. There are many research methods to promote the differentiation of stem cells into nucleus pulposus for the treatment of degenerative IVD, including growth factor intervention, coculture of stem cells and nucleus pulposus cells, hypoxia induction, stem cells planted in three-dimensional scaffolds, and applied stress, which promote the differentiation of stem cells into nucleus pulposus-like cells to a certain extent[11–15]. However, it is rare to use traditional Chinese medicine to promote the differentiation of stem cells into nucleus pulposus-like cells to repair a degenerative IVD.

Asperosaponin VI (ASA VI), also known as Akebia Saponin D (ASD), is the main bioactive component of the traditional Chinese medicine (TCM) Radix Dipsaci. The Chinese Pharmacopoeia specifies ASA VI content as Dipsacus asper Wall quality standard.

Dipsacus asper Wall, as an herbal medicine, has the effect of tonifying the liver and kidney, with a long history of safe use for strengthening the tendons and bones. The study found that Radix Dipsaci functions by inhibiting osteoclast differentiation, preventing osteoporosis and promoting fracture healing[16–18]. In addition, Radix Dipsaci can upregulate the expression of TGF-β1, increase collagen formation and promote Achilles tendon healing[19]. It has not yet been reported that ASA VI can promote the differentiation of stem cells into nucleus pulposus-like cells.

Numerous signaling pathways are regulated by the function of ASA VI, including PI3K/AKT, HIF-1α/VEGF, P38, and ERK1/2[20–22]. The mechanism by which ASA VI promotes the differentiation of stem cells into nucleus pulposus-like cells remains to be further studied. In this study, we evaluated how ASA VI affects the proliferation of stem cells and its possible potential mechanism of promoting the differentiation of HMSCs into nucleus pulposus-like cells.

**Materials And Methods**

**Materials**

ASA VI was purchased from Chengdu Must Bio-Technology Co. Ltd. (purity > 99%, China). Mesenchymal Stem Cell medium (MSCm) (Sciencell, 7501, USA), fetal bovine serum (FBS, Sciencell, 7552, USA) and dulbecco's phosphate buffered saline (DPBS, Sciencell, 0303, USA) were purchased from Sciencell. DMEM/F12 (Gibco, 21041025, USA) was purchased from Gibco. BeyoClick EdU-488 (Beyotime, C0071S,
China) was purchased from Beyotime Institute of Bio-Technology Co. Ltd. ProtoScript II cDNA (NEB, m3003L, USA) was purchased from NEB. DAPI (Sigma-Aldrich, D9542, USA), dimethylmethylene blue (Sigma-Aldrich, 341088, USA), glycine (Sigma-Aldrich, 410225, USA), glacial acetic acid (Sigma-Aldrich, S7653, USA) and bovine chondroitin 4-sulfate as standard (Sigma-Aldrich, C9819, USA) were purchased from Sigma-Aldrich. Primary antibodies for β-catenin (abcam, ab179467, USA) and paxillin1 (PAX1,abcam, ab32084, USA) were purchased from abcam. Aggrecan (Proteintech, 13880-1-AP, USA) was purchased from Proteintech, and Smad2/3 (Cell Signaling, 8685T, USA), phospho-Smad2/3 (Cell Signaling, 8828S, USA), ERK1/2 (Cell Signaling, 4695T, USA), and phospho-Erk1/2 (Cell Signaling, 4370T, USA) were purchased from Cell Signaling. Anti-rabbit secondary antibodies (abcam, ab150077, USA) were purchased from abcam.

