Treatment of rabbit intervertebral disc degeneration with co-transfection by adeno-associated virus-mediated SOX9 and osteogenic protein-1 double genes *in vivo*

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Abstract. Degeneration of the lumbar intervertebral disc is a common cause of low back pain and leg pain that affects the physical and mental health of the patient and increases the social burden. This study was performed to observe the biological effects of adeno-associated virus (AAV)-mediated osteogenic protein-1 (OP1) and SOX9 double gene co-transfection in rabbit intervertebral disc degeneration *in vivo*. The animals were randomly grouped into models of disc degeneration. After injecting 20 μl of double-gene mixed solution, OP1, SOX9, enhanced green fluorescent protein (EGFP) and PBS buffer into the disc of each group, X-ray analysis, magnetic resonance imaging (MRI), reverse transcription PCR (RT-PCR) and western blotting were performed on the 3rd, 6th and 9th week of surgery. On the 3rd, 6th and 9th week of the transfection, X-ray and MRI showed that the intervertebral height and T2-weighted signal intensity were restored significantly in groups A, B and C, whereas significant differences in intervertebral space and T2-weighted signal intensity were observed between group A and groups B and C ($P<0.05$). RT-PCR and western blotting showed that the expression of type II collagen and proteoglycan mRNA was upregulated in groups A, B and C. The expression in group A was significantly higher than that in the other groups ($P<0.05$). Recombinant AAV-mediated SOX9 and OP1 double-gene transfection significantly ameliorated the height of the degenerative intervertebral disc and significantly promoted the high expression of degenerative disc proteoglycan and type II collagen. It can therefore be concluded that dual-gene therapy has a synergistic effect.

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Introduction

Low back pain and leg pain caused by degenerative intervertebral disc are major problems in clinical orthopaedics. Although traditional surgery or non-surgical treatment can alleviate the clinical symptoms to some extent, they cannot fundamentally correct or prevent disc degeneration. With in-depth studies on intervertebral disc cells and developments in molecular biology, the delay and reversal of intervertabral disc degeneration have become the main objectives in orthopaedics, in which biological treatment of the degenerative disc, including cell therapy, molecular therapy and gene therapy, has been proposed (1). Previous studies (2) have focused on gene therapy of intervertebral disc degeneration. The gene is used to transfect the targeted gene, which activates the degenerative disc cells to continue their biological effects and increases the synthesis of the main matrix components of the disc (type II collagen and proteoglycan) for early prevention or reversal of disc degeneration.

In 1997, Wehling *et al* (2) proposed the idea of using a modified gene to reverse disc degeneration and successfully transfected the target gene into the *in vitro*-cultivated cartilage cells of bovine cartilage endplate. Sai *et al* (3) used transforming growth factor β1 (TGFβ1) and TGFβ3 as the targeted genes, adenovirus as the carrier and transfected rabbit nucleus pulposus cells. Their findings revealed that the synthesis of type II collagen and proteoglycan increased. Wallach *et al* (4) successfully constructed the tissue inhibitor of matrix metalloproteinase (TIMP)-carrying recombinant adenoviral vector (Ad-TIMP) and transfected the human degenerative disc cells *in vitro*, increasing proteoglycan synthesis.

Although the experimental studies of single-gene-transfected disc cells achieved good results, a large number of viral vectors were required, which increase the cytotoxic risks and immune response (5). Some investigators combined two or more factors for the transfection of intervertebral disc cells to reduce the amount of viral vectors and produce better biological effects. Xi *et al* (6) constructed adeno-associated virus (AAV)-hVEGF165 and AAV-TGFβ1, and transfected intervertebral disc cells with a single gene and combined genes. Their results showed that the expression level of annulus fibrosis type II collagen, which was transfected by the AV-hVEGF165 and

AAV-TGFβ1 double genes, was significantly higher than with transfection with a single gene, such as hVEGF165 or TGFβ1. Wallach *et al* (7) combined TGFβ1, bone morphogenetic protein-2 (BMP2) and insulin-like growth factor 1 (IGF1). This combination was transfected in disc cells, and their results showed that a three-factor-combination significantly increases the biological effects compared with only one or two factors combined.

