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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

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ABSTRACT. Intervertebral disc degeneration is a common condition that may lead to low back pain and radiculopathy. Understanding the pathophysiology and cellular and molecular events of degenerative disc disease has resulted in the proposal of a gene therapy approach to halt and reverse disc degeneration. We explored the feasibility of reversing intervertebral disc degeneration using human vascular endothelial growth factor$_{165}$ (hVEGF$_{165}$) and transforming growth factor-β1 (TGF-β1) gene therapy. hVEGF$_{165}$ complementary DNA was obtained from pcDNA3(+)-hVEGF$_{165}$ and cloned into adeno-associated virus (AAV)-pSNAV plasmids to construct the recombinant plasmid, AAV-pSNAV-hVEGF$_{165}$. After identification through restriction enzyme digestion and DNA sequencing, the AAV-

pSNAV-hVEGF$_{165}$ was transfected into HEK293 cells and vascular endothelial cells. Protein expression of hVEGF$_{165}$ was detected using a fluorescent immunohistochemical assay, and the effect of hVEGF$_{165}$ on vascular endothelial cell proliferation was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Packaged AAV-hVEGF$_{165}$ and AAV-TGF-β1 were co-transfected into the annulus fibrosus cells of degenerative intervertebral discs. hVEGF$_{165}$ and TGF-β1 expression by annulus fibrosus cells and the effect of the co-transfection on the level of collagen type I protein expression by annulus fibrosus cells were detected with Western blot. The results of restriction enzyme digestion and DNA sequencing confirmed that AAV-pSNAV-hVEGF$_{165}$ plasmids were constructed. The fluorescent immunohistochemical results confirmed hVEGF$_{165}$ protein expression. The MTT results showed that the hVEGF$_{165}$ protein promoted vascular endothelial cell proliferation. Biologically active AAV-hVEGF$_{165}$ and AAV-TGF-β1 were successfully constructed. Western blot confirmed hVEGF$_{165}$ and TGF-β1 expression in annulus fibrosus cells and demonstrated that the level of collagen type I protein expression was significantly higher in annulus fibrosus cells co-transfected with both AAV-hVEGF$_{165}$ and AAV-TGF-β1 compared with that in cells transfected with AAV-hVEGF$_{165}$ or AAV-TGF-β1 alone. hVEGF$_{165}$ has a synergistic effect with TGF-β1 that promotes the expression of collagen type I protein in annulus fibrosus cells from degenerative intervertebral discs.

Key words: Human vascular endothelial growth factor$_{165}$; Degeneration; Transforming growth factor-β1; Intervertebral disc; Collagen type I

INTRODUCTION

Intervertebral disc degeneration is a major cause of back pain (Rubin, 2007). In Western countries, intervertebral disc degeneration-related low back pain affects between 60 and 80% of the population at some point during life, making it one of the most important public health issues today (Urban and Winlove, 2007; Pellise et al., 2009). Disk degeneration is characterized by progressive loss of proteoglycans, which causes subsequent disk dehydration, disappearance of the nucleus pulposus, and defects, or collapse, of the annulus fibrosus, leading to degenerative disc diseases that include disc herniation, radiculopathy, myelopathy, spinal stenosis, instability, and low back pain (Zhao et al., 2006; Richardson et al., 2007).

Current methods for treating degenerative disc diseases include conservative methods and surgical therapies. However, these approaches address only the clinical symptoms of intervertebral disc degeneration and do not target pathophysiological pathways involved in the degenerative process. Biological therapy, a newly developed treatment approach, can regulate disease progression at the cellular and molecular levels and may also restore disc function.

Several growth factors have been used to stimulate cell proliferation and matrix synthesis within degenerated discs. These growth factors include transforming growth factor-beta1

(TGF-β1) (Thompson et al., 1991), bone morphogenetic protein-2 (Li et al., 2003), osteogenic protein-1 (Takegami et al., 1999; Matsumoto et al., 2002), growth and differentiation factor-5 (Walsh et al., 2004; Chujo et al., 2006), insulin-like growth factor-1, epidermal growth factor, and fibroblast growth factor (Thompson et al., 1991). *In vitro* studies have demonstrated that growth factors can upregulate extracellular matrix synthesis by intervertebral disc cells. However, the half-lives of these growth factors are short, and their effects do not last for significant periods of time (Franceschi et al., 2000).

Gene therapy is an exciting technology that overcomes the limited therapeutic duration of growth factors through continuous production of these therapeutic factors (Wang et al., 1999). Wehling et al. (1997) were the first to explore the option of gene transfer to the intervertebral disc. They used retrovirus-mediated gene transfer to successfully insert the beta-galactosidase gene into *in vitro* cultured chondrocytes from the bovine cartilage end-plate. Nishida et al. (1999) have reported success in delivering the TGF-β1 gene into rabbit nucleus pulposus cells with a 30-fold increase in active TGF-β1 production and a 100% increase in proteoglycan production in the treated discs. However, using adeno-associated virus (AAV)-mediated dual gene expression as a therapeutic strategy has seldom been reported.

In this study, we used the angiogenesis of vascular endothelial growth factor (VEGF) for improving the nutritional diffusion and matrix synthesis-promoting effects of TGF-β1. Annulus fibrosus cells of degenerative intervertebral discs were used as target cells. AAV was selected as the vector for gene transfer, and $VEGF_{165}$ and TGF-β1 were co-transfected into annulus fibrosus cells from degenerative rabbit intervertebral discs. The synthesis of collagen type I was assessed. Our study provides a theoretical basis for *in vivo* experiments and the reversal of early degenerative intervertebral disc therapy using multiple gene expression.

