Prevention of lumbar disc degeneration through co-manipulation of insulin-like growth factor 1 and vascular endothelial growth factor

Zuozhou Ye, Shan Zhao, Zuoqing Liu

Department of Orthopedics, Qingpu Branch of Zhongshan Hospital Affiliated to Fudan University, Shanghai, China

Contributions: (I) Conception and design: Z Liu; (II) Administrative support: Z Liu; (III) Provision of study materials or patients: Z Liu; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: Z Liu; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Zuoqing Liu. Department of Orthopedics, Qingpu Branch of Zhongshan Hospital affiliated to Fudan University, 1158 Gongyuan East Road, Shanghai 201700, China. Email: cxr67981@yeah.net.

Background: Associated with abnormal angiogenesis and disc dysfunction, lumbar disc degeneration (LDD) appears to be an important disease suffered by elderly people. Previous studies have highlighted the importance of insufficient insulin-like growth factor 1 (IGF1) and excessive vascular endothelial growth factor (VEGF) in the development and progression of LDD, though a practical therapeutic strategy is lacking.

Methods: The expression of IGF1 and VEGF was assessed in LDD specimens compared to normal disc tissue as a control. A gene therapy approach was performed, in which an adeno-associated virus (AAV) carrying both IGF1 and shVEGF (AAV-IGF1/shVEGF) was orthotopically injected to the rats that had undergone LDD. The alterations in IGF1 and VEGF levels in the treated disc tissue were confirmed by immunohistochemistry. The outcome of this therapy was assessed by disc cell death using an annexin V-FITC assay and by assessing lumbar proteoglycan and collagen II levels using ELISA.

Results: IGF1 expression was significantly downregulated in LDD, while VEGF expression was significantly upregulated in LDD, compared to normal controls. Combined AAV-IGF1/shVEGF treatment simultaneously corrected the insufficient IGF1 and the excessive VEGF in LDD rats. Moreover, AAV-IGF1/shVEGF significantly reduced disc cell death in the vertebral pulp and annulus fibrosus and significantly enhanced the lumbar proteoglycan and collagen II levels.

Conclusions: The simultaneous increase in IGF1 and depletion of VEGF effectively prevented the development of LDD, suggesting its potential as a novel therapeutic approach for LDD which is clinically translatable.

Keywords: Lumbar disc degeneration (LDD); gene therapy; insulin-like growth factor 1 (IGF1); vascular endothelial growth factor (VEGF); adeno-associated virus (AAV)

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Introduction

Many elderly people suffer from potent low back pain as a result of lumbar disc degeneration (LDD) (1-3). The lumbar disc has several components, including the nucleus pulposus (NP), annulus fibrosus, and cartilage end plates. The primary pathogenesis of LDD results from excessive cartilage-specific extracellular matrix production by apoptotic NP cells (4). Therefore, the current therapeutic strategy for treating LDD is focused on reducing the apoptosis of NP cells (5-9).

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Many factors play roles in the pathogenesis of LDD. The insulin-like growth factors (IGFs) have been shown to antagonize the progression of LDD by us (10-12) and by others (13-15). Specifically, we showed that IGF1 induced the phosphorylation of the IGF1 receptor (IGF1R), leading to phosphorylation of its downstream factor Akt and subsequently nuclear exclusion of FoxO1, to eventually suppress matrix metalloproteinase 3 (MMP3)-mediated LDD (10-12). Moreover, the importance of IGF1 in LDD has been acknowledged by other groups (16). On the other hand, abnormal activation of angiogenesis has also been shown to promote the progression of LDD (17-20). For example, degenerative intervertebral disc disorders have been associated with increased expression of vascular endothelial growth factor (VEGF), the most potent angiogenic factor (18). The VEGF family has 6 members, among which VEGF-A is the most important one and is referred to as VEGF in this article. Although these previous studies have highlighted the importance of IGF1 and VEGF in the development and progression of LDD, a practical therapeutic strategy is lacking.

Here, we reported a gene therapy approach using an adeno-associated virus (AAV) carrying IGF1 and shVEGF (AAV-IGF1/shVEGF), which corrected the insufficient IGF1 and the excessive VEGF in a rat model of LDD. We found that AAV-IGF1/shVEGF significantly reduced disc cell death in the vertebral pulp and annulus fibrosus and significantly improved the lumbar proteoglycan and collagen II levels. Thus, our study presents a novel therapeutic approach for LDD, which is clinically translatable.