**Cell culture**

Human mesenchymal stem cells (HMSCs) (Sciencell, 7500, USA) were purchased from Sciencell. Primary HMSCs were obtained by digestion. The cell line was cultured in mesenchymal stem cell medium (MSCm), supplemented with 10% FBS, 1% penicillin and 1% streptomycin at 37°C in a humidified atmosphere of 5% CO₂ in a T-75 flask for 48 hours before the first medium change. At 80% confluence, the cells were trypsinized (0.25% trypsin-EDTA) and passaged into T-25 flasks at a ratio of 1:3. HMSCs from the sixth passage were used in all experiments. For all subsequent experiments except the cell vitality and proliferation assays, the cultured medium was replaced with DMEM/F12 supplemented with FBS (Invitrogen, 1600044, USA), dexamethasone (Sigma-Aldrich, D1756, USA), ascorbic acid-2-phosphate (Sigma-Aldrich, A4544, USA) L-proline (Sigma-Aldrich, P0380, USA), ITS Supplement (Cyagen Biosciences Company, 10201, USA), and TGF-β1 (PeproTech, 96-100-21-10, USA).

**Assessment of cellular vitality**

Cell vitality was evaluated by cell counting XTT assays (Sigma-Aldrich, X4626, USA) according to the manufacturer’s instructions. In brief, HMSCs were seeded on 96-well plates (2 × 10^3 cells/well) at 37°C in a humidified atmosphere of 5% CO₂ for 24 hours. Then, 50 µl of XTT testing work fluid was added to each well, and the plates were cultured at 37°C in a humidified atmosphere of 5% CO₂ for 4 hours. After that, cells were treated with different concentrations of ASA VI (0, 0.01, 0.1, 1, 10, and 100 mg/l), the MSCm and ASA VI were changed every 72 hours, 50 µl of XTT test working solution was added to each well, and the plates were cultured at 37 °C in a humidified atmosphere of 5% CO₂ for 4 hours after 1, 3 and 5 days. The absorbance was measured at 450 nm using a microplate reader (Bio Tek, USA) after each addition of XTT test working solution.

**Assessment of cellular proliferation**

Cell proliferation was assessed by an EDU test using a BeyoClick EdU-488 cell proliferation kit combined with DAPI staining according to the manufacturer’s instructions. The HMSCs were plated on 96-well plates (2 × 10^3 cells/well) at 37°C in a humidified atmosphere of 5% CO₂ for 24 hours. Then, different doses of ASA VI (0, 0.01, 0.1, and 1 mg/L) were added to the wells, and the MSCm and ASA VI were
changed every 72 hours. Five days later, the EDU reagent (0.5 µL, 50 µmol/L) was added into each well containing 200 µL of MSCm and incubated at 37°C in a humidified atmosphere of 5% CO₂ for 2 hours. Then, cells were fixed with 4% paraformaldehyde and incubated with amino acetic acid for 5 minutes. After that, 100 µL of penetrant was added to each well and incubated for 5 minutes. Then, each well was supplemented with 100 µL of 1xEDU work fluid, and 30 minutes later, DAPI was used to stain cell nuclei for 10 minutes[23]. The stained cells were observed under a fluorescence microscope (OLYMPUS, USA).

**Quantitative Real-time Polymerase Chain Reaction**

The HMSCs were plated on 6-well dishes. Then, cells were treated with different concentrations of ASA VI (0, 0.01, 0.1, 1, 10, and 100 mg/L). The mixture was changed every 72 hours. Total RNA was extracted from cells after 3 and 7 days using TRIzol reagent (INV, 15596026, USA) according to the manufacturer’s instructions. Then, cDNA was synthesized by applying a cDNA reverse transcription kit according to the manufacturer’s instructions. In brief, first, an 8-µL reaction mixture containing 1 µg of total RNA, 2 µL of oligo d(T)23VN(50 µM) and RNase-free dH₂O was incubated at 65 °C for 5 minutes. Subsequently, 10 µL of ProtoScript II Reaction Mix (2X) and 2 µL of Proto-Script II Enzyme Mix (10X) were added to a final volume of 20 µL, and the mixture was incubated at 42 °C for 60 minutes. Finally, the mixture was incubated at 80 °C for 5 minutes. Gene expression was analyzed by quantitative real-time polymerase chain reaction (PCR, ABI Stepone Plus, USA). GAPDH was used to quantify PCR products and to confirm the use of equivalent RNA. Reactions were carried out in duplicate in a 96-well plate with a final volume of 20 µL. The PCR included an initial enzyme activation stage at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Products were quantified using a melting curve analysis. Results were calculated using the 2⁻ΔΔct method. The primers used in this study are shown in table 1.