MATERIAL AND METHODS

Plasmids, bacterial strains, viruses, and cells

The packaging plasmid AAV-pSNAV was purchased from Zhengyuan Vector Gene Technology Co., Ltd. (Beijing, China). pcDNA3(+)-$VEGF_{165}$ eukaryotic expression plasmids were a gift from Professor Yoshi Yonemitsu (Kyushu University, Japan). AAV-h$VEGF_{165}$, AAV-TGF-β1, and AAV-enhanced green fluorescent protein (EGFP) were packaged by the Zhengyuan Vector Gene Technology Co., Ltd.. *Escherichia coli* DH5α, human embryonic kidney HEK293 cells, vascular endothelial cells, and rabbit annulus fibrosus cells were available in our laboratory.

Construction and identification of AAV-pSNAV-$VEGF_{165}$

The PCR primers were designed based on the h$VEGF_{165}$ complementary DNA sequence of pcDNA3(+)-h$VEGF_{165}$. The forward primer contained the *Eco*RI restriction enzyme cutting site and the reverse primer contained the *Sal*I restriction enzyme cutting site. The sequences of primers were the following: forward primer (A): 5'-ATC GGA ATT CAT GAA CTT TCG CTG-3' and reverse primer (B): 5'-ATC TGT CGA CTC ACC GCC TCG GCT T-3'. PCR was performed using pcDNA3(+)-h$VEGF_{165}$ as the template. Amplified products underwent gel electrophoresis and a 596-bp band was considered positive. PCR products were digested using both *Eco*RI and *Sal*I enzymes, and h$VEGF_{165}$ fragments were collected accord-

ing to manufacturer instructions included with the DNA extraction and purification kits. After identification, pSNAV was digested with both *Eco*RI and *Sal*I enzymes and inserted into the collected $hVEGF_{165}$ fragments with T4 DNA ligase to obtain pSNAV-$hVEGF_{165}$. The pSNAV-$hVEGF_{165}$ was then transformed into DH5α competent *E. coli*. Positive clones were identified on flat-plate ALB culture medium. pSNAV-$hVEGF_{165}$ was extracted via alkaline lysis and identified through digestion and sequencing (Figure 1).

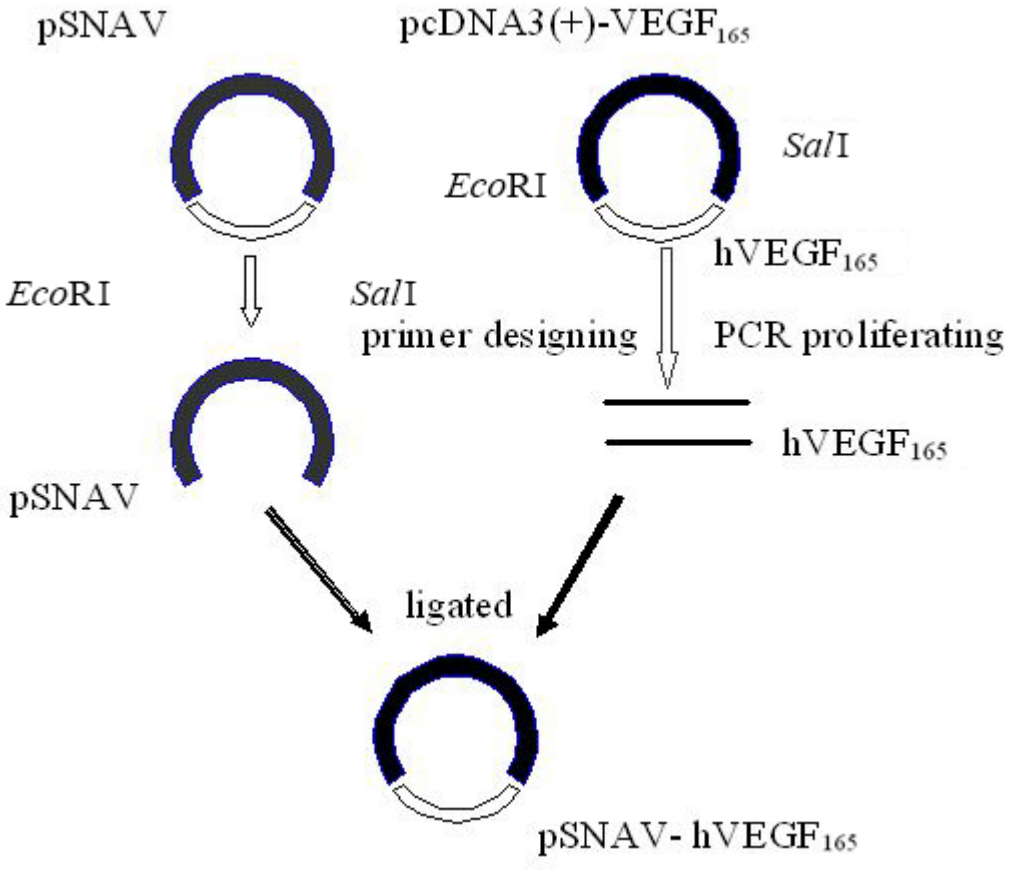

Figure 1. Construction and identification of pSNAV-hVEGF.

