·Basic Research·

Human β-NGF gene transferred to cat corneal endothelial cells

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Abstract

• AIM: To transfect the cat corneal endothelial cells (CECs) with recombinant human β-nerve growth factor gene adeno-associated virus (AAV-β-NGF) and to observe the effect of the expressed β-NGF protein on the proliferation activity of cat CECs.

• METHODS: The endothelium of cat cornea was torn under the microscope and rapidly cultivated in Dulbecco's modified Eagle's medium (DMEM) to form single layer CECs and the passage 2 endothelial cells were used in this experiment. The recombinant human AAV-β-NGF was constructed. The recombinant human AAV-β-NGF was transferred into cat CECs directly. Three groups were as following: normal CEC control group, CEC-AAV control group and recombinant CEC-AAV-β-NGF group. Forty-eight hours after transfection, the total RNA was extracted from the CEC by Trizol. The expression of the β-NGF target gene detected by fluorescence quantitative polymerase chain reaction; proliferation activity of the transfected CEC detected at 48h by MTT assay; the percentage of G1 cells among CECs after transfect was detected by flow cytometry method (FCM); cell morphology was observed under inverted phase contrast microscope.

• RESULTS: The torn endothelium culture technique rapidly cultivated single layer cat corneal endothelial cells. The self-designed primers for the target gene and reference gene were efficient and special confirmed through electrophoresis analysis and DNA sequencing. Forty-eight hours after transfect, the human β-NGF gene mRNA detected by fluorescence quantitative polymerase chain reaction showed that there was no significant difference between normal CEC control group and CEC-AAV control group (P>0.05); there was significant difference between two control groups and recombinant CEC-AAV-β-NGF group (P<0.05). MTT assay showed that transfect of recombinant AAV-β-NGF promoted the proliferation activity of cat CEC, while there was no significant difference between normal CEC control group and CEC-AAV control group (P>0.05). FCM result showed that the percentage of G1cells in CEC-AAV-NGF group was 76.8% while that in normal CEC control group and CEC-AAV control group was 46.6% and 49.8%.

• CONCLUSION: Recombinant AAV-β-NGF promotes proliferation in cat CECs by expressing bioactive β-NGF protein in high efficiency and suggests that its modulation can be used to treat vision loss secondary to corneal endothelial dysfunction.

• **KEYWORDS:** nerve growth factor; corneal endothelial cell; transfect; proliferation
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INTRODUCTION

Cornea is one of the most important refracting media, its transparency and integrity are necessary to maintain visual function. Due to defect of regeneration ability, the healing of trauma in cornea is limited. Genetic therapy is a relatively new method, the high efficiency adeno-associated virus (AAV) vectors greatly facilitated the clinical application of genetic therapy. AAV has great superiorities such as low pathogenicity, extensive host cell, high titre and bigger package capability and so on. Nerve growth factor (NGF) is one of the neurotrophin family, which could promote the proliferation and differentiation of neurocyte and many non-neuron cells [1]. In this study, we constructed AAV-β-NGF vector, transfered it to *in vitro* cultured cat corneal endothelial cells (CECs), expressed bioactive β-NGF protein in high efficiency and promoted the proliferation of CECs so as to lay a basis for the treatment of corneal endothelium blind.

MATERIALS AND METHODS

Design and Synthesize of Primers Specifically designed probe primers and fluorescent probes with ABI Primer

Express software and analyzed with BLAST. 1) Human *β-NGF* gene: the forward and reverse primers were as follows 5'-CAC ACT GAG GTG CAT AGC GT -3', 5'-TGA TGA CCG CTT GCT CCT GT -3'. Fluorescent probes: 5'-ATC TGG ACT TCG AGG TCG GTG GTG C -3', the length of the target gene is 182 bp. 2) Cat glyceraldehyde-3-phosphate dehydrogenase (endo-reference): the forward and reverse primers were as follows 5'-CTT AGC ACC CCT GGC CAA G -3', 5'-GAT GTT CTG GAG AGC CCC G -3'. Fluorescent probes: 5'-CAT GCC ATC ACT GCC ACC CAG AAG A -3', the length of the target gene is 146 bp.

