Original Article

Thymosin Beta-4 Recombinant Adeno-associated Virus Enhances Human Nucleus Pulposus Cell Proliferation and Reduces Cell Apoptosis and Senescence

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Abstract

Background: Thymosin beta-4 (TB-4) is considered key roles in tissue development, maintenance and pathological processes. The study aimed to prove TB-4 positive biological function on nucleus pulposus (NP) cell apoptosis and slowing the process of cell aging while increasing the cell proliferation.

Methods: TB-4 recombinant adeno-associated virus (AAV) was constructed and induced to human NP cells. Cell of same group were cultured without gene modification as controlled group. Proliferation capacity and cell apoptosis were observed during 6 passages of the cells. Morphology and expression of the TB-4 gene were documented as parameter of cell activity during cell passage.

Results: NP cells with TB-4 transfection has normal TB-4 expression and exocytosis. NP cells with TB-4 transfection performed significantly higher cell activity than that at the control group in each generation. TB-4 recombinant AAV-transfected human NP cells also show slower cell aging, lower cell apoptosis and higher cell proliferation than control group.

Conclusions: TB-4 can prevent NP cell apoptosis, slow NP cell aging and promote NP cell proliferation. AAV transfection technique was able to highly and stably express TB-4 in human NP cells, which may provide a new pathway for innovation in the treatment of intervertebral disc degenerative diseases.

Key words: Biological Treatment; Cell Aging; Degeneration of Intervertebral Disc; Thymosin Beta-4

Introduction

Degeneration of intervertebral discs (IVDs) is a significant contributor to low back pain, which is one of the most common musculoskeletal complaints in world-wide.[1,2] In most IVD degeneration cases, patients have low back pain, IVD herniation and different levels of limited activity, which significantly reduces their quality of life.[3] Anatomically, IVDs consist of the surrounding ligamentous annulus fibrosus (AF), the core nucleus pulposus (NP) and the capping endplate (EP), which work together to compose the semi-hard tissue that connects adjacent vertebral bodies.[4] From birth, IVDs contain a nourishing vascular system in AF and EP tissue. However, atrophy of the vessels usually takes place with increasing age in adults.[5] Insufficient nutrient levels place cells within IVDs, and especially the NP, at risk of cell death.[6]

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Degeneration of IVDs can result in instability of the spine, disc height loss, compression of nerve roots, and even degenerative lumbar scoliosis.[7] Starting as early as the 2nd decade of life and progressing with age, degeneration of IVDs is considered a chronic type of extracellular matrix (ECM) degradation.[8] Although, the etiology is still not completely known, it is believed that cell loss caused by cell death is one of the main contributors to the degeneration of IVDs and ECM.[9] In particular, increasing evidence indicates that IVD degeneration is mainly caused by cell death, including programmed apoptosis and nonapoptotic autophagy.[10] Due to the distance separating the nourishing vessels from the core of this anatomical niche, NP cells were shown to be the most degenerated amongst cells in IVD tissue.[11]

Thymosin beta-4 (TB-4) is a tiny 43 amino acid intracellular peptide and the most important member of the TB family. TB-4 is found in high concentrations in the cytoplasm of most types of cells and tissues, including cell fragments, platelets, leukocytes and the

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spleen.[12] TB-4 is considered to be a moonlighting peptide that exhibits various biological functions,[13] including key roles in tissue development, maintenance and pathological processes.[14,15] TB-4 has identical actin binding sites, and by forming a 1:1 complex with G-actin.[16] TB-4 inhibits polymerization to F-actin, which promotes cell migration and adhesion.[17] TB-4 also promotes angiogenesis by stimulating the differentiation of umbilical vein endothelial cells and the migration of other endothelial cells,[18] and this peptide functions as an anti-inflammatory factor in many disease models.[19,20] TB-4 not only down-regulates inflammatory mediators but also up-regulates preinflammatory cytokine suppressors, such as interleukin-10, and reduces the infiltration and adhesion of inflammatory cells.[12] Recently, increasing evidence has shown that TB-4 has a strong effect on preventing apoptosis and promoting tissue regeneration.[12] In particular, Sosne *et al*. found that down-regulating TB-4 expression *in vitro* using a gene-silencing approach reduced cell survival and induced hypoxia-induced cell apoptosis.[21] TB-4 has also been known as a potential target for many clinical diseases and is gaining attention in many medical fields.[22-25] Because of its ability to enhance Akt and integrin-linked kinase activation and suppress NF-kB activation, collagen synthesis and cardiomyocyte apoptosis, TB-4 has been discussed for its effect on improving therapeutic cardiac function and protecting the heart from damage following administration during the remodeling period postmyocardial ischemia.[24,26] Meanwhile, Morris *et al*. observed oligodendrogenesis and functional improvement in a rat model of multiple sclerosis and traumatic brain injury after TB-4 treatment.[13] In the field of IVD degeneration, Tapp *et al*. reported that TB-4 treatment reduced annulus cell apoptosis *in vitro*, which suggests that TB-4 may have therapeutic applications in therapies for IVD degeneration,[22] although the biological effect of TB-4 on NP cells has not yet been investigated.