Previous studies (14,18) confirmed that SOX9 or osteogenic protein-1 (OP1) single-gene therapy delays disc degeneration. However, their combined therapy for the treatment of intervertebral disc degeneration has not yet been reported.

In this experiment, AAV-mediated OP1 and SOX9 double-gene co-transfection was applied to transfect the rabbit degenerative intervertebral disc. OP1 was used to promote the synthesis of the matrix components of nucleus pulposus cells and to upregulate SOX9 expression. SOX9 was used for its matrix component-promoting function to observe the effects on rabbit intervertebral disc degeneration.

Materials and methods

Construction of vector and reagents. Recombinant AAV (rAAV)-OP1, rAAV-SOX9 and rAAV-EGFP were provided by Biowit Technologies Co., Ltd. (Shenzhen, China). The recombinant AAV vector was diluted with phosphate buffer to a concentration of $5x10^5$ plaque-forming units (PFU) per 1 μl.

The other reagents used in this study included rabbit anti-collagen II, rabbit anti-proteoglycan and goat anti-rabbit antibodies from Bioss (Beijing, China); TRIzol A+ reagent from Tiangen (Beijing, China); and reverse transcription-PCR kit from Takara Inc. (Dalian, China).

Animals. Thirty healthy adult New Zealand white rabbits (provided by the Experimental Animal Centre of Shandong Province) were used, without any limitation of gender, with an average age of 4 months and an average weight of 2.5 kg. The rabbits with congenital spine deformity and disc disease were excluded through magnetic resonance imaging (MRI). Adaptive feeding was performed for 4 weeks, and then feeding was prohibited the day prior to the surgery. The animals were anaesthetised with a mixture of ketamine and chlorpromazine (1:1) through intramuscular injection. Following anaesthesia, the right side of the lower back was shaved, and the skin (20x15 cm) was prepared for future experimental use. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Qingdao Medical University.

Animal modelling methods. Subsequent to anaesthesia, the animal was placed in a lateral position, and the four limbs were fixed. A vertical incision of ~2 cm was made from the iliac crest to the 12th rib margin via the right extraperitoneal approach. The peritoneum was bluntly dissected before directly reaching the anterolateral position of the lumbar transverse process, exposing the transverse processes of L3, L4, L5 and L6. Based on the iliac crest position (parallel to the L6 vertebral plane) and the exposed L3/4, L4/5 and L5/6 intervertebral discs, a 24-gauge needle was used to pierce into the intervertebral disc centre from the side front but parallel to the upper and lower endplates. The piercing depth was controlled at ~5 mm and continued for 5 sec, then the needle was removed and the opening was sutured (8). Penicillin (800,000 units) was intramuscularly injected pre- and post-operatively. Post-operative bleeding was observed under natural standard conditions. No incidences of infection or death were reported.

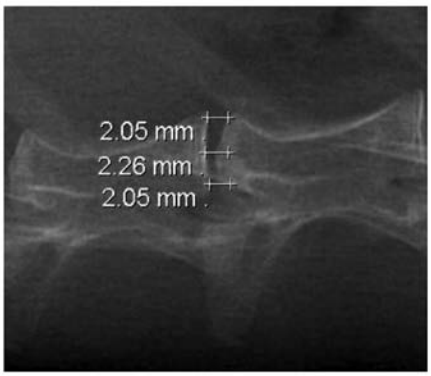

Figure 1. Computed radiography (CR) lateral intervertebral height measured with three midline methods.

Grouping and gene transfection. The 30 successfully modelled experimental animals were randomly divided into groups A, B, C, D and E. We injected 20 μl of rAAV-OP1 and rAAV-SOX9 mixture (1:1) (the virus titre as $5x10^5$ PFU), 20 μl of rAAV-OP1, 20 μl of rAAV-SOX9, 20 μl of rAAV-EGFP and 20 μl of PBS into each group, respectively. At the 4th week of modelling, post-operative freedom of movement and eating was allowed.

X-ray. The spine computed tomography (CT) examination was performed on the 3rd, 6th and 9th week of the surgery towards each rabbit. Attention was given to ensure that the animals were in the same depth of anaesthesia. The CT scan parameters were 50 kV, 100 mA and 50 msec. The 'three midline' approach was adopted to measure the intervertebral height of the experimental animals. Centricity DICOM viewer 3.1.1 was used to enlarge all images four times. The disc height of each surgical section ($L_{3,4}$, $L_{4,5}$ and $L_{5,6}$) was measured (Fig. 1).