Primary Culture of Cat Corneal Endothelial Cells All animal procedures performed in this study complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care and Use Committee of the Affiliated Hospital of Qingdao University. Two months old cats, with no limit to sexuality, healthy without medical history were used in this study. Cats were anesthetized by chloral hydrate. Totally 120 cat eye balls were enucleated and immersed in D-Hanks solution with 100 μg/mL penicillin and 100 μg/mL phytomycin for 30min and then rinsed with sterile water, ruled out abnormality with examination. The corneas were excised under sterile condition and placed in a petri dish containing DMEM. Under a dissecting microscope, Descemet's membrane attached with endothelium was stripped from the stroma and placed in a 15-mL centrifuge tube containing 0.25% trypsin, incubated for 10min at 37℃. Cells were detached by vigorous disruption with a flame-polished pipette, centrifuged and resuspended in culture medium DMEM with 0.5% fetal bovine serum then were incubated in tissue culture bottles at 37℃ in a 5% CO_2 humidified atmosphere. Medium was changed every other day. Cells reached confluence in 10-14d. Monolayer cultures of cat endothelial cells were harvested using 0.05% trypsin/0.02% EDTA solution.

Immunocytochemistry Stainning of Cat Corneal Endothelium Cells Neuron specific enolase (NSE) is the specific mark protein of cat corneal endothelial cell, which could effectively distinguish endothelial cell from keratocyte. CEC could keep expressing NSE even after 20 passages while keratocyte never express NSE. Immunocytochemistry stainning was performed to identify the corneal endothelium cells with anti-NSE antibodies. Briefly, 1×10^4 cells growing in chamber slides (Nalge Nunc International, Rochester, NY, USA) were fixed with 4% paraformaldehyde, rinsed with phosphate buffered saline (PBS) and permeabilized with ice-cold acetone. Non-specific binding was blocked by incubating cells in 1% bovine serum albumin (BSA) for 30min at room temperature. Added with anti-NSE antibodies (1:250 in PBS, Invitrogen Molecular Probes), cells were incubated overnight at 4℃, then rinsed with PBS. The second antibody was then applied for 1h at room temperature. After rinsed with PBS, cells were applied with ABC elite and DAB. Rinsed with ddH_2O for 3 times, cells were then coverslipped with Geltol (Thermo Electron Corp., Waltham, MA, USA) as a mounting media and viewed under inverted phase contrast microscope.

Transfer *β-NGF* Gene to *in Vitro* Cultured Corneal Endothelial Cells There are three groups in the study: normal CEC control group, CEC-AAV control group and recombinant CEC-AAV-β-NGF group. The adeno-associated virus was diluted with DMEM to 1×10^9 in tite, discard the supernatant liquid 4h later, then was added to DMEM with 10% blood serum. Two repeated wells were designed for each group. All the cells were harvested at 12h, 1, 3, 5 and 7d after transfection.

***β-NGF* Gene Expression Assay** Total CEC RNA in three groups was isolated by Trizol reagent. 1) The purified RNA was analyzed by agrose gel electrophoresis and quantified spectraphotometrically. The *β-NGF* cDNA was synthesized in 10 μL real-time polymerase chain reaction (RT-PCR) mixture: 5×ExScript™ Buffer 2 μL, 10 mmol/L dNTP Mixture 0.5 μL, 100 μmol/L Random 6mers 0.5 μL, ExScript™ Rtase (200 U/μL) 0.25 μL, RNase Inhibitor (40 U/μL) 0.25 μL, total RNA 0.5 μg. Reverse transcription at 42℃ for 12min, then reverse transcriptase was deactivation at 95℃ for 2min. 2) Synthesize with specific primers: specifically designed probe primers of humanβ-NGF gene and cat glyceraldehyde-3-phosphate dehydrogenase were applied to PCR reaction. The reaction mixture was firstly pre-denatured at 95℃ for 10s, denatured at 95℃ for 5s, and 45s extension at 60℃, amplified in 40 cycles. 3) fluorescent quantitation PCR reaction: the reaction mixture was 20 μL includeμ L: 2×Premix ExTaqTM buffer 10 μL, primers concentration 0.2 μmol/L, probes concentration 0.05 μmol/L, 50×ROX Reference Dye Ⅱ 0.4 μL, cDNA 2 μL. The reaction mixture was firstly pre-denatured at 95℃ for 10s, denatured at 95℃ for 5s, and 45s extension at 60℃, amplified in 40 cycles. The mean CT value of the two values in each group was analyzed by $2^{-\triangle\triangle CT}$ method to get the relative express quantity. △△CT= (CTtarget gnen-CTendo-reference gene)transfected-(CTtarget gnen-CTendo-reference gene) control. Here the CT value means the cycles for the reaction mixture to reach the fluorescent threshold in PCR reaction.