**Glycosaminoglycans (GAGs) assay**

The HMSCs were plated on 6-well plates. After 24 hours, cells were treated with the appropriate concentration of ASA VI (1 mg/L), while cells in the control group were not treated with ASA VI. The medium was changed every 72 hours. To investigate the effect of ASA VI on the secreted ECM proteins, a dimethylmethylene blue assay (DMMB) was used to quantify the soluble GAGs in the cell culture media. After 7 and 14 days, the cell culture media were collected. A portion of the media was mixed with DMMB dye and incubated with moderate agitation at room temperature for 30 minutes. Upon incubation, the solution was centrifuged to form a pellet of GAGs that bound to the dye. The pellet was washed in ice-cold acid-salt solution, centrifuged and resuspended in 10% SDS for the DMMB assay. The DMMB assay was quantified at 525 nm using a microplate reader (Bio Tek, USA). Absorbance was converted to GAG concentrations using a calibration curve obtained using different concentrations of bovine chondroitin 4-sulfate as the standard. The GAGs were normalized using the total protein in the media quantified at UV 280 nm using a microplate reader (Bio Tek, USA)[24].
The HMSCs were plated on 96-well plates (2 × 10^3 cells/well) at 37°C in a humidified atmosphere of 5% CO₂ for 24 hours. Then, cells were treated with the appropriate concentration of ASA VI (1 mg/L), while control cells were not treated with ASA VI. The medium was changed every 72 hours. After 14 days, cells were fixed with 4% paraformaldehyde for 5 minutes. After that, each well was treated with 0.2% Triton X-100 in 1X PBS for 5 minutes at room temperature. Cells were then blocked with 5% blocking serum from specific species in 1X PBS at room temperature for 1 hour. Subsequently, cells were incubated with primary antibody (1:50) diluted in antibody dilution buffer for 1 hour at room temperature, followed by incubation with the corresponding fluorochrome-labeled secondary antibodies diluted in antibody dilution buffer (1:200) for 1 hour at room temperature in the dark. Finally, DAPI was used to stain cell nuclei for 10 minutes[25]. The stained cells were observed under a fluorescence microscope (OLYMPUS, USA).

**Western blotting analysis**

The HMSCs were plated on 6-well plates. After 24 hours, cells were treated with the appropriate concentration of ASA VI (1 mg/L), while control cells were not treated with ASA VI. After 48 hours, cells were washed with PBS and lysed with lysis buffer mixed with PMSF for 30 minutes on the ice, followed by ultrasonic fragmentation (400W, Ultrasound 15 seconds, stop 15 seconds) for 10 minutes on an ice bath. After centrifuging for 10 minutes at 10,000xg and 4 °C, the soluble was added with Loading Buffer (5:1) and Boiled in boiling water for 5 minutes. Protein samples were separated by 10% SDS-PAGE under 80 V for 30 minutes and 100 V for 90 minutes and then transferred to nitrocellulose membranes. The membranes were blocked with TBS buffer for 1 hour at room temperature. The primary antibodies (rabbit polyclonal anti-ERK1/2, anti-phosphospecific ERK1/2, anti-Smad2/3, anti-phosphospecific Smad2/3, 1/500 dilution; rabbit monoclonal anti-β-actin, 1/5000 dilution) were added to the nitrocellulose membranes and incubated overnight at 4 °C. Subsequently, the membranes were washed thrice for 5 minutes each with TBS buffer and incubated with anti-rabbit secondary antibodies (1:5000) for 1 hour at room temperature. The membranes were washed thrice for 5 minutes each again, and detection was performed using a dual-color infrared imaging system (Odyssey, LI-COR, USA).