We present the following article in accordance with the ARRIVE reporting checklist (available at https://dx.doi.org/10.21037/atm-21-4977).

Methods

Protocol approval and animal treatments

The present study was approved by the Research and Animal Ethics Association of Zhongshan Hospital affiliated to Fudan University under a license number No. GDR452, in compliance with the institutional guidelines for the care and use of animals. A protocol was prepared before study without registration. A total of 40 1-year-old female Sprague-Dawley rats (140–160 g) were purchased from the Animal Laboratory of the Academy of Medical Sciences (Beijing, China) and used in strict accordance with the guidelines for the Care and Use of Laboratory Animals. For each experiment, power calculations (P<0.05) were used to include an adequate number of animals for the observed effects to be legitimate. An allocation concealment method was used for randomization in allocating experimental units to the control and treatment groups. The potential confounders were minimized. No criteria were used for excluding animals (or experimental units) during the experiment, and no data were excluded during the analysis. The study did not have humane endpoints. The rats were kept in separate cages with free access to food and water, and in a 12/12-hour light/dark cycle (temperature, 24 ℃; humidity, 50%). Rats were randomly divided into 4 groups of 10. The sham group received only a skin incision, which was then sutured to close the wound. The rats in the other 3 groups received removal of the sacrospinal muscles, spinous processes, supraspinous ligaments, interspinous ligaments, and posterolateral halves of the bilateral zygapophysial joints of the lumbar spine (a model for LDD). Afterwards, the rats in the LDD group received an orthotopic injection of 150 µL normal saline, rats in the LDD + AAV-scr group received an orthotopic injection of 10^{11} AAV-scr in a 150 µL volume, and rats in the LDD + AAV-IGF1/VEGF group received an orthotopic injection of 10^{11} AAV-IGF1/VEGF in a 150 µL volume. Rats were kept for 8 weeks before analysis.

Cell culture and AAV preparation

A human disc cell line, nucleus pulposus SV40 (HNPSV), has been described previously (21). The HNPSV cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco; Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Sigma-Aldrich, St Louis, MO, USA), penicillin (100 µg/mL), and streptomycin (250 ng/mL) at 37 ℃ in a 5% CO_2 atmosphere. AAV serotype 2 vectors were generated by transfection of human embryonic kidney 293 cells. Rat IGF1 was prepared by PCR using cDNA from primary rat hepatic cells. The respective scramble sequence was used as a control for rat IGF1. The sequence for shVEGF was 5'-TGTGAATGCAGACCAAAGA-3', and its scramble was 5'-GGTATCTACTAGATGTACT-3' (22). Transfection was performed with Lipofectamine 3000 reagent (Invitrogen, CA, Carlsbad, USA), according to the instructions of the manufacturer. The prepared virus was stored at −80 ℃. Titration of viral vectors was determined using a dot blot assay.

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Table 1 Primer sequences

Primers	Forward sequence	Reverse sequence
GAPDH	5'-TGATTCTACCCACGGCAAGTT-3'	5'-TGATGGGTTTCCCATTGATGA-3'
VEGF	5'-CAAGCCAAGGCGGTGAGCCA-3'	5'-TCTGCCGGAGTCTCGCCCTC-3'
IGF1	5'-TCTTGAAGGTGAAGATGCACACCA-3'	5'-CCTGAGGTTCTTCACAG-3'

Sampling and analysis by immunohistochemistry and ELISA

After being euthanized by an intraperitoneal overdose of pentobarbital sodium, the lumbar spines of the rats, including the L4 to L6 discs, were removed. The vertebral pulp and annulus fibrosus were isolated for immunohistochemical and ELISA analysis. For immunohistochemistry, the metaphysis of the vertebral pulp and annulus fibrosus specimens were fixed in 4% paraformaldehyde for 2 hours, then paraffin embedded and cut into 5 µm thick sections. The immunohistochemistry followed the ABC method, while DBA was used to develop IGF1 signals and fast red was used to develop VEGF signals. The primary antibodies were mouse anti-IGF1 (ab176523; Abcam, Cambridge, MA, USA; dilution: 1:300) and rabbit anti-VEGF (ab52917; Abcam; dilution: 1:800). The secondary antibodies were HRP-conjugated anti-mouse and anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). ELISA was performed using the IGF1 kit (MG100; R&D System, Los Angeles, CA, USA), VEGF kit (RRV00; R&D System), proteoglycan kit (EKC39620; Biomatik, Wilmington, DE, USA), and collagen II kit (LS-F23885; LSBio, Seattle, WA, USA).