Transfection

A 12-well plate was inoculated with HEK293 cells and incubated at 37°C in a humidified 5% CO_2 atmosphere for 18-24 h. When cell confluency reached 50-60%, pSNAV-$hVEGF_{165}$ was transfected into the HEK293 cells with Lipofectamine™ 2000 according to manufacturer instructions. After a 48-h incubation, the cellular supernatant was stored, and the remaining cells were used to detect the presence of $hVEGF_{165}$ protein via fluorescent immunohistochemistry. The cells were incubated with mouse anti-human VEGF monoclonal antibody

(1:200) at 4°C overnight, and then immunoglobulin G-tetramethylrhodamine-5-(and 6)-isothiocyanate was added to react for 30-45 min at room temperature. The cells were washed with Tris-buffered saline buffer in darkness and then observed under a fluorescent microscope.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

A 96-well plate was inoculated with vascular endothelial cells and incubated at 37°C in a 5% CO_2 humidified atmosphere for 24 h. When cell confluency reached 60-70%, we added 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 µL Lipofectamine TM 2000. After a 24-h incubation, 20 µL MTT was added and incubated for 4 h, followed by the addition of 150 µL dimethyl sulfoxide per well. The optical density (OD) of each well at 570 nm was determined using a plate reader. The cell viability curve was derived, and $hVEGF_{165}$ protein activity was determined.

Transfection of rAAV-$hVEGF_{165}$ and Western blot

After identification, pSNAV-$hVEGF_{165}$ was used by the Vector Gene Technology Co. to construct AAV-$hVEGF_{165}$. A 6-well plate was inoculated with fourth-passage cultured rabbit annulus fibrosus cells and incubated at 37°C for 16-18 h. When cell confluency reached 60%, cells in each well were counted and rAAV-$hVEGF_{165}$ was added according to a multiplicity of infection of 1×10^5. Concurrently, AAV-EGFP was added to a well at the same multiplicity of infection to observe the transfection efficiency of AVV. After transfection, cells were incubated for an additional 72 h; Western blot was then performed to detect $hVEGF_{165}$ expression in annulus fibrosus cells.

Transfection of AAV-TGF-β1 and Western blot

The procedures for AAV-TGF-β1 transfection were the same as those for rAAV-$hVEGF_{165}$, and the identification of TGF-β1 expression in annulus fibrosus cells was the same as that for $hVEGF_{165}$.

Co-transfection of rAAV-$hVEGF_{165}$ and AAV-TGF-β1 and Western blot

The co-transfection of rAAV-$hVEGF_{165}$ and AAV-TGF-β1 was performed as previously described. The level of expression of collagen type I protein in rabbit annulus fibrosus cells was determined by Western blot.

RESULTS

Construction and identification of pSNAV-$VEGF_{165}$

The PCR blank control showed no band (lane 1, Figure 2). pSNAV-$VEGF_{165}$ digested with endonuclease *Eco*RI showed one band after electrophoresis, confirming that the pSNAV plasmid was successfully obtained (lane 2, see Figure 2). PCR products were digested with both *Eco*RI and *Sal*I enzymes to obtain a 581-bp $hVEGF_{165}$ fragment (lane 3, see Figure 2).

PCR products of 596 bp containing the $hVEGF_{165}$ gene proved the successful amplification of $hVEGF_{165}$ (lane 4, see Figure 2). After digestion with both *Eco*RI and *Sal*I enzymes, pSNAV was inserted into the 581-bp $hVEGF_{165}$ fragments with T4 DNA ligase to obtain pSNAV-$hVEGF_{165}$, which was then transformed into DH5α competent *E. coli*. The DH5α competent *E. coli* was plated on ALB culture medium at 37°C overnight, and 10 bacterial colonies were selected for amplification. pSNAV-VEGF plasmids were then extracted and identified.