Recombinant adeno-associated virus (AAV) vectors have emerged as a new vector for gene therapy, and they have been widely used in clinical trials to treat a variety of diseases.[27-29] Recently, these vectors have been evolved and diversified to appeal to a variety of gene delivery needs with excellent safety records and success rates.[30,31] Compared to other types of viral vectors, recombinant AAV vectors show the capacity for long-term gene expression and can autonomously replicate without a helper virus. In addition, their low pathogenicity and immunogenicity make recombinant AAV vectors a preferred model for researchers in the field of gene therapy.[32]

In this study, we hypothesized that TB-4 administration would decrease cell apoptosis and slow the process of cell aging while increasing the cell proliferation of NP cells. To this end, we constructed recombinant AAVs that encoded TB-4 and transfected them into NP cells. The target cells were obtained from a male patient who had undergone L4-5 NP resection.

Methods

Synthesis of thymosin beta-4 cDNA and cell culture of 293 cells

We synthesized TB-4 cDNA according to the human TB-4 coding sequence, which can be found in the nucleic acid database NM-021109.3. The synthesized gene sequence was transduced into the pGEM T easy vector to check the validity of the sequence. The 293 human embryonic kidney cell line was derived from the HEK 293 cell line obtained from the American Type Culture Collection. Two hundred and ninety-three cells were adapted to grow at 37°C in 5% CO_2 in Dulbecco's Modified Eagle's Medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibco, USA).

Construction of thymosin beta-4 recombinant adeno-associated virus

To gain access to the furin propeptide identification point located downstream of the PHG-NT4 signaling peptide, the *Nae* I enzyme sequence was added to the 5' end of the TB-4 synthesis sequence following the validity check of the TB-4 cDNA. Next, a *Bam*HI sequence was added to the 3' end, and the cDNA sequence was inserted into the AAV recombinant plasmid vector. Then, pAAV-helper, pAAVRC and NT4-Thymosin were co-transfected into 293 human embryonic kidney cells using the PEI transfection agent to package and obtain TB-4 recombinant AAV.[33]

Harvest and purification of thymosin beta-4 recombinant adeno-associated virus

After transfection for 72 h, the cell medium was removed from 293 cells, which were then rinsed once using phosphate-buffered saline (PBS) containing 2 mmol/L $CaCl_2$ and 2 mmol/L $MgCl_2$. The cells were then physically harvested into PBS containing calcium and magnesium using a cytobrush. The cells were harvested by centrifugation at 3000 r/min and suspended in pH 7.6 10 mmol/L HEPES buffer containing 150 mmol/L NaCl. The cells were frozen and thawed 3 times and then centrifuged at 8000 r/min; the supernatant was then harvested with 50% ammonium sulfate and stored overnight. The next day, the virus was extracted with sediment after being centrifuged at 10,000 r/min, dissolved in a PBS solution containing calcium and magnesium and dialyzed for 72 h with the dialysis agent changed every 12 h. After centrifuging the virus suspension at a speed of 10,000 r/min and discarding the undissolved sediment, a negative and positive ion double column-bed was used to purify and isolate the virus, which was stored at −80°C.

Titer and infection ability of the thymosin beta-4 recombinant adeno-associated virus

The virus sample suspension was digested with proteases, and the nucleic acid was extracted using the phenol-chloroform method. After separating with ethanol, the nucleic acids were dissolved in TE buffer. Then, a cytomegalovirus (CMV) promoter-area primer was used to measure viral copy concentration (pfu/ml) by fluorescein quantitative polymerase chain reaction (qPCR).

To test infection capacity, we packaged the TB-4 recombinant AAV with a green fluorescent protein (GFP) reporter gene into 293 cells. After 72 h of infection, flow cytometry was used to calculate the number of cells with GFP expression. The infection ability was calculated as the ratio of GFP-expressing cells to the total amount of virus used (pfu).

Cultivation and passage of nucleus pulposus cells

Harvest of nucleus pulposus tissue

The IVD tissue sample was obtained from an informed male participant with IVD herniation under the approval of the orthopedic department and the hospital's Ethics Committee. Fresh NP tissue was harvested via an IVD discectomy surgical procedure. The tissue was immediately washed with PBS to remove blood cells. The surrounding AF was carefully removed, and the NP was cut into pieces approximately 1 mm^3 in size.

Nucleus pulposus cell isolation and cultivation

The NP cells were isolated from the tissue sample by treatment with 0.2% collagenase type II in Dulbecco's Modified Eagle's Medium/F12 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA) and 1% penicillin/streptomycin at 37°C in a 5% CO_2 humidified incubator for 3 h. After replenishing the tissue with fresh collagenase type II and culture medium, the NP cells were further digested under the same conditions overnight to ensure that the cells were completely isolated from the tissue.