Apoptosis assay

Cells were labeled with annexin V-FITC and propidium iodide (PI) using an apoptosis detection kit (KeyGEN Biotech, Nanjing, China), and analyzed by flow cytometry using CellQuest software (Becton-Dickinson Biosciences, San Jose, CA, USA).

RNA isolation and quantitative polymerase chain reaction (RT-qPCR)

RNA extraction and cDNA synthesis were routinely performed. The RT-qPCR primers were shown in *Table 1*. Values were normalized against GAPDH, which proved to be stable across the samples, and then compared to experimental controls.

Statistical analysis and bioinformatics

All statistical analyses were carried out using the SPSS 20.0 statistical software package. Data were investigated using one-way ANOVA with a Bonferroni correction, followed by Fisher's exact test to compare 2 subgroups. All values are shown as mean ± standard deviation (SD) and were considered significant if $P<0.05$ and not significant (NS) if $P>0.05$. For bioinformatics analysis, transcriptome RNA-sequencing (RNA-seq) data of human disc specimens from normal and LDD patients were downloaded from the GEO data portal (https://www.ncbi.nlm.nih.gov/geo/). RNA-seq data of specimens in a published database (GSE124272) were used for analysis by the R software Linear Models for Microarray and RNA-Seq Data (Limma) package.

Results

Bioinformatics analyses of IGF1 and VEGF levels in LDD patients

Since previous studies have highlighted the importance of IGF1 and VEGF in the development and progression of LDD, we first analyzed the levels of IGF1 and VEGF in disc specimens from normal control and LDD patients. We performed GEO database mining and reanalyzed the data from a public database. The Limma R package was used to identify genes that were significantly altered in LDD, compared to normal controls. As shown in a volcano plot (*Figure 1A*), we found that IGF1 expression was significantly downregulated in LDD, while VEGF expression was significantly upregulated in LDD, compared to normal controls (*Figure 1B*). Thus, these data indicate that downregulation of IGF1 and upregulation of VEGF may both contribute to LDD development and suggest that reversal of these features may attenuate or even inhibit the process.

Preparation of AAVs overexpressing IGF1 and depleting VEGF

In order to determine whether our hypothesis was correct,

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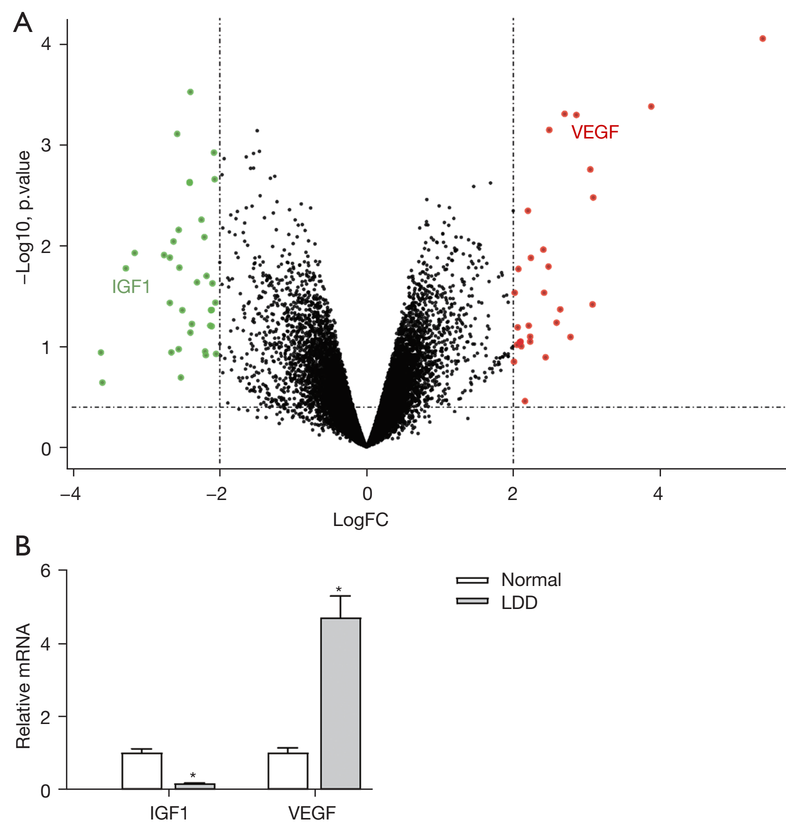