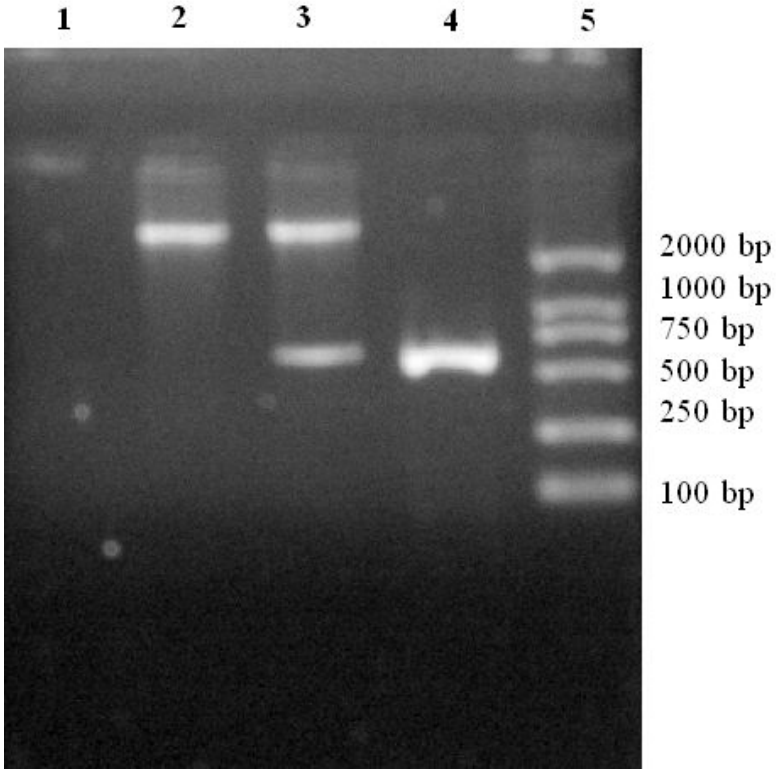

Figure 2. Presence of the $hVEGF_{165}$ cDNA and construction and identification of pSNAV-$VEGF_{165.}$ *Lane 1* = PCR blank control; *lane 2* = pSNAV-$VEGF_{165}$ digested with endonuclease *Eco*R1 showing one band; *lane 3* = pSNAV-$VEGF_{165}$ confirmed following digestion with endonuclease *Eco*R1 and *Sal*1, and the 581-bp band is the gene fragment of interest; *lane 4* = PCR product of $VEGF_{165}$ is about 596 bp; *lane 5* = DL2000 DNA marker.

The results showed that two of the 10 colonies contained the pSNAV-$VEGF_{165}$, which was digested with *Sma*I enzyme to obtain 812-, 839-, 1113-, and 4810-bp fragments. Only three bands appeared on the electrophoresis gel because the phase difference between the 812- and 839-bp bands was too small to be distinguishable (lane 1, Figure 3). pSNAV was digested with endonuclease *Sma*I, and 839-, 1113-, and 4810-bp segments appeared (lane 2, Figure 3). These results confirmed successful construction of pSNAV-$VEGF_{165}$. Further sequencing results were consistent with a published $VEGF_{165}$ sequence, demonstrating the successful production of a recombinant plasmid.

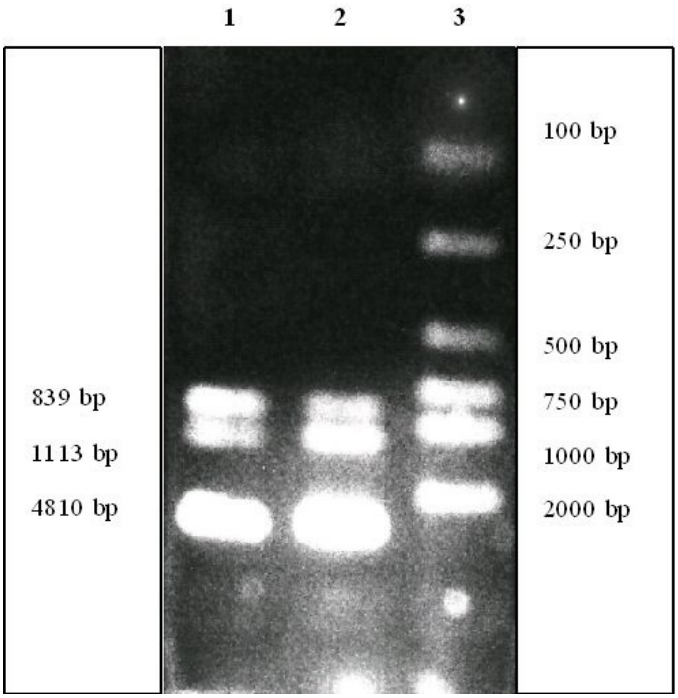

Figure 3. Identification of pSNAV-VEGF$_{165}$ by endonuclease digestion. *Lane 1* = pSNAV-VEGF$_{165}$ digested with the endonuclease *Sma*I. Although there should be four fragments (812, 839, 1113, and 4810 bp), because the phase difference between 812- and 839-bp bands was too small to be distinguishable, there were only three bands on the electrophoresis gel; *lane 2* = pSNAV digested with endonuclease *Sma*I, and 839, 1113, and 4810 bp segments are seen; *lane 3* = DL2000 DNA marker.

Transfection of pSNAV-VEGF$_{165}$ and fluorescence immunohistochemistry

Twenty-four hours after pSNAV-VEGF$_{165}$ transfection into HEK293 cells with Lipofectamine TM 2000, a few dead cells were present; 48 h later, cell growth was robust and cell confluency reached 95% (Figure 4A). The supernatant was then collected, and the remaining cells were used to determine the protein expression of pSNAV-hVEGF$_{165}$. The number of HEK293 cells in the control group was greater than that in the experimental group (Figure 4B). Using a fluorescent microscope, we observed VEGF$_{165}$ protein-positive fluorescence in the transfected cells (Figure 5A), whereas no fluorescence occurred in non-transfected control cells (Figure 5B). These results demonstrated that pSNAV-hVEGF$_{165}$ can express hVEGF$_{165}$.

Effect of transfection of vascular endothelial cells with pSNAV-hVEGF$_{165}$ on cell viability

The supernatant was added to vascular endothelial cells at 60-70% fusion and incubated for 24 h. After the addition of MTT, the cells appeared blue. With the addition of supernatant, the color became more marked. Dimethyl sulfoxide was added to dissolve the

MTT, and OD values at 570 nm were determined to produce a cell viability curve. The results indicated that $VEGF_{165}$ promoted cell proliferation and viability (Figure 6).