The enzymatically digested cells were filtered through 80-micron mesh cell sieves and suspended in the culture medium described above. The culture medium was changed every 2 days. When the cells reached 90% confluence after 10 days, they were treated with a 0.25% trypsin-ethylene diamine tetra acetic acid solution and subcultured in a 25-ml culture flask (1×10^5/ml, 5 ml per flask). This method was repeated until the NP cells stopped growing or could not reach confluence.[34,35]

Gene transduction of thymosin beta-4 and green fluorescent protein recombinant adeno-associated virus

To transfect recombinant AAV into human NP cells, passage 2 NP cells were inoculated to coverslips in uncovered six well plates preloaded with poly-L-lysine (1×10^5/ml, 1 ml per well). After allowing the cells to adhere for 24 h, TB-4, the control solution, and the GFP co-packaged recombinant AAV were added to the cells (100 μl, 1×10^{11} pfu/ml). After transfection for 72 h, the coverslips were removed from the 6 well plates. The morphology of the NP cells was observed under a phase-contrast microscope (Eclipse Ni-U, Nikon). NP cells were rinsed with PBS, fixed with acetone for 10 min, and then kept at 4°C for immunochemistry and immunofluorescent staining. The coverslips that were loaded with NP cells transfected with the GFP recombinant AAV were rinsed with PBS and then observed and photographed directly without fixation.

SA-β-Gal staining of human nucleus pulposus cells

Cell passage, the cell duplication period and intracellular SA-β-Gal were studied to evaluate the senescence of human NP cells using Severino's method.[36] NP cells were fixed at room temperature for 15 min using fixation buffer, washed with PBS and incubated in SA-β-Gal working solution at 37°C without CO_2 for 12 h. After washing thoroughly, the NP cells were dehydrated with ethanol at different concentrations and clarified with dimethylbenzene. The NP cells were observed using an optical microscope, and the progression of senescence was determined by calculating the ratio of SA-β-Gal-positive cells to the total number of cells.[37]

Determination of cell apoptosis

Nucleus pulposus cells were loaded onto coverslips as described above. Following transfection using TB-4 or control recombinant AAV for 72 h, transferase dUTP nick end labeling (TUNEL) staining was performed to measure cellular apoptosis. The cells were washed with PBS for 5 min at room temperature and were then treated with proteinase K (200 μg/ml) for 45 s and rinsed with PBS twice. Next, the cells were incubated with terminal deoxynucleotidyl transferase and dUTP labeled with DIG, and the cells were kept in a moisturizing chamber at 37°C for 2 h, which allowed the 3'-OH to be labeled by dUTP-DIG. After washing with TBS twice, the NP cells were blocked with 5% goat serum at 37°C for 30 min. Then, the cells were treated with anti-DIG-biotin at 37°C for 1 h and then washed with TBS 3 times. Following treatment with SABC-AP, BCIP/NBT was added to reveal the staining color. TUNEL-positive cells presented with blue color in the nucleus, and the positive cells could be observed and counted under an optical microscope as discussed above.

Determination of cell proliferation capacity

Cell proliferation capacity was determined by MTT staining. The NP cells were inoculated to a 96-well flask (5×10^3 per well). After adherence, TB-4 or control recombinant AAV (100 MOI) and an equal volume of medium were added to the NP cells. After incubation at 37°C for 72 h, the NP cells were treated with the MTT agent (20 μl per well). After shaking for 5 min, the cells were incubated at 37°C for another 2 h. Then, the solution in the wells was removed, 200 μl of dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) was added, and the absorbance at 490 nm was measured. Six wells were left without cells to function as a blank control; the absorbance values in the other wells are presented as mean ± standard deviation (SD).

Results

Accuracy of the recombinant vector gene sequence and recombinant adeno-associated virus vector infection

Identifying the gene sequence and testing the infection capacity was an important component of this study. After transducing the cDNA of TB-4 into the pGEM-T easy vector, restriction enzymes were used to identify

the plasmid vector. The recombinant vector had a band length of 400 bp, which demonstrated that the correct vector was constructed successfully [Figure 1a]. The CMV promoter of the purified recombinant AAV vector was amplified by qPCR. The upstream primer sequence is 5'-GCGTGTACGGTGGGAGGTCT-3', and the downstream primer sequence is 5'-CAATGTTGAGGGTGGGGGTT-3'. The concentration of the recombinant AAV was 1.2×10^{11} per ml. When the reporter gene had been identified in HEK 293 cells, flow cytometry analysis was conducted, which demonstrated that the infection rate of the recombinant AAV was 4.56% [Figure 1b].

Passage and morphology of human nucleus pulposus cells

The passage and activity of cells as well as the morphology of the cell group with and without TB-4 recombinant AAV transfection were observed and recorded. The NP cells in the control group generally showed polygon or rounded shapes with a few fusiform cells. The P2 generation of control NP cells was clearly larger in size [Figure 2a]. As the NP cells passed from P2 to P3, they experienced decreased cell activity, an extended cell duplication period, slowed cell growth, and irregular cell outlines. Signs of aging appeared at P2, and growth retardation appeared at P3 [Figure 2b].

Regarding TB-4 recombinant AAV-transfected NP cells, morphological changes involving cell aging and a lagging cell duplication period also appeared as the cells were passaged. However, the outline of the cell changed slowly, and growth retardation did not appear until the P6 generation. Importantly, the cellular activity at the P5 generation of TB-4 AAV-transfected cells was significantly higher than that at the P3 generation of the control group [Figure 2c-e].