Figure 1 Bioinformatics analyses of IGF1 and VEGF levels in LDD patients. (A) Volcano plot for the GEO database (GSE124272) to compare the gene profiling of disc cells between LDD patients and normal controls. Data were analyzed by R language. (B) Levels of IGF1 and VEGF in disc cells in LDD patients compared to normal controls. *P<0.05. N=16. IGF1, insulin-like growth factor 1; VEGF, vascular endothelial growth factor; LDD, lumbar disc degeneration.

we used an AAV technique to enhance IGF1 signaling and suppress VEGF-mediated angiogenesis in one construct. The experimental AAV used an AAV serotype 2 carrying recombinant IGF1 and shVEGF under a CMV promoter, while 2 respective scramble controls were used in the control AAV (*Figure 2A*). We transduced a disc cell line, HNPSV, with the 2 AAVs and compared these cells to untreated cells. We found that AAV-IGF1/shVEGF-transduced cells expressed significantly higher levels of IGF1 than controls, as determined by RT-qPCR for mRNA expression (*Figure 2B*) and ELISA for protein expression (*Figure 2C*). Moreover, AAV-IGF1/shVEGF-transduced cells expressed significantly lower levels of VEGF than controls, as determined by RT-qPCR for mRNA expression (*Figure 2D*) and ELISA for protein expression (*Figure 2E*). Thus, the quality of the AAVs was validated.

AAV-IGF1/shVEGF treatment increases IGF1 in spinal tissue after LDD

Next, we used a rat model of LDD to examine the effects of the AAV-IGF1/shVEGF gene therapy. Forty rats were randomly divided into 4 groups of 10. Group 1 was sham, group 2 was LDD and an orthotopic saline injection, group 3 was LDD and an orthotopic AAV-scr injection, and group 4 was LDD and an orthotopic AAV-IGF1/shVEGF injection. Rats were kept for 8 weeks before analysis. First, we examined the levels of IGF1 in the rat vertebral pulp and annulus fibrosus by immunohistochemistry (*Figure 3A*) and ELISA (*Figure 3B*). We found that IGF1 signaling was modestly increased in groups 2 and 3 compared to group 1, while IGF1 signaling was increased much more in group 4 (*Figure 3A,3B*). Moreover, the number of NP cells in groups 2 and 3 appeared to reduce compared to group 1. However,