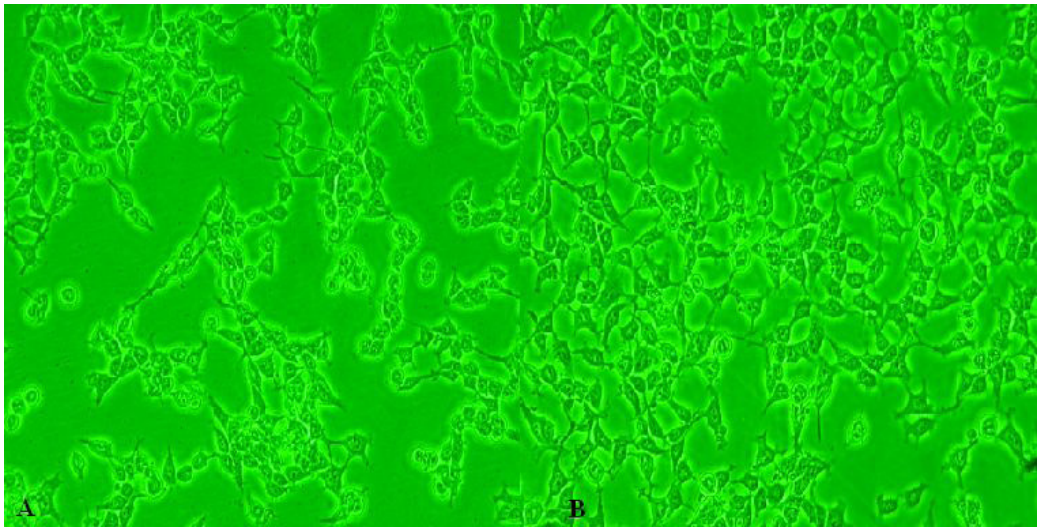

Figure 4. HEK293 cells transfected with pSNAV-$VEGF_{165}$ under fluorescence microscopy (100X). **A.** HEK293 cells transfected with pSNAV-$VEGF_{165}$; **B.** control group: HEK293 cells not transfected with pSNAV-$VEGF_{165}$. The number of HEK293 cells in the control group was greater than that of the experimental group.

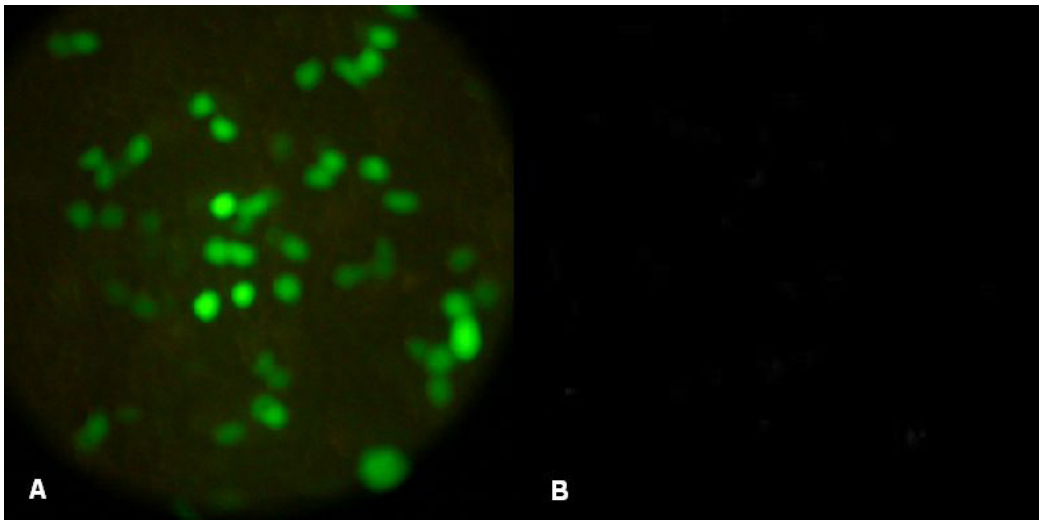

Figure 5. Detection of the $VEGF_{165}$ protein by fluorescence immunocytochemistry. **A.** $VEGF_{165}$-positive staining in HEK293 cells transfected with pSNAV-$VEGF_{165}$ (40X); **B.** $VEGF_{165}$-negative staining in HEK293 cells not transfected with pSNAV-$VEGF_{165}$ (40X).

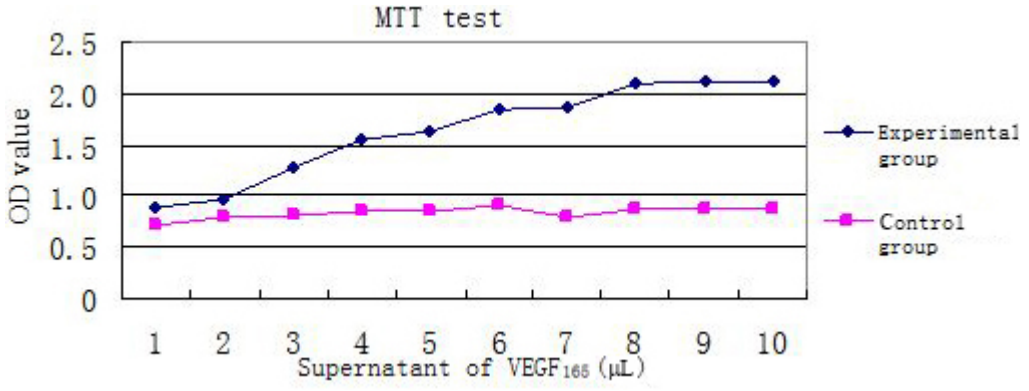

Figure 6. Cell viability curve in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test indicate that $VEGF_{165}$ promotes cell proliferation and viability.