Expression of the thymosin beta-4 gene in human nucleus pulposus cells posttransfection

Immunohistochemistry was used to detect the expression of transduced TB-4 along with the recombinant AAV construct. TB-4 expression was detected in both control and TB-4 recombinant AAV-transfected NP cells. In control NP cells, a small amount of endogenous TB-4 expression was detected, and TB-4 expression decreased as the cells were passaged [Figure 3a and b]. Meanwhile, a notable amount of TB-4 expression was detected in recombinant AAV-transfected NP cells in most generations. NP cell growth tended to assemble toward TB-4 high-expressing areas. We also discovered that TB-4 recombinant AAV-transfected cells secreted large amounts of TB-4 through the P6 generation [Figure 3c-e]. Under high-power magnification, we observed positively staining granules that were exocytosed from NP cells [Figure 3f].

To further demonstrate the exocytosis of TB-4 in recombinant AAV-transfected NP cells, we extracted mRNA and used the NT4 forward primer and TB-4 reverse primer to conduct reverse transcription-PCR to evaluate transcription of the NT4 signaling peptide-TB-4 fusion gene. We found that TB-4 recombinant AAV-transfected cells at the P2, P4 and P6 generations showed an amplification band at 375 bp, whereas no band was observed in control NP cells [Figure 3g], which indicated that TB-4 recombinant AAV-transfected NP cells undergo significant NT4-TB-4 transcription.

Thymosin beta-4 recombinant adeno-associated virus-transfected human nucleus pulposus cells show slower cell aging, lower cell apoptosis and higher cell proliferation

Thymosin beta-4 recombinant AAV-transfected NP cells were observed to undergo more passage generations and

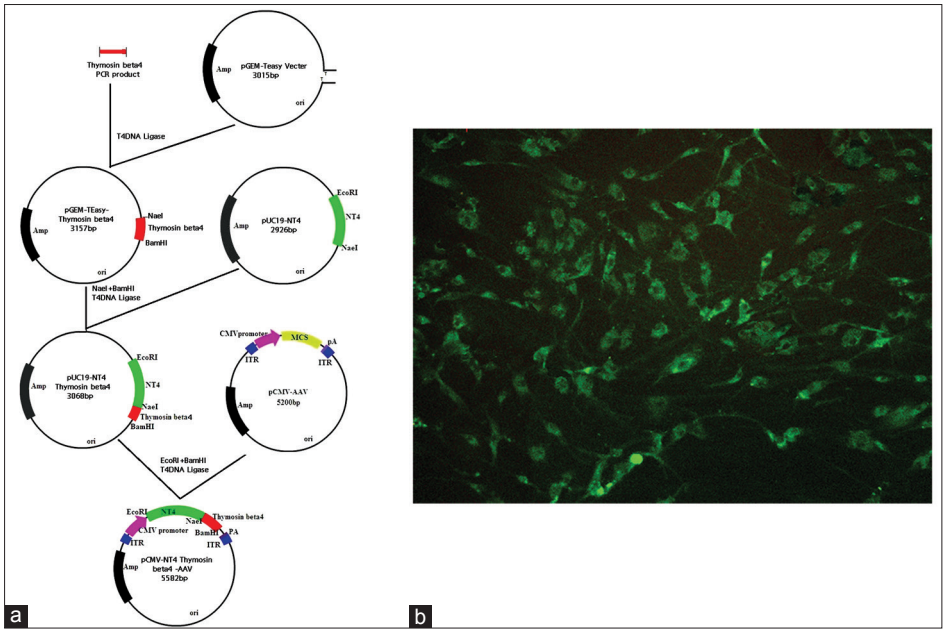

Figure 1: (a) Technical route of adeno-associated virus (AAV) recombinant vector construction; (b) Flow cytometry analysis showed infection ability of the recombinant AAV was 4.56%.

have shorter cell duplication periods than the control group *in vitro*, which suggested that the transfected cells had improved cellular activity. Furthermore, cell aging and apoptosis were also investigated. *In situ* SA-β-Gal cell staining was conducted for both control and transfected cells, and the P3 generations of both cell groups were compared.