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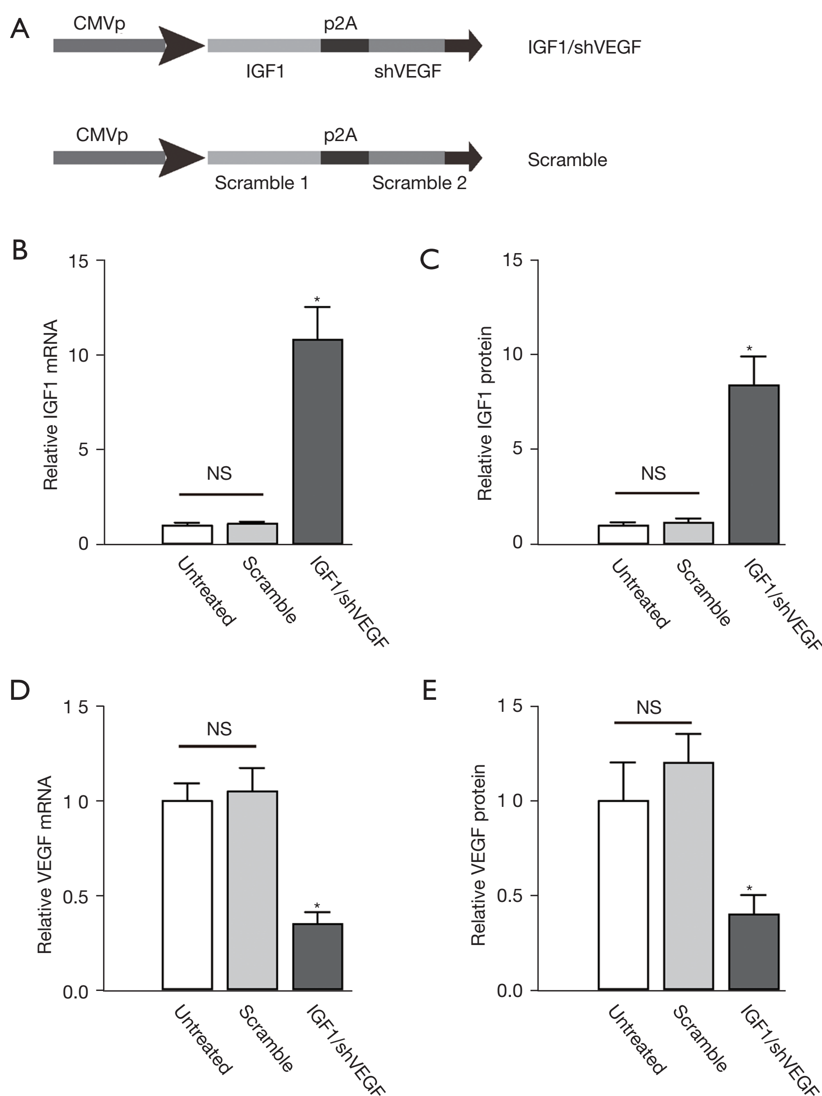

Figure 2 Preparation of AAVs overexpressing IGF1 and depleting VEGF. (A) Schematic to show the structure of AAV-IGF1/shVEGF and AAV-scramble viruses. The experimental AAV used an AAV serotype 2 carrying recombinant IGF1 and shVEGF (connected by a p2A structure) under a CMV promoter, while 2 respective scramble controls were used in the control AAV-scramble. (B-E) A disc cell line, HNPSV, was transduced with the 2 AAVs and compared to untreated cells. (B-C) IGF1 levels determined by RT-qPCR (B) and ELISA (C). (D,E) VEGF levels determined by RT-qPCR (D) and ELISA (E). *P<0.05. NS: non-significant. N=5. AAV, adeno-associated virus; IGF1, insulin-like growth factor 1; VEGF, vascular endothelial growth factor.

this number improved in group 4 (*Figure 3A*). Together, these data confirm that AAV-IGF1/shVEGF treatment increases IGF1 in spinal tissue after LDD.

AAV-IGF1/shVEGF treatment decreases VEGF in spinal tissue after LDD

Next, we examined the levels of VEGF in the rat vertebral pulp and annulus fibrosus by immunohistochemistry (*Figure 4A*) and ELISA (*Figure 4B*). We found that VEGF signaling was dramatically and significantly increased in groups 2 and 3 compared to group 1, while this increase in VEGF was significantly attenuated in group 4 (*Figure 4A,4B*). Thus, these data confirm that AAV-IGF1/shVEGF treatment decreases VEGF in spinal tissue after LDD.