Transfection of rAAV-hVEGF$_{165}$ and hVEGF$_{165}$ expression

Cell confluency reached 60% 16-18 h after rabbit annulus fibrosus cells were inoculated into a 6-well plate. At this time, rAAV-$hVEGF_{165}$ and rAAV-EGFP were transfected into the rabbit annulus fibrosus cells. Seventy-two hours later, cell fusion reached more than 80%. Fluorescent microscopy showed a high level of fluorescence excitation in rAAV-EGFP-transfected cells (Figure 7), demonstrating that AAV effectively transfected degenerative annulus fibrosus cells with an efficiency of 40%. Western blot confirmed the expression of $hVEGF_{165}$ protein with a relative molecular weight of 45×10^3 in the cells (Figure 8).

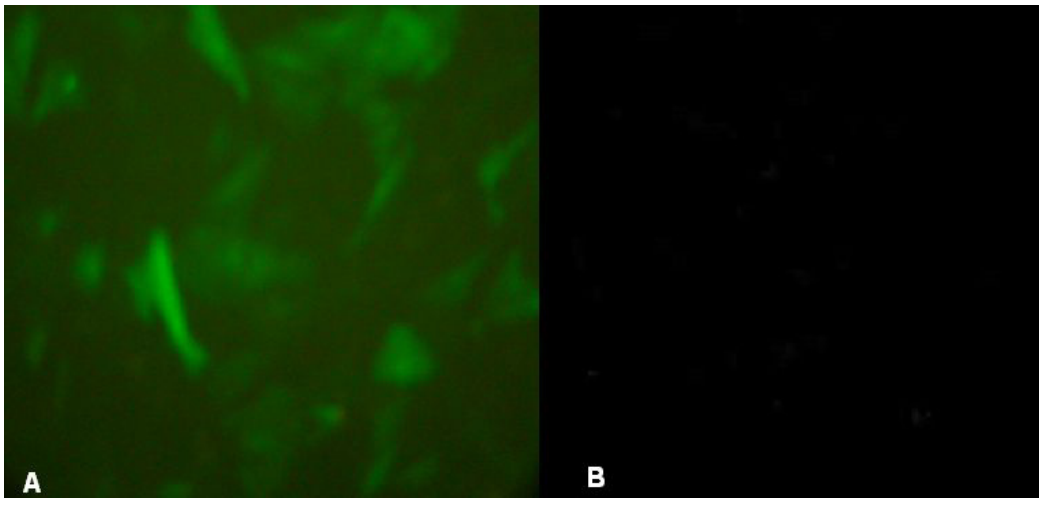

Figure 7. Fluorescence excitation in rAAV-EGFP-transfected rabbit degenerative annulus fibrosus cells. **A.** Green fluorescence in rAAV-EGFP-transfected cells (experimental: 400X). **B.** No fluorescence excitation in cells non-transfected with rAAV-EGFP (control: 400X).

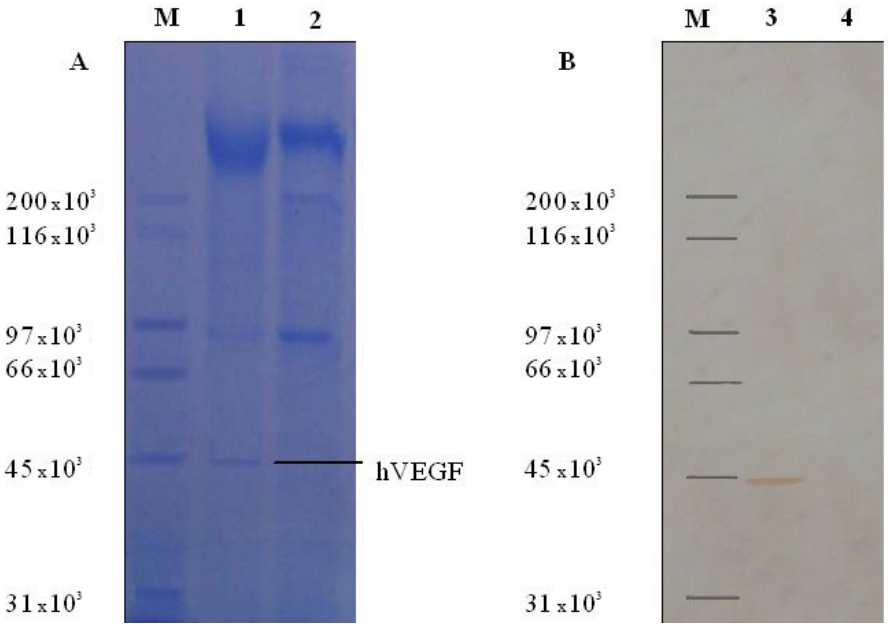

Figure 8. hVEGF$_{165}$ protein in rabbit annulus fibrosus cells transfected with rAAV-hVEGF$_{165}$. **A.** SDS-PAGE electrophoresis. *Lane M* = marker of the BIO-RAD protein; *lane 1* = AAV-hVEGF$_{165}$-transfected cell protein; *lane 2* = control group (cells not transfected with AAV-hVEGF$_{165}$). **B.** Western blot for the VEGF protein. *Lane M* = marker of the BIO-RAD protein; *lane 3* = positive results of Western blot of AAV-hVEGF$_{165}$-transfected cells; *lane 4* = negative expression of the hVEGF$_{165}$ protein in the control group.