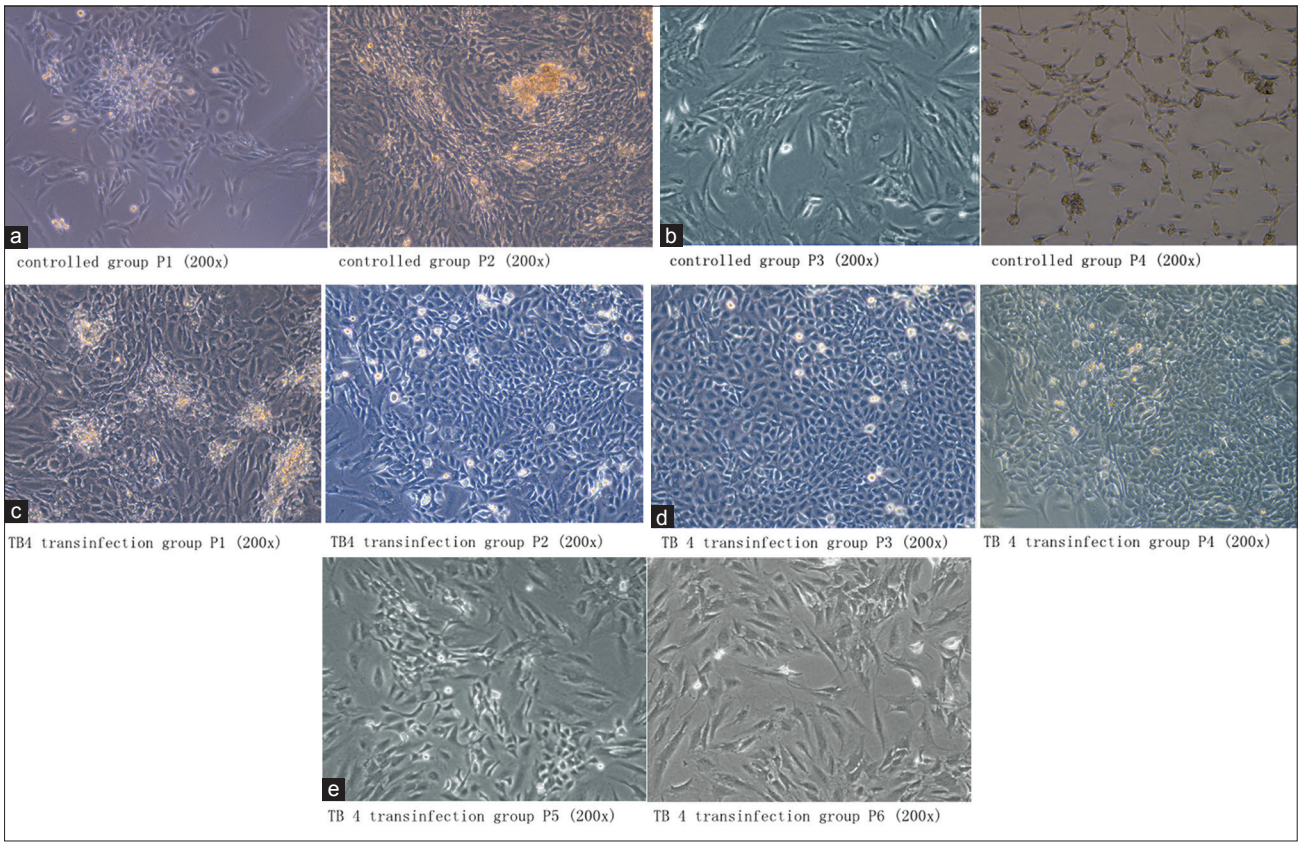

Figure 2: (a and b) Passage and the morphology of the human nucleus pulposus of controlled group P1-P4. Cell count and shape of the cell appeared degeneration in controlled group P2 and cessation of growth in P3; (c-e) Passage and the morphology of the human nucleus pulposus of thymosin beta-4 transinfection group P1-P6. Original magnification ×200.