Cell Proliferation Assay Cell proliferation was tested by modified MTT method. Forty-eight hours after being transfected, CECs in three groups were subcultured in 5.0×10^4/mL to 96-well tissue culture plate for another 24h. For MTT assay, cells were switched to MTT solutions (5 mg/mL) 20 μL, 37℃, 5% CO_2 for 4h, then solution was discarded and 150 μL dimethyl sulfoxide (DMSO) was added to each well, traced blender shock for 10min, then detected the OD value in 490 nm.

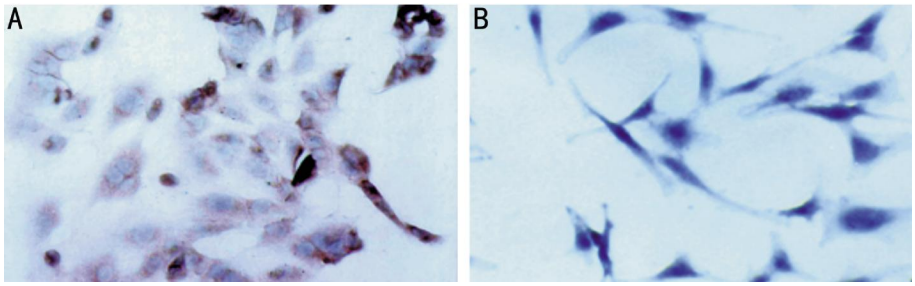

Figure 1 NSE staining of cat endothelial cells and keratocyte A: NSE staining was positive in cat endothelial cells (×400); B: Keratocyte had no specific antigen staining (×400).

Detect percentage of G1 cells by flow cytometry method Twenty-four hours after transfect passaged the CEC by 1:2 and subcultured for 48h. Digested cells with 0.05% trypsin, centrifuged and resuspended cells in ice cold PBS to remove cell debris in medium. Filetered the cell suspension with 500 mesh nylon network to get cell in 1×10^6/mL. Stained cells according to the study plan and detected the cell cycle with FCM.

Morphology observation Keep observing the cells growth condition under inverted phase contrast microscope after transfection. Pick up 10 fields randomly, count the cell numbers and get the mean values. At 24, 48h and 5d after transfection, evaluated the differences of CECs proliferation between recombinant CEC-AAV-β-NGF group and two control groups .

Statistical Analysis Statistical analyses of quantity-PCR and MTT results were performed with SigmaStat 11.5 (SPSS) software. Differences between groups were assessed with a t-test and followed by a Tukey (Student-Neumann-Keuls) multiple comparisons of means tests. Data are expressed as means ±SD. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

Primary Cultured Cell Morphology Observation CECs adhered in 2-3d primary culture, two weeks later expanded to massive single layer cells in shapes of similar circular and polygon. Cells were passaged and inoculated in 96-well board, 24h later most of them adhered.

Immunocytochemistry Stainning of Cat Corneal Endothelial Cells NSE staining show buffy macrobead could be found in the cytoplasm of corneal endothelium cells with over 98% positive rate (Figure 1A), while NSE was negative in keratocyte control group (Figure 1B).

Synthesize with Specific Primers The total RNA of all groups is 1.8-2.0 in A_{260}/A_{280}. The electrophoresis result of PCR product shows a line in 182 bp and 146 bp, which is consist with human *β-NGF* gene and cat glyceraldehyde-3-phosphate dehydrogenase gene (Figure 2). The sequencing result shows the 182 bp line is human *β-NGF* gene which is totally consist with genebank.

Fluorescent Quantitation Polymerase Chain Reaction The results show that fluorescent quantitation PCR in every group got classic S shape curve. The Ct value of glyceraldehyde-3-phosphate dehydrogenase group diversified trifle which means the initiate quantity of templates in each group is almost the same. At 12h, 1, 3, 5 and 7d after transfection, the human *β-NGF* gene mRNA detected by fluorescence quantitative polymerase chain reaction showed: there were no significant differences between normal CEC control group and CEC-AAV control group ($P>0.05$); there were significant differences between the two control groups and recombinant CEC-AAV-β-NGF group in all the five time point ($P<0.05$); there were significant difference of *β-NGF* gene mRNA express in recombinant CEC-AAV-β-NGF group of different time points (Table 1, Figure 3).