Transfection of AAV-TGF-β1 and TGF-β1 expression

Cell confluency reached 60% 18 h after rabbit degenerative annulus fibrosus cells were inoculated into a 6-well plate. At that time, rAAV-TGF-β1 was transfected into the cells, and 72 h later, cell confluency was more than 80%. Western blot confirmed the expression of TGF-β1 protein with a relative molecular weight of 25 x 10^3 in the degenerative annulus fibrosus cells (Figure 9).

Co-transfection of rAAV-hVEGF$_{165}$ and AAV-TGF-β1 and collagen type I protein expression

The cell growth status before and after transfection was the same as that previously described. The level of expression of collagen type I protein per well was determined using Western blot. The relative molecular weight of collagen type I was 300 x 10^3. The expression of collagen type I protein was highest in cells co-transfected with AAV-hVEGF$_{165}$ and AAV-TGF-β1, second highest in cells transfected only with AAV-TGF-β1, and lowest in cells transfected with AAV-hVEGF$_{165}$ alone or in control cells. These results indicated that TGF-β1 promoted the expression of collagen type I protein in rabbit degenerative annulus fibrosus cells. hVEGF$_{165}$ alone does not directly promote the expression of collagen type I protein but has a synergetic effect with TGF-β1 in promoting the expression of this protein by these cells (Figure 10).

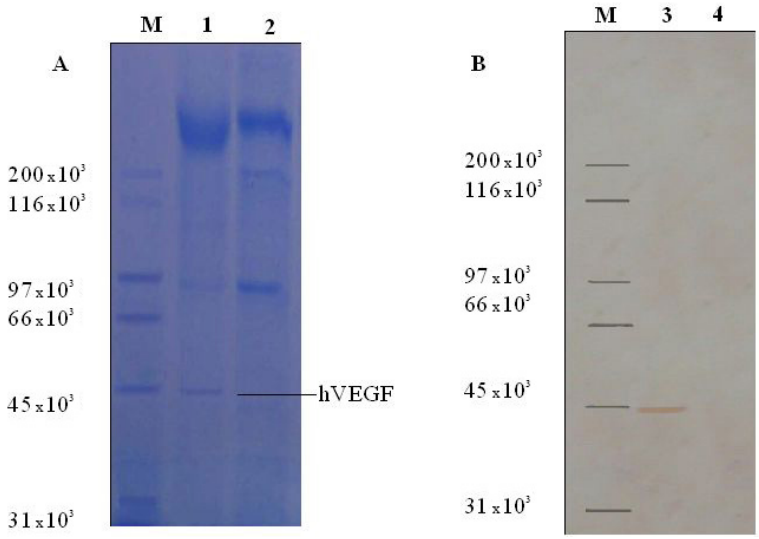

Figure 9. TGF-β1 protein in rabbit degenerative annulus fibrosus cells transfected with rAAV-TGF-β1. **A.** SDS-PAGE electrophoresis. *Lane M* = marker of the BIO-RAD protein; *lane 1* = nAAV-TGF-β1-transfected cell protein; *lane 2* = control group (cells not transfected with AAV-TGF-β1). **B.** Western blot for the TGF-β1 protein. *Lane M* = marker of the BIO-RAD protein; *lane 3* = positive results of Western blot of AAV-TGF-β1-transfected cells; *lane 4* = negative expression of the TGF-β1 protein in the control group.

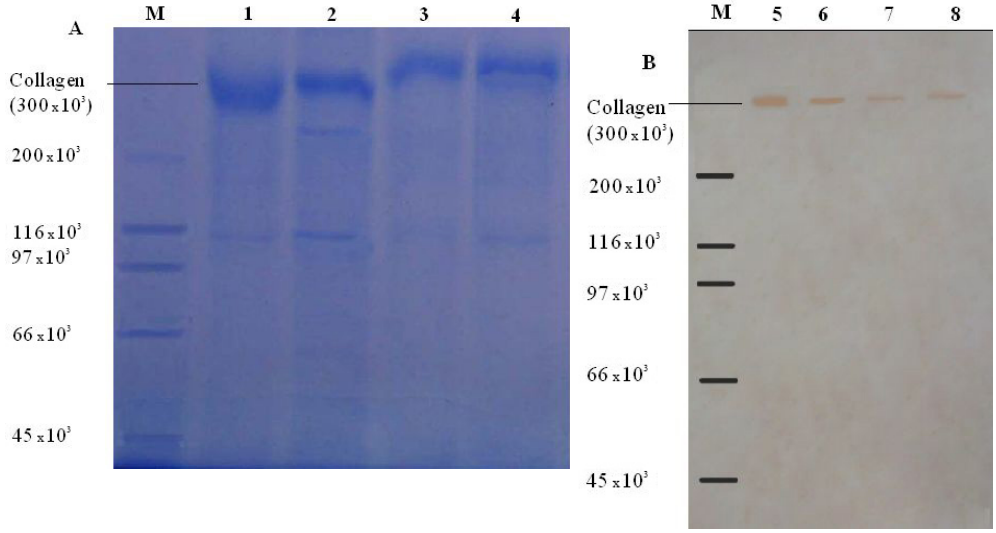

Figure 10. Collagen type I protein expression by rabbit degenerative annulus fibrosus cells co-transfected with rAAV-hVEGF$_{165}$ and AAV-TGF-β1. **A.** SDS-PAGE electrophoresis. *Lane M* = Marker of the BIO-RAD protein; *lane 1* = AAV-TGF-β1- and AAV-hVEGF$_{165}$-co-transfected cell protein; *lane 2* = AAV-TGF-β1-transfected cell protein; *lane 3* = AAV-hVEGF$_{165}$-transfected cell protein; *lane 4* = control (cells not transfected with AAV-hVEGF$_{165}$ or AAV-TGF-β1). **B.** Western blot for collagen type I. *Lane M* = marker of the BIO-RAD protein; *lane 5* = positive results of Western blot of AAV-TGF-β1- and AAV-hVEGF$_{165}$-co-transfected cells; *lane 6* = AAV-TGF-β1-transfected cells; *lane 7* = AAV-hVEGF$_{165}$-transfected cells; *lane 8* = control group.