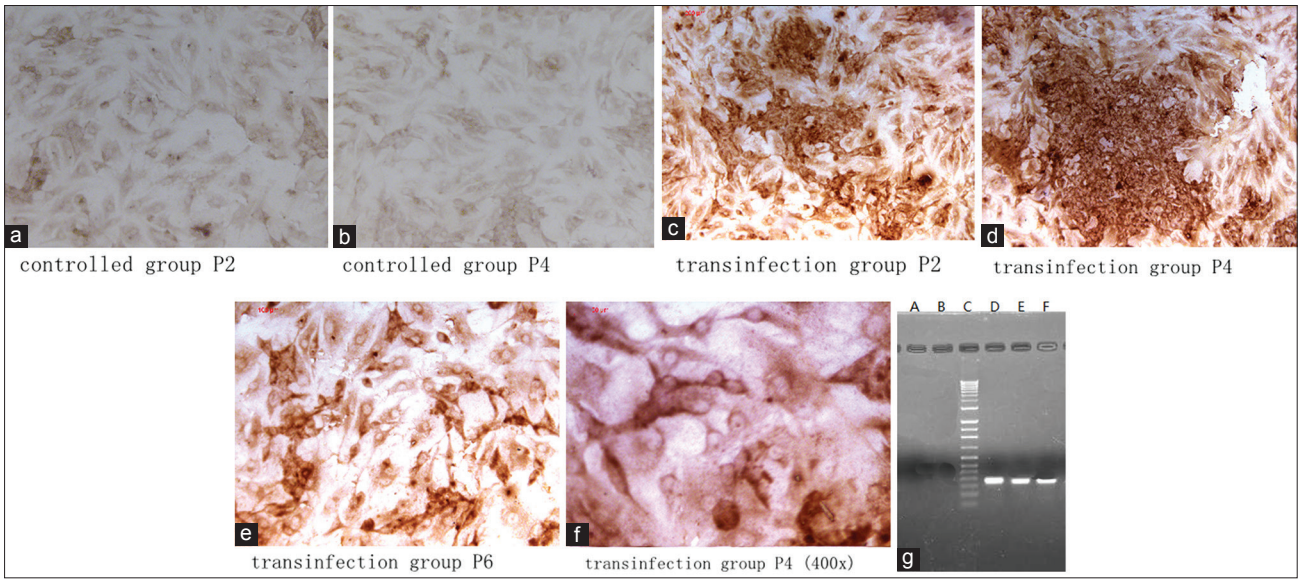

Figure 3: (a and b) Immunohistochemistry of thymosin beta-4 (TB-4) gene expression in human nucleus pulposus (NP) cells controlled group; (c-e) Immunohistochemistry of TB-4 gene expression in TB-4 transinfected human NP cells; (f) High power field of the microscope of transinfection group P4, positive staining granule exocytosed can be observed; (g) Reverse transcription polymerase chain reaction of TB-4 expression in cell groups, band of 375bp could be identified in transinfection group while no band was observed in controlled NP cells (A: Controlled group P2; (B: Controlled group P3; C: Marker; D: Transinfection group P2; E: Transinfection group P4; F: Transinfection group P6). a-f: Original magnification ×400.

The TB-4 recombinant AAV-transfected cells showed less staining than cells from the control group, which indicated that the transfected cells underwent slower cellular aging.

Regarding cell apoptosis, which is considered one of the main causes of IVD degeneration,[9] terminal deoxynucleotidyl TUNEL assays were performed for the P3 generations of cells with or without TB-4 recombinant AAV transfection. Compared to control NP cells, there were significantly fewer stained cells among the transfected cells, suggesting that TB-4 recombinant AAV transfection reduced apoptosis in human NP cells.

Cell proliferation represents direct evidence of cellular activity and has a strong effect on cell survival. The MTT method was used to evaluate the proliferative ability of transfected and control cells. After measuring the absorbance of the cell suspension, we found that TB-4 recombinant AAV-transfected cells showed elevated cell proliferation and more cell passages than normal human NP cells.

Discussion

Similar to other degenerate diseases, research on IVD degeneration therapy has blossomed as the development of cytobiology and molecular biology.[10] Because of the unique anatomical structure and stress distribution of the human spine, IVD degeneration and its complications have become quite common among the senior population. In the niche established by AF, NP and EP tissue, atrophy of the vessels along with increasing age results in vasculature that is only present in EP tissue, which means that the NP tissue in the center can only obtain nutrients via fluid flow or diffusion through the EP and AF tissues. As a result, the oxygen tension is reduced as the distance from the vasculature to the NP center increases. In NP tissue, hypoxia, low pH from high lactic acid concentrations due to long-term anaerobic metabolism and low nutrition caused by the distance between the NP tissue and nourishing vasculature significantly impact the survival of resident cells.[5,9,38] Cell death, including programmed cell death and necrosis, has been demonstrated to be the main contributor to IVD degeneration, and cell apoptosis, which is known as type I programmed cell death, has been identified as one of the main causes of IVD degeneration. Modulating levels of cytokines have also been shown to alter the pathways involved in cell apoptosis and aging, which highlights a potential therapeutic avenue for IVD degeneration.

Thymosin beta-4 is a tiny, naturally occurring 5 kDa peptide that was first isolated from the thymus in 1981 and has multiple biological functions. In corneal cells, TB-4 has been shown to suppress the activation of caspases,[23] which are involved in many aspects of cell apoptosis.[39] Moreover, TB-4 plays a significant role in wound healing and ECM remodeling in corneal cells. TB-4 is also involved in the synthesis of the cell skeleton and microtubules and in the differentiation of hair follicle and tooth enamel stem cells.[14] Furthermore, regenerative medicine studies have highlighted the potential for TB-4 to serve as a clinical target in a variety of diseases.

In this study, we constructed a recombinant AAV that encodes TB-4 using biological techniques and transfected this vector into NP cells obtained from the L1-2 segment of a patient with IVD degeneration. First, we ensured that the TB-4 synthesis sequence was correctly constructed, and our results showed that the recombinant AAV had high transfection efficiency and provided stable, continuous transcription in human NP cells. We also found that recombinant AAV transfection decreased cell apoptosis and cell aging and increased cell proliferation and the number of cell passages of human NP cells. Although abundant evidence has previously demonstrated that TB-4 can prevent cell apoptosis, our study is the first to demonstrate the effect of endogenous TB-4 expression transfected into degenerating human NP cells.