**Statistical analysis**

The results are expressed as the mean ± standard deviation. Statistical significance was determined using a one-way ANOVA test and a T-test to compare the different groups in SPSS 19.0 statistical software. For each test, at least three independent parallel experiments were performed. P < 0.05 was considered to be statistically significant.

**Results**

**Effect of ASA VI on HMSC proliferation**

The effect of ASA VI on the cell proliferation of HMSCs was evaluated by the XTT and EDU assays. Cell numbers were increased after incubation with different concentrations of ASA VI (0.01, 0.1, 1, 10, and 100 mg/L) for 1, 3 and 5 days and peaked at 1 mg/mL (Fig. 1A). The EDU assay results showed that the
proliferation of HMSCs can be enhanced with the increase of ASA VI dose (Fig. 1B and 1C), which was similar to the XTT results.

**Nucleus Pulposus Cells Gene Expression under Different Concentrations of ASA VI**

To study the effects of different concentrations of ASA VI on the biosynthesis of HMSCs, ECM expression was analyzed by RT-PCR. The gene expression profiles were investigated after 3 and 7 days of ASA VI cultivation at 0 (control), 0.01, 0.1, 1, 10 and 100 mg/L. Figure 2 shows the relative gene expression of the nucleus pulposus markers (type II collagen, aggrecan, SOX9, KRT19, and PAX1) for the ASA VI and control groups. The results showed that the concentrations of 0.01, 0.1 and 1 mg/l ASA VI upregulated the gene expression levels of the markers, which peaked at 1 mg/L. However, when the concentration of ASA VI increased to 10 mg/l and 100 mg/l, the gene expression did not upregulate with the increased concentration and even appeared to be inhibited.

These findings indicated that 1 mg/L was the optimal concentration of ASA VI for stimulating HMSCs differentiation into nucleus pulposus like-cells. Thus, we adopted this concentration for the subsequent experiments.

**GAGs expression under ASA VI**

GAGs expression levels were investigated after 7 and 14 days of ASA VI cultivation at 0 (control) and 1 mg/l. Figure 3 shows that the GAGs contents in the cell supernatants significantly increased with time in the experimental and control groups. The increase in the rate of GAGs contents in the cell supernatant of the experimental group was higher than that of the control group at 7 and 14 days.

**ASA VI accelerated aggrecan and PAX1 deposition**

Aggrecan and PAX1 immunofluorescence staining revealed stronger green staining in ASA VI (1 mg/l)-treated groups compared with the control groups after being cultured for 14 days, which suggests that there was more abundant regenerated aggrecan and PAX1 deposition (Fig. 4).

**ASA VI upregulated P-ERK1/2 and P-Smad2/3 expression**

To investigate the mechanism of action of ASA VI in promoting the differentiation of HMSCs into nucleus pulposus like-cells, we explored the effect of ASA VI on P-Smad2/3 and P-ERK1/2 expression using Western blotting. The results indicated that ASA VI (1 mg/l) could better upregulate both P-Smad2/3 and P-ERK1/2 protein levels than in the control group (Fig. 5).

**Discussion**
Due to the increasing aging of the population, the incidence of degenerative IVD is becoming higher and higher, and the treatment of degenerative IVD has received wide attention. Compared with traditional therapies, biotherapy may be more beneficial to relieve pain, repair degenerative nucleus pulposus and restore the biomechanical function of IVD[26]. Stem cells with high-efficiency self-renewal and pluripotency can be differentiated into various cell lines, including cartilage cell-like cells and nucleus pulposus-like cells. Therefore, inducing stem cells to differentiate into nucleus pulposus-like cells for IVDD has become the focus of biotherapy for IVDD. How to induce stem cells to differentiate into nucleus pulposus phenotype more effectively becomes a key problem in the treatment of IVDD[27]. Promoting the phenotypic differentiation of stem cells into nucleus pulposus cells is the basis of nucleus pulposus regeneration.