MRI. T2-weighted signal strength in MRI is a sensitive indicator of the degree of degeneration in clinical imaging. MRI was performed on the experimental animals on the 3rd, 6th and 9th week of the second surgery for the T2-weighted signal strength of each test disc. All the animals were intramuscularly injected with a mixture of ketamine and chlorpromazine (1:1) for anaesthesia. A Siemens Avanto 3.0T medical superconductive MRI scanner was used to scan the sagittal lumbar spine of animals with a cervical coil. The parameters of the scanner were: T2-weighted sequence repetition time, 2,000-6,000 msec; echo time, 80-120 msec; bandwidth, 25 Hz; microwave, 16; matrix 384X224; Nex = 4; field of vision, 16x16 cm; thickness, 3 mm; and spacing, 0.2 mm. The degenerative disc mainly emitted a low signal, i.e., T2-weighted signal intensity decreased and the disc became thinner. Based on the changes in the T2-weighted signal, the improved Thompson classification method was

Table I. Specific primer sequences.

Primer name	Primer sequence (5'-3')
Upstream rabbit aggrecan	GACCGAGGTCAGTGGATTGT
Downstream rabbit aggrecan	CCAGGTCATTTATTCTGTGT
Upstream rabbit type II collagen	GGCTCCCAGAACATCACCTA
Downstream rabbit type II collagen	GATGACAGTCTTGCCCCACT
Upstream β-actin	TCCCTGGAGAAGAGCTACGA
Downstream β-actin	GTGTTGGCGTACAGGTCCTT

Table II. Intervertebral height of each group at each time point.

Time	A	B	C	D	E
3W	1.21±0.06	1.10±0.03	1.13±0.10	1.09±0.01	1.06±0.01
6W	1.42±0.09	1.18±0.05	1.19±0.07	1.05±0.02	1.03±0.01
9W	1.63±0.10	1.31±0.05	1.36±0.09	0.99±0.03	0.95±0.04

Compared with single gene groups, respectively, dual-gene group enhanced the intervertebral height significantly (P<0.05). However, the intervertebral height showed no increase in D and E group (P>0.05). W, week.

used as a standard. The MRI data were blindly assessed by senior radiologists, and the disc T2-weighted signals were divided into four levels.

RT-PCR. At the 3rd, 6th and 9th week of the transfection, the purity and concentration of disc tissue for TRIzol extraction of the total RNA of each group were measured. The RT kit was used for reverse transcription. PCR was used to amplify type II collagen, proteoglycans and internal reference β-actin. The PCR products were separated by 1.5% agarose gel electrophoresis, observed and then photographed. The Quantity One gel image analysis system was used for statistical analysis. All the experimental primers were prepared following the primer design principles. These primers were identified as the specific primers according to the basic local alignment research tool (Table I).

Western blotting. The collected degenerative intervertebral disc of each group was added into the cell lysate to lyse the cells completely and was centrifuged at 12,000 rpm for 10 min. After adding 100 μl of supernatant into 20 μl of sample buffer of 6X sodium dodecyl sulphate (SDS), the mixture was degenerated in a boiling water bath for 10 min. Then, the mixture was electrophoresis-isolated with 10 and 8% SDS-polyacrylamide gels. The products were transferred to nitrocellulose and blocked with blocking solution for 30 min. The primary antibody (anti-proteoglycan, 1:500 and anti-type II collagen, 1:500) and secondary antibody (anti-rabbit IgG) were added at 37˚C for hybridization and diaminobenzidine staining. A digital gel imager ChemiImager 4000 was used to scan the stripes, with Quantity One v4.62 software to analyse the striped grey values.

Statistical analysis. Data are presented as mean ± standard deviation, using the SPSS 19.0 statistical package. The data between groups were compared using one-way ANOVA. Pairwise comparisons were analysed by the least significant difference method. MRI data were analysed by the hierarchical rank-sum test. P<0.05 was considered to indicate statistical significance.