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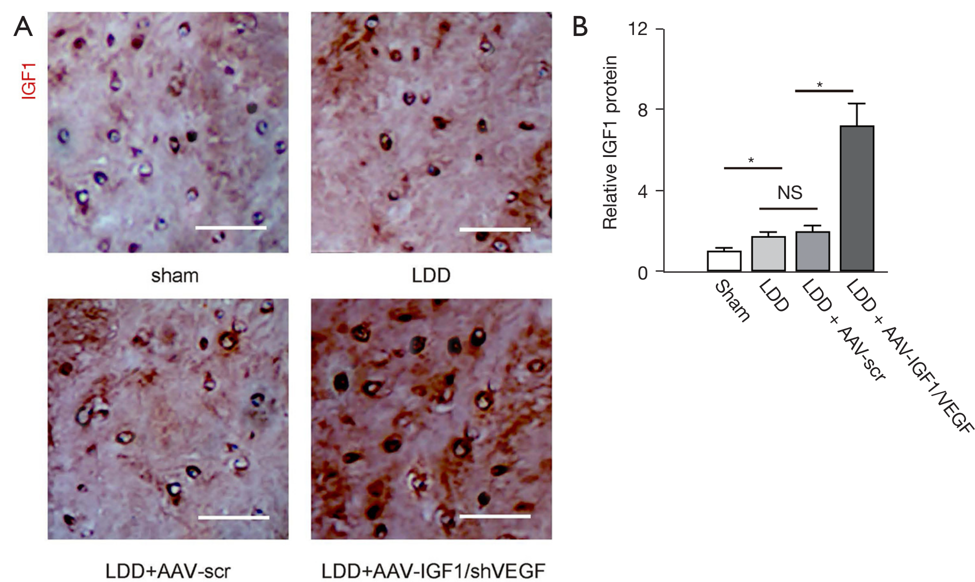

Figure 3 AAV-IGF1/shVEGF treatment increases IGF1 in spinal tissue after LDD. We used a rat model of LDD to examine the effects of the AAV-IGF1/shVEGF gene therapy. Forty rats were randomly divided into 4 groups of 10. Group 1 was sham, group 2 was LDD and an orthotopic saline injection, group 3 was LDD and an orthotopic AAV-scr injection, and group 4 was LDD and an orthotopic AAV-IGF1/shVEGF injection. Rats were kept for 8 weeks before analysis. (A,B) The levels of IGF1 in the rat vertebral pulp and annulus fibrosus 8 weeks after treatment were examined by immunohistochemistry (A) and ELISA (B). *P<0.05. NS: non-significant. N=10. Scale bars are 100 µm. AAV, adeno-associated virus; IGF1, insulin-like growth factor 1; VEGF, vascular endothelial growth factor; LDD, lumbar disc degeneration.

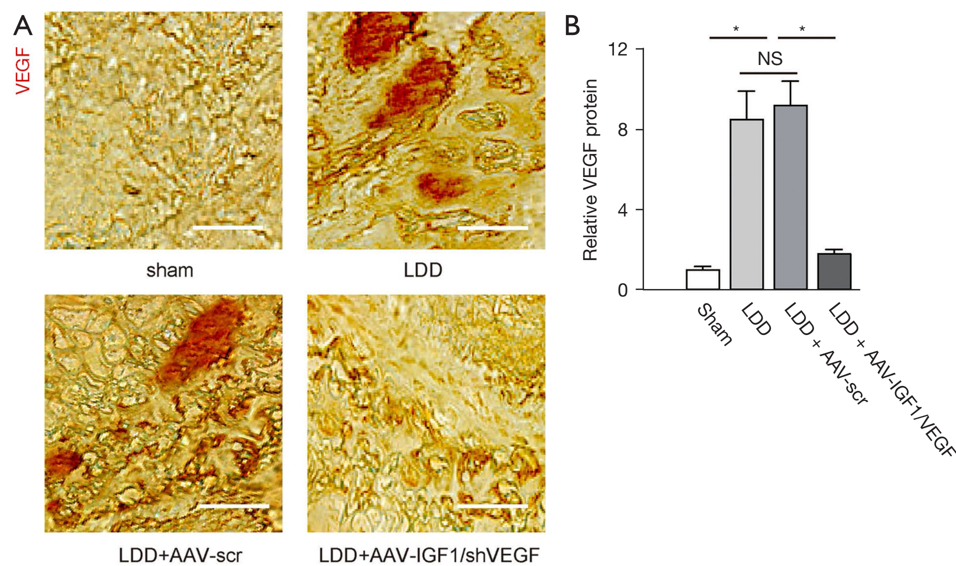

Figure 4 AAV-IGF1/shVEGF treatment decreases VEGF in spinal tissue after LDD. (A,B) The levels of VEGF in the rat vertebral pulp and annulus fibrosus 8 weeks after treatment were examined by immunohistochemistry (A) and ELISA (B). *P<0.05. NS, non-significant. N=10. Scale bars are 100 µm. AAV, adeno-associated virus; IGF1, insulin-like growth factor 1; VEGF, vascular endothelial growth factor; LDD, lumbar disc degeneration.