DISCUSSION

The intervertebral disc comprises the cartilage end-plate, nucleus pulposus, and annulus fibrosus. With increasing age and the influence of various factors, such as smoking and drinking habits (Podichetty, 2007; Hangai et al., 2008), degenerative changes gradually occur in intervertebral discs. The pathological process of degenerating intervertebral discs involves micro-damage that occurs in the annulus fibrosus and subsequent dehydration of the nucleus pulposus. Permeability of the cartilage end-plate decreases, and the number of intervertebral disc cells is reduced, leading to functional degeneration (Riches and McNally, 2005; Vernon-Roberts et al., 2008; Guehring et al., 2009). Biochemical studies have indicated that in degenerative intervertebral discs, the level of collagen type II is reduced, and levels of collagen types I and III increase, whereas proteoglycan content (especially aggrecan) decreases with the proportion of non-aggrecan increase, and levels of chondroitin sulfate decrease and keratan sulfate increases (Fujita et al., 1993). One factor in intervertebral disc degeneration is stress injury of the annulus fibrosus leading to micro-disruption and herniation of the nucleus pulposus. Although spontaneous repair of annulus fibrosus disruption is rare, gene therapy may be useful in effecting this repair. Gene therapy uses transfected target genes to activate degenerating intervertebral disc cells to promote the synthesis of type I and II collagen and proteoglycans and reduce their degradation, which can delay or reverse early degenerative changes and promote regeneration (Shimer et al., 2004).

Gene therapy for degenerative intervertebral discs has been studied for less than 10 years. In 1997, Wehling et al. first proposed that transgenes could reverse intervertebral disc degeneration, and they transfected target genes into chondrocytes from bovine vertebral cartilage end-plates *in vitro*. In 1999, Nishida et al. transfected human TGF-β1 cDNA into nucleus pulposus cells of rabbit intervertebral discs, demonstrating an increased synthesis of proteoglycans. Wallach et al. (2003) have successfully transfected inhibitors of metalloproteinase-1 into human degenerative intervertebral disc cells, which revealed increased synthesis of proteoglycans in the nucleus pulposus. Chen and Yang (2001) have transfected TGF-β1 into rabbit degenerative nucleus pulposus cells, showing that TGF-β1 expression persists for 12 weeks and collagen and proteoglycan contents in the nucleus pulposus increased. Previous studies on gene therapy for degenerative intervertebral discs have mainly involved human TGF-β1 transfected into nucleus pulposus cells with adenovirus as the vector. In our study, AAV served as the vector, and our results indicated that it was superior to adenovirus. AAV has the following advantages: i) slight immune reaction and high safety; ii) achievement of "latent" infection status without helper viruses; iii) site-directed integration, avoiding the potential risk of host cell mutations caused by random integration; iv) extensive host range, including mitotic cells and quiescent cells; and v) stable and long-term expression of exogenous genes carried. Our results demonstrate that AAV has a higher transfection efficiency (about 40%) for annulus fibrosus cells from degenerative intervertebral discs, and that the transfected genes, $VEGF_{165}$ and TGF-β1, were expressed and played a biological role in reversing degeneration; these results confirm that AAV is a better viral vector for gene therapy.

In this study, $hVEGF_{165}$ and TGF-β1 were co-transfected into degenerative annulus fibrosus cells. $hVEGF_{165}$, a highly specific vascular endothelium mitogen, can increase vascular permeability and promote angiogenesis and has synergetic effects with cytokines such as TGF-β1 and bone morphogenetic protein. Our results indicated that the level of collagen type I in $hVEGF_{165}$-

transfected annulus fibrosus cells was similar to that of control cells, demonstrating that $hVEGF_{165}$ specifically affects only vascular endothelial cells and has no direct effect on other cells.

TGF-β1, a cytokine with extensive biological activity, is the most important cytokine in gene therapy for degenerative intervertebral discs because it positively regulates the biological functions of nucleus pulposus and annulus fibrosus cells. Many studies have confirmed that TGF-β1 promotes the synthesis of proteoglycans and collagen type II by nucleus pulposus cells. Chen et al. (1999) have found that TGF-β1 enhances the expression of collagen type I in a dose-dependent manner, reflecting the close association of TGF-β1 with fibration in degenerative discs. Our results also confirmed that the level of expression of collagen type I protein in TGF-β1-transfected annulus fibrosus cells was significantly increased compared with that in $hVEGF_{165}$-transfected and control annulus fibrosus cells, suggesting that TGF-β1 can repair the disrupted annulus fibrosus. In this study, the level of collagen type I protein expression was significantly higher in cells co-transfected with both $hVEGF_{165}$ and TGF-β1 than in cells transfected with TGF-β1 alone, indicating the biological effects of $hVEGF_{165}$ combined with TGF-β1 are greater than those of TGF-β1 alone. Our study provides a theoretical basis for *in vivo* experiments and the reversal of early degeneration of the intervertebral disc using gene therapy.

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