At present, there is no specific cell phenotype to identify nucleus pulposus cells. By comparing the phenotypes of cartilage cells and nucleus pulposus cells, it was found that type II collagen, aggrecan and SOX9 expression was shared by nucleus pulposus cells and cartilage cells[28, 29]. However, nucleus pulposus cells are significantly different from chondrocyte cells in terms of composition and biological function. Thus, to ensure the accumulation of proper ECM, it is necessary to identify the cell phenotype of differentiated cells. KRT19 as a specific marker of human chordae was used to identify positive markers in nucleus pulposus cells[30]. Thorpe AA et al have found that KRT19 can be used as a unique gene for the identification of nucleus pulposus cells[31]. PAX1 is involved in the regulation of IVD formation in the embryonic stage and has been verified in human nucleus pulposus cells. Moreover, it is widely used as a new positive phenotype for the identification of nucleus pulposus cells in the study of stem cell differentiation into nucleus pulposus[32]. Thus, genes such as type II collagen, aggrecan, SOX9, KRT19 and PAX1 can be used as a genetic phenotype to identify nucleus pulposus cells.

As the main bioactive component of the Radix Dipsaci, previous studies have shown that ASA VI has the effects of protecting the nerves, heart, and liver and resisting osteoporosis[33]. In recent years, it has been reported that ASA VI has the effects of regulating intestinal microflora, preventing atherosclerosis, resisting inflammation, reducing cortisol, and promoting angiogenesis and wound healing[20, 34–37]. In this study, we found that compared with the control group, ASA VI had more potential to promote differentiation of HMSCs into nucleus pulposus-like cells, especially when the concentration of ASA VI was 1 mg/L. To better understand the possible mechanism of ASA VI promoting differentiation of HMSCs into nucleus pulposus-like cells, we studied its effects on the ERK1/2 and Smad2/3 signaling pathways. Previous studies have found that the ERK and Smad signaling pathways are involved in the proliferation and differentiation of stem cells[38–40]. Moreover, in recent years, it has been found that the ERK1/2 and Smad2/3 signaling pathways regulate the differentiation of stem cells into nucleus pulposus-like cells and cartilage cell-like cells[41–42]. In this study, we found that ASA VI may promote the differentiation of HMSCs into nucleus pulposus cells by upregulation of P-ERK1/2 and P-smad2/3. However, the exact mechanism remains to be further verified.

Conclusions
We found that ASA VI promoted the differentiation of HMSCs into nucleus pulposus-like cells, probably by activating ERK1/2 and Smad2/3 signaling pathways. Our research increases our understanding of the potential mechanism of ASA VI promoting the differentiation of HMSCs into nucleus pulposus-like cells and suggests that ASA VI has therapeutic potential in the treatment of IVDD with stem cells.

**Abbreviations**

IVD  
intervertebral disc  
IVDD  
intervertebral disc degeneration  
ASA VI  
Asperosaponin VI  
TCM  
traditional Chinese medicine  
HMSC  
human mesenchymal stem cell  
XTT  
2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2 \( \text{H} \)-tetrazolium-5-carboxanilide  
EDU  
5-ethynyl-2-deoxyuridine  
COL2A1  
type II collagen  
SOX9  
sry-related high mobility group-box gene9  
PAX1  
paxillin1  
KRT19  
cytokeratin19  
GAGs  
glycosaminoglycans  
ERK  
extracellular regulated protein kinases  
Smad  
small mothers against decapentaplegic  
LBP  
Low back pain  
ECM  
extracellular matrix  
IVD
intervertebral disc
PI3K
phosphatidylinositol 3 kinase
AKT
protein kinase B
HIF-1a
hypoxia-inducible factor 1-alpha
VEGF
vascular endothelial growth factor
P38
protein 38
MSCm
Mesenchymal Stem Cell medium
FBS
fetal bovine serum
PBS
phosphate buffered saline
DPBS
dulbecco's phosphate buffered saline
DMEM
dulbecco's modification of eagle's medium
DAPI
4,6-diamino-2-phenyl indole
EDTA
ethylene diamine tetraacetic acid
TGF-β1
transforming growth factor-beta 1
PCR
polymerase chain reaction
RNA
ribonucleic acid
DNA
deoxyribonucleic acid
GAPDH
glyceraldehyde-3-phosphate dehydrogenase
DMMB
dimethylmethylene blue assay
PMSF
phenylmethanesulfonyl fluoride
SDS
sodium dodecyl sulfate
SDS-PAGE
sodium dodecyl sulfate -polyacrylamide gel electrophoresis