Thymosin beta-4 has varying effects on cell proliferation. Previously, it was reported that the presence of TB-4 could inhibit the proliferation and migration of human hepatic stellate cells.[40] In this study, we found that TB-4 recombinant AAV transfection increased NP cell proliferation and the number of cell passages rather than the absence of altering AF cell proliferation reported in other studies. We speculate that these different results are due to functional mechanistic differences between recombinant AAV-transfected eukaryotic- and prokaryotic-sourced cytokines. Indeed, we cannot ignore the issues regarding the use of cytokines secreted by prokaryotic cells or those artificially synthesized; regardless of whether they function *in vitro* or *in vivo*, all of these oligopeptides may hydrolyze under the influence of proteases and peptidases. Furthermore, the dose application is also an inevitable problem that will need to be addressed in future studies. However, under most circumstances, transfected cells are able to avoid these issues. Compared to the artificial application of medicine from other sources, the secreted products of transfected cells are more stable and continuous, and they closely resemble normal cellular secretion conditions in many ways. In this study, we believe that the use of recombinant AAV strongly overshadowed synthesis by prokaryotic cells or chemical protocols.

Our work demonstrates that TB-4 can prevent NP cell apoptosis, slow NP cell aging and promote NP cell proliferation. The NP cells used in this study were obtained directly from a patient with IVD degeneration, which is more biologically relevant than using cells obtained from cell lines or animal models. However, the NP cells are originated from a single individual due to the difficulty of obtaining human tissue samples, but we believe with other researchers paying more attention to the apoptosis/senescence preventability of TB-4, stronger evidence shall be revealed before long. We constructed and used TB-4 recombinant AAV to transfect human NP cells directly and have clearly demonstrated that our recombinant AAV transfection technique was able to highly and stably express TB-4 in human NP cells, which may provide a new pathway for innovation in the treatment of IVD degenerative diseases in the field of practical regenerative medicine.