Results

X-ray. After modelling, the lumbar spine X-ray revealed that the post-operative rabbit intervertebral space narrowed, with bone sclerosis under the endplate and vertebral bone hyperplasia. Different treatment methods and post-operative time

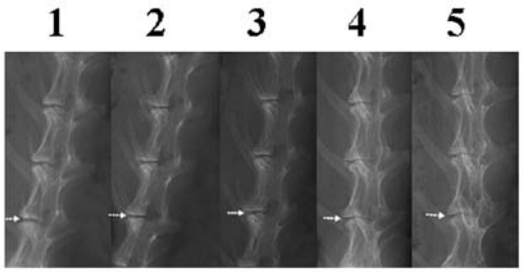

Figure 2. Lateral lumbar spine computed radiography (CR) results of each group at the 9th week. Lane 1, PBS group; lane 2, SOX9 group; lane 3, OP1 group; lane 4, double gene group; lane 5, EGFP group. OP1, osteogenic protein-1; EGFP, enhanced green fluorescent protein.

periods affected the disc height, with statistically significant differences among the single gene, double genome and vacant virus groups (P<0.05). A statistically significant difference was observed between the single- and dual-gene groups (P<0.05). By contrast, the difference between PBS and the vacant virus group was not statistically significant (P>0.05), indicating that the needlestick injuries caused intervertebral disc degeneration and appeared as a reduction in intervertebral height. The double- and single-gene injections restored the intervertebral height. Thus, the recovery effects of the double-gene group were better than those of the single-gene group (Fig. 2, Table II).

MRI. The preoperative MRI T2-weighted images showed that the T2-weighted signal of each disc of the experimental animals was uniform and high (Fig. 3), with no signs of degeneration. At post-operative 3rd, 6th and 9th weeks, the T2-weighted signal intensity and intervertebral disc height of the double-gene group were not significantly different from their pre-operative values, showing uniform high intensity signals. The T2-weighted signal intensities of the rAAV-OP1 and r-SOX9 groups were moderate, whereas the T2-weighted signal intensity decreased in the control and vacant virus groups (Fig. 4).

The statistical results (Table III) show that at the 3rd, 6th and 9th weeks of the transfection, statistically significant differences were observed among the double-gene, vacant virus and PBS control groups (p1=0.000, <0.05). Statistically significant differences were also observed among the double-gene, SOX9 and OP1 groups (p2=0.007, p3=0.013, <0.05), whereas no significant difference was found between the SOX9 and OP1 groups (p4=0.308, >0.05). Statistically significant differences

Table III. MRI T2-weight signal intensity of each group at each time point.

Group	Pre-operative	Post-operative 3rd week	Post-operative 6th week	Post-operative 9th week
A	1.00±0.00	1.27±0.60	1.13±0.35	1.20±0.41
B	1.00±0.00	2.13±0.74	1.80±0.56	2.00±0.65
C	1.00±0.00	2.33±0.72	1.93±0.60	2.20±0.68
D	1.00±0.00	2.87±0.52	3.07±0.60	3.27±0.59
E	1.00±0.00	2.93±0.87	3.13±0.64	3.33±0.99

Compared with single-gene groups, respectively, the dual-gene group enhanced the MRI T2-weighted signal intensity significantly (P<0.05). However, the MRI T2-weighted signal intensity showed no increase in groups D and E (P>0.05). MRI, magnetic resonance imaging.

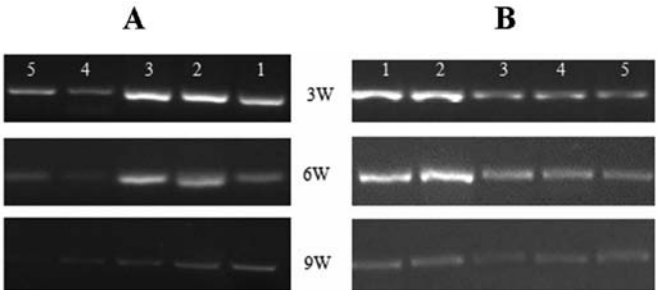

Figure 3. Expression of type II collagen mRNA and proteoglycan mRNA in each group at different time points. (A) Type II collagen mRNA. (B) Proteoglycan mRNA. Lane 1, PBS group; lane 2, SOX9 group; lane 3, OP1 group; lane 4, double gene group; lane 5, EGFP group. OP1, osteogenic protein-1; EGFP, enhanced green fluorescent protein. W, week.