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

All authors have provided consent for publication in the Journal of Chinese Medicine.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

YN conducted the study and drafted the manuscript. LX supervised the study and revised the manuscript. RD and XZ provided the technical support and advices for the study. all authors read and approved the final manuscript.
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Tables

Table 1.

| Name     | 5→sequence→3                  | Product size | NCBI Reference Sequences(Ref Seq) |
|----------|-------------------------------|--------------|----------------------------------|
| GAPDH    | Sense: CCAGAACATCAT CCCTGCCT  | 185          | NM_00125679                      |
|          | Antisense: CCTGCTTCACCA CCTTCTTG |              |                                  |
| COL2A1   | Sense: TCCACGGAAGGC TCCCAGAA | 141          | NM_001844.5                      |
|          | Antisense: CCTGCTATTGCC CTCTGCC |              |                                  |
| Aggrecan | Sense: CCTCTGGACAAC CAGGTATTAG | 97           | NM_001135                        |
|          | Antisense: CCAGATGTTTCT CCACTCAGAT |          |                                  |
| SOX9     | Sense: GAGCTGAGCAG CGACGTCAT  | 130          | NM_000346.4                      |
|          | Antisense: CGTAGCTGCCCG TGTAGGTG |          |                                  |
| KRT19    | Sense: GGAAGACACACT GGCAGAAA   | 112          | NM_002276.5                      |
|          | Antisense: CTCACTATCAGC TCGCACATC |          |                                  |
| PAX1     | Sense: CCGCTCGCTATG GAGCAGAC  | 204          | NM_001257096.1                   |
|          | Antisense: GGAGCCGGTCT CGTTGTAGC |          |                                  |

Figures
ASA VI promoted the proliferation of HMSCs. (Figure 1A) Cell viability results of HMSCs treated with different concentrations of ASA VI for 1, 3 and 5 days. (Figure 1B) EDU staining test of HMSCs after being treated with different concentrations of ASA VI (scale bar: 200 µm). (Figure 1C) The relative number of HMSCs stained by EDU in each group. *p<0.05 vs the controls, **p<0.01 vs the controls.
Figure 2

Effects of different concentrations of ASA VI on gene expression. The expression levels of selected genes were assessed by RT-PCR after cells were treated with different concentrations of ASA VI for 3 and 7 days. *P<0.05 vs the controls, **p<0.01 vs the controls.
Figure 3

Effect of ASA VI on the amount of GAGs in the culture media. Treatment with ASA VI (1 mg/l) showed higher amounts of secreted GAGs in the culture media. **p<0.01 vs the controls.
The expression levels of aggrecan and PAX1 genes were verified by immuno-staining. The immunofluorescence signals were stronger (green staining) compared with the controls (scale bar: 200 µm).

Figure 4
Figure 5

The effects of ASA VI on the protein expression levels of P-ERK1/2 and P-Smad2/3. Western blot analysis of P-ERK1/2 and P-Smad2/3 expression levels after treatment with ASA VI for 48 hours. P-ERK1/2 and P-Smad2/3 were normalized to ERK1/2 and Smad2/3.** p<0.01 vs the controls.