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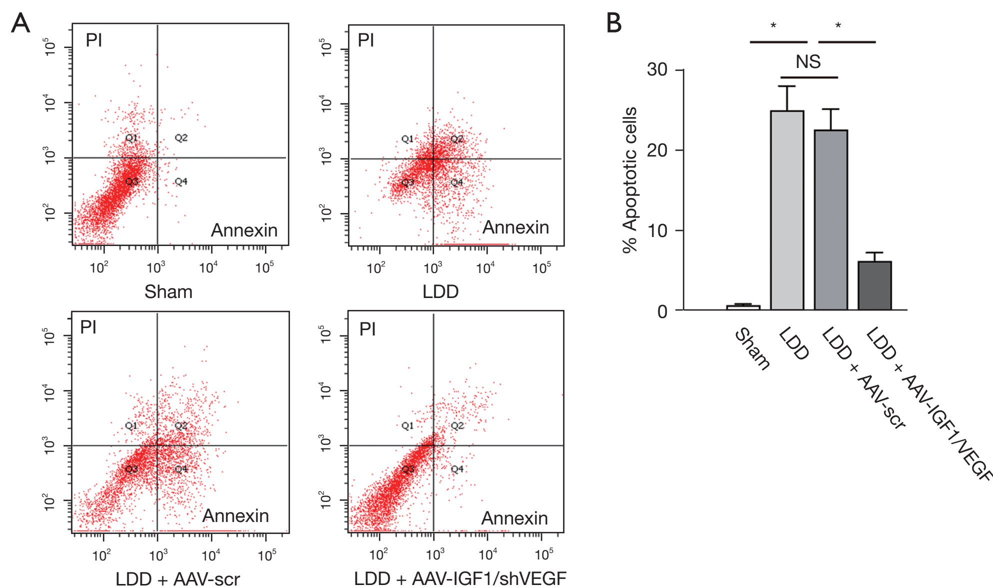

Figure 5 AAV-IGF1/shVEGF treatment reduces cell apoptosis in spinal tissue after LDD. (A,B) Disc cell apoptosis was assessed by examining dissociated single cell suspensions from the rat vertebral pulp and annulus fibrosus 8 weeks after treatment by a flow cytometry-based apoptosis assay, shown by representative flow plots (A) and by quantification (B). *P<0.05. NS, non-significant. N=10. AAV, adeno-associated virus; IGF1, insulin-like growth factor 1; VEGF, vascular endothelial growth factor; LDD, lumbar disc degeneration.

AAV-IGF1/shVEGF treatment reduces cell apoptosis in spinal tissue after LDD

After validation of the AAV-IGF1/shVEGF treatment, we examined the effect of AAV-IGF1/shVEGF treatment on LDD. Disc cell apoptosis is the most important feature of LDD, thus the cells from the rat vertebral pulp and annulus fibrosus were dissociated into single cell suspensions for a flow cytometry-based apoptosis assay. We found that the apoptotic cells were dramatically and significantly increased in groups 2 and 3 compared to group 1, while this increase in apoptotic cells was significantly attenuated in group 4 (*Figure 5A,5B*). Thus, these data suggest that AAV-IGF1/shVEGF treatment reduces cell apoptosis in spinal tissue after LDD.

AAV-IGF1/shVEGF treatment enhances spinal proteoglycan and collagen II levels after LDD

The proteoglycan levels in the spine decrease with the progression of LDD and faithfully represent the disease severity (23). Moreover, levels of collagen II similarly decrease during LDD (24). We therefore examined the levels of spinal proteoglycan and collagen II after treatment. Our data showed that the levels of proteoglycan and collagen II were dramatically and significantly decreased in groups 2 and 3 compared to group 1, while this decrease in proteoglycan and collagen II levels was significantly attenuated in group 4 (*Figure 6A,6B*). These data suggest that AAV-IGF1/shVEGF treatment enhances spinal proteoglycan and collagen II levels after LDD. Thus, AAV-IGF1/shVEGF treatment significantly antagonizes the development of LDD.