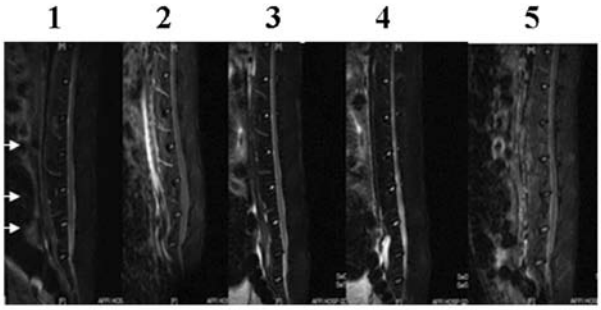

Figure 4. Lumbar magnetic resonance imaging (MRI) T2-weighted images of the experimental animals at the 3rd week. Lane 1, PBS group; lane 2, SOX9 group; lane 3, OP1 group; lane 4, dual-gene group; lane 5, EGFP group. OP1, osteogenic protein-1; EGFP, enhanced green fluorescent protein.

were found among the SOX9, OP1, EGFP and PBS groups (P<0.01), indicating that the needlestick injuries are capable of reducing the disc signal intensity in T2-weighted MRI, whereas the post-injury injection of SOX9, OP1 and AAV co-transfection vectors are able to improve the disc signal intensity of T2-weighted MRI. The recovery effects of the double-gene co-transfection group were better compared with those of the single-gene transfection group.

RT-PCR. At the post-operative 3rd, 6th and 9th weeks, the expression levels of type II collagen and proteoglycan mRNA in the double-gene group were significantly different from the other groups (P=0.000, <0.05). No statistically significant difference was noted in the expression of type II collagen between the SOX9 and OP1 groups (P=0.598, >0.05), whereas the expression of proteoglycan mRNA showed a significant difference (P=0.006, <0.05). These results indicate that OP1 and SOX9 double-gene co-transfection of the rabbit intervertebral disc degeneration are able to positively regulate the expression of type II collagen and proteoglycan mRNA, and delay or reverse disc degeneration. Dual-gene therapy had a synergistic effect towards disc tissue degeneration. The SOX9 gene significantly improved the expression of type II collagen mRNA but did not increase the expression of proteoglycan mRNA (Fig. 3, Table IV).

Western blotting. At the 3rd, 6th and 9th weeks of post-transfection, the electrophoresis results of type II collagen and proteoglycan expression showed statistically significant differences among the single-gene, double-gene and control groups (P<0.05). The expression levels of type II collagen and proteoglycan were not significantly different among the vacant virus and control groups (P>0.05). The single- and double-gene groups exhibited significantly statistical differences in the expression of type II collagen and proteoglycan (P<0.05). These results suggest that BMP7 and SOX9 are able to promote the synthesis of type II collagen and proteoglycan and that double-gene therapy can promote better the significant expression of type II collagen and proteoglycan compared with single-gene therapy (Fig. 5, Table V).

Discussion

Degeneration of the lumbar intervertebral disc is a common cause of low back pain and leg pain, seriously affecting the physical and mental health of the patient and increasing the social burden. Although conservative scientific treatments and surgical treatment strategies have been utilized towards the secondary disorders of lumbar disc degeneration, these measures cannot fundamentally retard or cease the process of disc degeneration. With the continuous development of molecular biological techniques, gene therapy is now the focus of intervertebral disc degeneration investigations with the aim to increase the synthesis of the main matrix components, type II collagen and proteoglycan, and to decrease their degradation in order that early prevention or reversal of disc degeneration can be achieved (9). Currently, commonly used genes

Table IV. Expression of type II collagen mRNA and proteoglycan mRNA of rabbit intervertebral disc degeneration in different groups at various time points.