References

1. Kepler CK, Ponnappan RK, Tannoury CA, Risbud MV, Anderson DG. The molecular basis of intervertebral disc degeneration. Spine J 2013;13:318-30.
2. Liu Y, Li JM, Hu YG. Transplantation of gene-modified nucleus pulposus cells reverses rabbit intervertebral disc degeneration. Chin Med J 2011;124:2431-7.
3. Hammill RR, Beazell JR, Hart JM. Neuromuscular consequences of low back pain and core dysfunction. Clin Sports Med 2008;27:449-62, ix.
4. Humzah MD, Soames RW. Human intervertebral disc: Structure and function. Anat Rec 1988;220:337-56.
5. Smith LJ, Nerurkar NL, Choi KS, Harfe BD, Elliott DM. Degeneration and regeneration of the intervertebral disc: Lessons from development. Dis Model Mech 2011;4:31-41.
6. Guehring T, Wilde G, Sumner M, Grünhagen T, Karney GB, Tirlapur UK, *et al.* Notochordal intervertebral disc cells: Sensitivity to nutrient deprivation. Arthritis Rheum 2009;60:1026-34.
7. Ding WY, Yang DL, Cao LZ, Sun YP, Zhang W, Xu JX, *et al.* Intervertebral disc degeneration and bone density in degenerative lumbar scoliosis: A comparative study between patients with degenerative lumbar scoliosis and patients with lumbar stenosis. Chin Med J 2011;124:3875-8.
8. Boos N, Weissbach S, Rohrbach H, Weiler C, Spratt KF, Nerlich AG. Classification of age-related changes in lumbar intervertebral discs: 2002 Volvo Award in basic science. Spine (Phila Pa 1976) 2002;27:2631-44.
9. Ding F, Shao ZW, Xiong LM. Cell death in intervertebral disc degeneration. Apoptosis 2013;18:777-85.
10. Freemont AJ. The cellular pathobiology of the degenerate intervertebral disc and discogenic back pain. Rheumatology (Oxford) 2009;48:5-10.
11. Gruber HE, Hanley EN Jr. Analysis of aging and degeneration of the human intervertebral disc. Comparison of surgical specimens with normal controls. Spine (Phila Pa 1976) 1998;23:751-7.
12. Crockford D, Turjman N, Allan C, Angel J. Thymosin beta4: Structure, function, and biological properties supporting current and future clinical applications. Ann N Y Acad Sci 2010;1194:179-89.
13. Morris DC, Zhang ZG, Zhang J, Xiong Y, Zhang L, Chopp M. Treatment of neurological injury with thymosin ß4. Ann N Y Acad Sci 2012;1269:110-6.
14. Philp D, St-Surin S, Cha HJ, Moon HS, Kleinman HK, Elkin M. Thymosin beta 4 induces hair growth via stem cell migration and differentiation. Ann N Y Acad Sci 2007;1112:95-103.
15. Hinkel R, El-Aouni C, Olson T, Horstkotte J, Mayer S, Müller S, *et al.* Thymosin beta4 is an essential paracrine factor of embryonic endothelial progenitor cell-mediated cardioprotection. Circulation 2008;117:2232-40.
16. Carlier MF, Didry D, Erk I, Lepault J, Van Troys ML, Vandekerckhove J, *et al.* Tbeta 4 is not a simple G-actin sequestering protein and interacts with F-actin at high concentration. J Biol Chem 1996;271:9231-9.
17. Huff T, Müller CS, Otto AM, Netzker R, Hannappel E. Beta-Thymosins, small acidic peptides with multiple functions. Int J Biochem Cell Biol 2001;33:205-20.
18. Sribenja S, Wongkham S, Wongkham C, Yao Q, Chen C. Roles and mechanisms of ß-thymosins in cell migration and cancer metastasis: An update. Cancer Invest 2013;31:103-10.
19. Malinda KM, Sidhu GS, Mani H, Banaudha K, Maheshwari RK, Goldstein AL, *et al.* Thymosin beta4 accelerates wound healing. J Invest Dermatol 1999;113:364-8.
20. Sosne G, Christopherson PL, Barrett RP, Fridman R. Thymosin-beta4 modulates corneal matrix metalloproteinase levels and polymorphonuclear cell infiltration after alkali injury. Invest Ophthalmol Vis Sci 2005;46:2388-95.
21. Sosne G, Qiu P, Goldstein AL, Wheater M. Biological activities of thymosin beta4 defined by active sites in short peptide sequences. FASEB J 2010;24:2144-51.
22. Tapp H, Deepe R, Ingram JA, Yarmola EG, Bubb MR, Hanley EN Jr, *et al.* Exogenous thymosin beta4 prevents apoptosis in human intervertebral annulus cells *in vitro*. Biotech Histochem 2009;84:287-94.
23. Sosne G, Qiu P, Kurpakus-Wheater M, Matthew H. Thymosin beta4 and corneal wound healing: Visions of the future. Ann N Y Acad Sci 2010;1194:190-8.
24. Gupta S, Kumar S, Sopko N, Qin Y, Wei C, Kim IK. Thymosin ß4 and cardiac protection: Implication in inflammation and fibrosis. Ann N Y Acad Sci 2012;1269:84-91.
25. Zhu T, Park HC, Son KM, Kwon JH, Park JC, Yang HC. Effects of thymosin ß4 on wound healing of rat palatal mucosa. Int J Mol Med 2014;34:816-21.
26. Thatcher JE, Welch T, Eberhart RC, Schelly ZA, DiMaio JM. Thymosin ß4 sustained release from poly (lactide-co-glycolide) microspheres: Synthesis and implications for treatment of myocardial ischemia. Ann N Y Acad Sci 2012;1270:112-9.
27. Mitchell AM, Nicolson SC, Warischalk JK, Samulski RJ. AAV's anatomy: Roadmap for optimizing vectors for translational success. Curr Gene Ther 2010;10:319-40.
28. Grieger JC, Samulski RJ. Adeno-associated virus vectorology, manufacturing, and clinical applications. Methods Enzymol 2012;507:229-54.
29. Ferla R, Claudiani P, Savarese M, Kozarsky K, Parini R, Scarpa M, *et al.* Prevalence of anti-adeno-associated virus serotype 8 neutralizing antibodies and arylsulfatase B cross-reactive immunologic material in mucopolysaccharidosis VI patient candidates for a gene therapy trial. Hum Gene Ther 2015;26:145-52.
30. Xiao PJ, Lentz TB, Samulski RJ. Recombinant adeno-associated virus: Clinical application and development as a gene-therapy vector. Ther Deliv 2012;3:835-56.
31. Ye GJ, Scotti MM, Thomas DL, Wang L, Knop DR, Chulay JD. Herpes simplex virus clearance during purification of a recombinant adeno-associated virus serotype 1 vector. Hum Gene Ther Clin Dev 2014;25:212-7.
32. Petrs-Silva H, Linden R. Advances in recombinant adeno-associated viral vectors for gene delivery. Curr Gene Ther 2013;13:335-45.
33. Reed SE, Staley EM, Mayginnes JP, Pintel DJ, Tullis GE. Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors. J Virol Methods 2006;138:85-98.
34. Tsai TT, Lai PL, Liao JC, Fu TS, Niu CC, Chen LH, *et al.* Increased periostin gene expression in degenerative intervertebral disc cells. Spine J 2013;13:289-98.
35. Iwasaki K, Sudo H, Yamada K, Higashi H, Ohnishi T, Tsujimoto T, *et al.* Effects of single injection of local anesthetic agents on intervertebral disc degeneration: *Ex vivo* and long-term *in vivo* experimental study. PLoS One 2014;9:e109851.
36. Severino J, Allen RG, Balin S, Balin A, Cristofalo VJ. Is beta-galactosidase staining a marker of senescence *in vitro* and *in vivo*? Exp Cell Res 2000;257:162-71.
37. Zheng Y, Xu Z. MicroRNA-22 induces endothelial progenitor cell senescence by targeting AKT3. Cell Physiol Biochem 2014;34:1547-55.
38. Sakai D, Grad S. Advancing the cellular and molecular therapy for intervertebral disc disease. Adv Drug Deliv Rev 2014; doi:10.1016/j.addr.2014.06.009.
39. Zhang L, Niu T, Yang SY, Lu Z, Chen B. The occurrence and regional distribution of DR4 on herniated disc cells: A potential apoptosis pathway in lumbar intervertebral disc. Spine (Phila Pa 1976) 2008;33:422-7.
40. Xiao Y, Qu C, Ge W, Wang B, Wu J, Xu L, *et al.* Depletion of thymosin ß4 promotes the proliferation, migration, and activation of human hepatic stellate cells. Cell Physiol Biochem 2014;34:356-67.

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