Discussion

Many factors and signaling pathways contribute to the development of LDD. Among these factors and pathways, IGF1 signaling and the VEGF pathway appear to play critical roles. Interference with IGF1/IGFR signaling or the VEGF pathway alone may not exert optimal effects on the prevention of LDD, since their downstream signaling cascades share some identical factors and can affect each other (25-29). For example, we have shown that IGF1 induced the phosphorylation of IGF1R, leading to the phosphorylation of its downstream factor Akt and subsequently nuclear exclusion of FoxO1, to eventually suppress matrix metalloproteinase 3 (MMP3)-mediated LDD (10). On the other hand, VEGF has been shown to signal partially through Akt/mTOR signaling (30).

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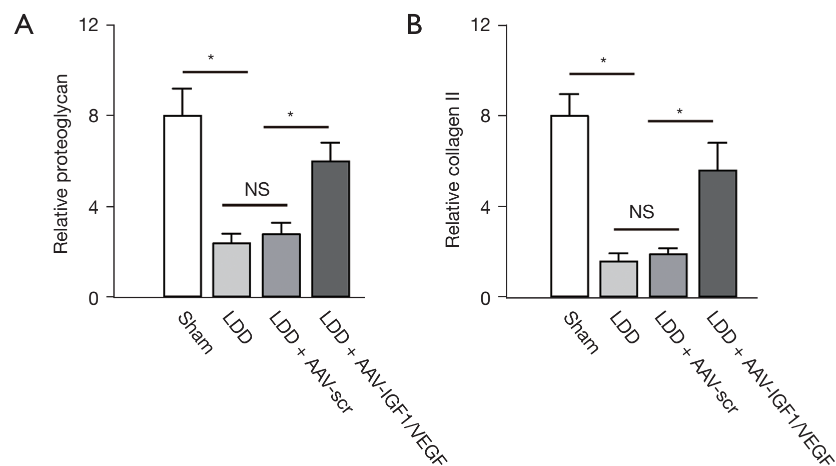

Figure 6 AAV-IGF1/shVEGF treatment enhances spinal proteoglycan and collagen II levels after LDD. (A,B) ELISA analysis of the levels of proteoglycan (A) and collagen II (B) in the rat vertebral pulp and annulus fibrosus 8 weeks after treatment. *P<0.05. NS: non-significant. N=10. AAV, adeno-associated virus; IGF1, insulin-like growth factor 1; VEGF, vascular endothelial growth factor; LDD, lumbar disc degeneration.

Hence, co-manipulation of both factors may be more effective for achieving therapeutic needs. Indeed, a previous study targeted both IGF1 and VEGF to treat a rodent model of amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease characterized by motor neuron cell death in the cortex, brainstem, and spinal cord (31). Therefore, in this study, we generated an AAV vector to specifically target both factors and evaluated its effects on LDD.

After *in vitro* and *in vivo* validation of the viral vectors, we showed that gene therapy co-manipulating IGF1 and VEGF inhibits LDD progression and severity. Decreases in both proteoglycan and collagen II that occur in disc matrix molecules are known to contribute to loss of disc function with ageing and disc degeneration (32,33). Moreover, the loss of biosynthetic capability of disc cells is important for the pathogenesis of LDD, which may be an important target for a successful therapy (32,33). Here, our intervention significantly attenuated changes in these molecules, and thus provides further promise for translating it into a treatment for patients.

To the best of our knowledge, this strategy using orthotopic AAV-based gene therapy targeting both the IGF1 pathway and angiogenesis is the first approach that successfully and effectively prevented LDD and is readily translatable to the clinic. AAVs are now used in clinical trials and this orthotopic injection can be easily performed in human patients. Hence, we have shown a novel strategy that can prevent LDD progression in a highly translatable way. Although this study provided a novel method to effectively treat LDD, the exact molecular mechanisms that underlie the cure are not fully understood. Moreover, the analysis on mouse behavior post treatment should provide additional confidence for this approach to be applied to human patients in the future. Thus, following studies may address the molecular changes in disc cells, endothelial cells, and inflammatory cells, since our approach may alter the interaction among these cells to lead to a therapeutic effect.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/atm-21-4977). The authors have no conflicts of interest to declare.

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The present study was approved by the Research and Animal Ethics Association of Zhongshan Hospital affiliated to Fudan University under a license number No. GDR452, in compliance with the institutional guidelines for the care and use of animals. A protocol was prepared before study without registration.

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