	Type II collagen mRNA			Proteoglycan mRNA		
Group	Post-operative 3rd week	Post-operative 6th week	Post-operative 9th week	Post-operative 3rd week	Post-operative 6th week	Post-operative 9th week
A	2.62±0.52	3.36±0.20	2.13±0.11	2.63±0.48	3.73±0.23	2.20±0.10
B	1.35±0.11	2.07±0.10	1.03±0.05	1.70±0.09	2.61±0.27	1.52±0.13
C	1.66±0.94	2.11±0.10	1.06±0.07	0.88±0.07	0.64±0.08	0.63±0.11
D	0.86±0.07	0.54±0.14	0.32±0.29	0.81±0.05	0.57±0.04	0.35±0.01
E	0.86±0.08	0.50±0.08	0.29±0.31	0.83±0.06	0.55±0.09	0.31±0.03

Compared with single-gene groups, respectively, the dual-gene group enhanced the expression of collagen II mRNA and proteoglycan mRNA significantly (P<0.05), while the expression of collagen II mRNA and proteoglycan mRNA showed no increase in groups D and E (P>0.05). Compared with groups B and C, the expression of type II collagen mRNA did not exhibit a statistically significant difference (P=0.598, >0.05), while proteoglycan mRNA expression exhibited a significant difference (P=0.006, <0.05).

Table V. Type II collagen and proteoglycan expression results in each group at different time points.

	Type II collagen			Proteoglycan		
Group	Post-operative 3rd week	Post-operative 6th week	Post-operative 3rd week	Post-operative 6th week	Post-operative 3rd week	Post-operative 6th week
A	1.33±0.02	1.61±0.06	1.83±0.03	1.18±0.08	1.49±0.07	1.67±0.05
B	1.02±0.03	1.24±0.04	1.36±0.06	1.01±0.05	1.15±0.03	1.31±0.08
C	1.13±0.04	1.43±0.05	1.62±0.08	0.98±0.06	1.13±0.07	1.25±0.03
D	0.91±0.03	0.85±0.02	0.77±0.03	0.93±0.06	0.82±0.06	0.74±0.03
E	0.89±0.07	0.81±0.03	0.79±0.04	0.91±0.04	0.82±0.08	0.74±0.06

Compared with single-gene groups, respectively, the dual-gene group enhanced the expression of collagen II and proteoglycan significantly (P<0.05). However, the expression of collagen II and proteoglycan showed no increase in groups D and E (P>0.05).

include TGFβ (10), SOX9, TIMP (11), BMP and IGF1 (12). Related studies have shown that the combination therapy of two or more growth factors in the treatment of degenerative intervertebral disc cells is better than single-gene therapy because multi-gene co-transduction has stronger biological control towards nucleus pulposus cells (13). Although some studies have shown that individual genes such as SOX9 and OP1 are capable of relieving disc degeneration, SOX9 and OP1 double-gene co-transfection in the treatment of intervertebral disc degeneration has never been conducted.

BMP7, also known as OP1, belongs to the Dpp/Vgl subsets of the TGF superfamily. Given its significant promoting effect towards the anabolic cartilage, annulus and nucleus pulposus cells (14), BMP7 is preferred among the basic orthopaedic investigators examining gene therapy research for disc degeneration. OP1 functions in virtually all tissues and organs in animal growth and development. Its functions are widely related to embryology, evolutionology and pathology. BMP7 (OP1), as an important factor to promote bone fusion, has already been approved by the Food and Drug Administration and is widely used in clinical practice. Takegami *et al* (15) investigated the effects of BMP7 on the rabbit intervertebral disc. Masuda *et al* (16) found that recombinant human OP1 is

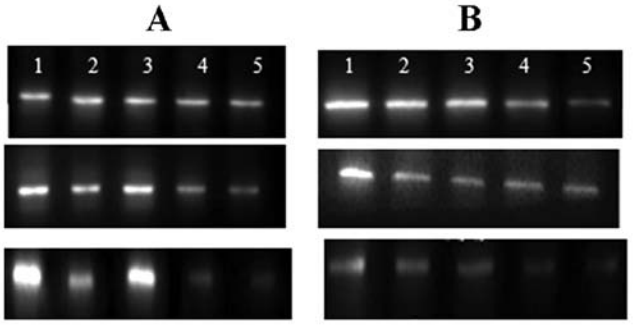

Figure 5. Type II collagen and proteoglycan expression in each group at different time points. (A) Collagen type II. (B) Polysaccharide. Lane 1, PBS group; lane 2, SOX9 group; lane 3, OP1 group; lane 4, double gene group; lane 5, EGFP group. OP1, osteogenic protein-1; EGFP, enhanced green fluorescent protein.

able to stimulate proteoglycan and type II collagen synthesis in a dose-dependent manner, increasing the expression levels of proteoglycan and type II collagen mRNA. Zhang *et al* (17) found that OP1 stimulates the synthesis of cattle annulus fibrosus cells, nucleus pulposus cells and proteoglycan in

the embryo, adult and mature stages. In the OP1 stimulation, they found that almost the same amount of proteoglycan is produced under the same conditions.

SOX9 is a crucial transcription factor in chondrogenesis that activates a series of signal transduction pathways to promote the expression of gene products. Studies found that SOX9 is closely associated with disc degeneration. RT-PCR, western blotting and immunological detection methods were applied to different time periods of disc nucleus cells. The expression of SOX9 was gradually reduced from newborns to patients with severe disc degeneration. The expression of type II collagen gene was also found to have the same decreasing tendency as SOX9 mRNA in nucleus pulposus cells, indicating that the occurrence of disc degeneration is associated with the reduction of SOX9 expression. Thus, SOX9 has an important function in the positive regulation of type II collagen synthesis (18). Paul *et al* (19) found that in *in vitro* experiments, the adenovirus-mediated SOX9 transfection of degenerative disc cells can increase intracellular SOX9 and type II collagen expression. Gruber *et al* (20) demonstrated that the SOX9 expression levels of intervertebral disc tissue are inversely proportional to age by immunolocalisation analysis. Lefebvre *et al* (21) investigated the positive regulation mechanism of SOX9 on type II collagen and found that a minimal DNA element of intron I of type II collagen gene (Col2a1) causes cartilage-specific cell expression in transgenic mice. This element is also a chondrocyte-specific enhancer in transient transfection experiments. The expression of Col2al is closely correlated with the high expression levels of SOX9 RNA and proteins in chondrocytes. The experiment confirmed that this minimal Co12 enhancer was a target sequence of SOX9. Shintani *et al* (22) showed that BMP7 positively regulates the gene expressions of critical factors in chondrogenesis, such as, SOX9 and type II collagen, in a dose-dependent manner.

In this study, the combined use of OP1 to promote the synthesis of matrix components in annulus fibrosus and nucleus pulposus cells, as well as to promote matrix component synthesis, were studied to reverse intervertebral disc degeneration. The adenovirus-mediated BMP7 and SOX9 double genes were transfected into rabbit intervertebral disc degeneration by different approaches. The expression and imaging conditions of type II collagen and proteoglycan were detected. The results show that the AAV-mediated double gene had obvious therapeutic effects on degenerative disc and was able to reverse disc degeneration. Its treatment was therefore better than that of single-gene therapy. Additionally, double-gene therapy has a synergistic effect. In conlusion, this study combined the specific regulation effect of SOX9 gene towards type II collagen; the upregulation effects of BMP7 on proteoglycan and type II collagen; the positive regulation function of SOX9 gene; and SOX9 and OP1, two genes with unidirectional positive regulation functions in animal degeneration experiments.

References

1. Evans C: Potential biologic therapies for the intervertebral disc. J Bone Joint Surg Am 88 (Suppl 2): 95-98, 2006.
2. Wehling P, Schulitz KP, Robbins PD, Evans CH and Reinecke JA: Transfer of genes to chondrocytic cells of the lumbar spine. Proposal for a treatment strategy of spinal disorders by local gene therapy. Spine (Phila Pa 1976) 22: 1092-1097, 1997.
3. Sai JM, Hu YG and Wang DC: Constructing adeno-associated virus-TGFbeta3 and comparing its biological effect on proteoglycan synthesis in dedifferentiated nucleus pulpous cells with adenovirus-TGFbeta1. Chin Med Sci J 22: 113-118, 2007.
4. Wallach C, Sobajima S, Watanabe Y, *et al*: Gene transfer of the catabolic inhibitor TIMP-1 increases measured proteoglycans in cells from degenerated human intervertebral discs. Spine (Phila Pa 1976) 28: 2331-2337, 2003.
5. Lohr F, Huang Q, Hu K, Dewhirst MW and Li CY: Systemic vector leakage and transgene expression by intratmorally injected recombinant adenovirus vectors. Clin Cancer Res 7: 3625-3628, 2001.
6. Xi YM, Dong YF, Wang ZJ, Liu Y, Diao ZZ and Hu YG: Co-transfection of adeno-associated virus-mediated human vascular endothelial growth factor 165 and transforming growth factor-β1 into annulus fibrosus cells of rabbit degenerative intervertebral discs. Genet Mol Res: Feb 28, 2013 (Epub ahead of print).
7. Wallach CJ, Gilbertson LG and Kang JD: Gene therapy applications for intervertebral disc degeneration. Spine (Phila Pa 1976) 28: 93-98, 2003.
8. Xi Y, Kong J, Liu Y, *et al*: Minimally invasive induction of an early lumbar disc degeneration model in rhesus monkeys. Spine (Phila Pa 1976) 38: E579-E586, 2013.
9. Nishida K, Suzuki T, Kakutani K, *et al*: Gene therapy approach for disc degeneration and associated spinal disorders. Eur Spine J 17 (Suppl 4): 459-466, 2008.
10. Nishida K, Kang JD, Gilbertson LG, *et al*: Modulation of the biologic activity of the rabbit intervertebral disc by gene therapy: an in vivo study of adenovirus-mediated transfer of the human transforming growth factor beta1 encoding gene. Spine (Phila Pa 1976) 24: 2419-2425, 1999.
11. Haro H, Grawford HC, Fingleton B, *et al*: Matrix metalloprotemase-3-dependent generation of a macrophage chemoattractant in model of herniated disc resorption. J Clin Incest 105: 133-141, 2000.
12. Osada R, Ohshima H, Ishihara H, *et al*: Autocrine/paracrine mechanism of insulin-like growth factor-1 secretion, and the effect of insulin-like growth factor-1 on proteoglycan synthesis in bovine intervertebral discs. J Orthop Res 14: 690-699, 1996.
13. Moon S, Nishirla K, Gilbertson LG, *et al*: Biologic response of human interverterbral disc cell to gene therapy cocktail. Spine (Phila Pa 1976) 33: 1850-1855, 2008.
14. Imai Y, Miyamoto K, An HS, Thonar EJ, Andersson GB and Masuda K: Recombinant human osteogenic protein-1 upregulates proteoglycan metabolism of human anulus fibrosus and nucleus pulposus cells. Spine (Phila Pa 1976) 32: 1303-1309, 2007.
15. Takegami K, Thonar EJ, An HS, Kamada H and Masuda K: Osteogentic protein-1 enhances matrix replenishment by intervertebral disc cells previously exposed to interleukin-1. Spine (Phila Pa 1976) 27: 1318-1325, 2002.
16. Masuda K, Imai Y, Okuma M, *et al*: Osteogenic protein-1 injection into a degenerated disc induces the restoration of disc height and structural changes in the rabbit anular puncture model. Spine (Phila Pa 1976) 31: 742-754, 2006.
17. Zhang Y, An HS, Song S, *et al*: Growth factor osteogenic protein-1: differing effects on cells fron three distinct zones in the bovine intervertebral disc. Am J Phys Med Rehabil 83: 515-521, 2004.
18. Sive JI, Baird P, Jeziorsk M, Watkins A, Hoyland JA and Freemont AJ: Expression of chondrocyte markers by cells of normal and degenerate intervertebral disc. Mol Pathol 55: 91-97, 2002.
19. Paul R, Haydon RC, Cheng HW, *et al*: Potential use of S0X9 gene therapy for intcrvcrtcbral degenerative disc disease. Spine (Phila Pa 1976) 28: 755-763, 2003.
20. Gruber HE, Norton HJ, Ingram JA and Hanley EN Jr: The S0 transcription factor in the human disc: decreased immunolocalization with ageand disc degeneration. Spine (Phila Pa 1976) 30: 625-630, 2005.
21. Lefebvre V, Huang W, Harley VR, Goodfellow PN and de Crombrugghe B: Sox9 is a potent activator of the chondrocyte-specific enhancer of the proalphal(II) collagen gene. Mol Cell Biol 17: 2336-2346, 1997.
22. Shintani N and Hunziker EB: Chondrogenic differentiation of bovine synovium: bone morphogenetic proteins 2 and 7 and transforming growth factor beta1 induce the formation of different types of cartilaginous tissue. Arthritis Rheum 56: 1869-1879, 2007.