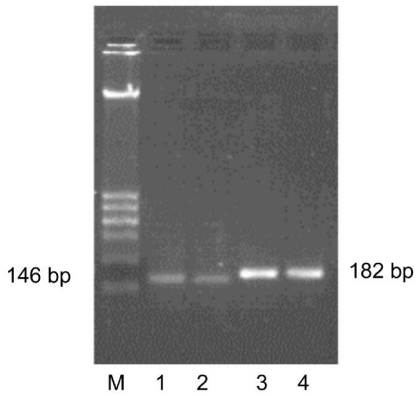

Figure 2 Agarose gel electrophoresis of polymerase chain reaction products of target genes M: 100-600 bp marker; 1,2: cat glyceraldehyde-3-phosphate dehydrogenase gene (146 bp); 3,4: human *β-NGF* gene (182 bp).

Biological Activities of the Expressed *β-NGF* In this study, the bioactivity of expressed *β-NGF* was detected based on the fact that it could promote CEC cells survival and proliferation. The MTT results showed 3d after transfection, the expressed *β-NGF* could obviously promote survival and proliferation of cat endothelial cells in CEC-AAV-β-NGF group ($P<0.01$ *vs* the two control groups). Two control groups showed no significant differences ($P>0.05$) in between. Blank AAV did not change the proliferation activity of CEC and there was no toxic effect of AAV to CEC (Table 2).

Detect Percentage of G1 Cells by Flow Cytometry Method FCM showed 3d after transfection, percentage of G1 cells in normal CEC control group is 46.6% , in

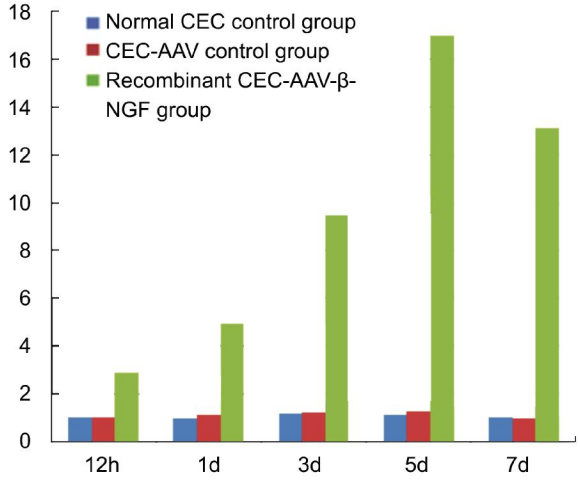

Figure 3 Relative quantitative analysis of human β–NGF mRNA.

Table 1 Relative quantitative analysis of human β-NGF mRNA ($2^{-\Delta\Delta Ct}$) $\bar{x} \pm s$

Group	Time	$2^{-\Delta\Delta Ct}$
A1	12h	1.0044±0.1812
A2	1d	0.9612±0.2031
A3	3d	1.1329±0.3062
A4	5d	1.1265±0.2843
A5	7d	0.9857±0.1328
B1	12h	1.0142±0.2039
B2	1d	1.1032±0.2121
B3	3d	1.1858±0.2962
B4	5d	1.2533±0.3127
B5	7d	0.9766±0.2051
C1	12h	2.8481±0.3985
C2	1d	4.9018±0.5290
C3	3d	9.5574±0.9528
C4	5d	16.9411±2.1384
C5	7d	13.0653±1.8124

A: Normal CEC control group; B: CEC-AAV control group; C: Recombinant CEC-AAV-β-NGF group.

Table 2 MTT result of 3d after transfection

Blank medium	Normal CEC control	CEC-AAV control	CEC-AAV-β-NGF
0.255	1.625	1.502	2.638
0.242	1.641	1.531	2.753
0.273	1.614	1.624	2.687
0.234	1.586	1.543	2.861
0.263	1.609	1.590	2.569
0.257	1.592	1.649	2.826

C EC-AAV control group is 49.8% while in recombinant CEC-AAV-β-NGF group is 76.8%. The result means that transfection of AAV-β-NGF could put more cells to G1 stage while blank AAV hadn't show this function (Figure 4).

Morphologic Changes of Corneal Endothelial Cells After Being Transfected CEC being transfected with recombinant CEC-AAV-β-NGF group proliferated obviously faster compared to the two control groups. After the first 24h, cells in all three groups proliferated in shapes of roundness and polygon in small scales. However, 48h later, cells transfected with recombinant CEC-AAV-β-NGF proliferated into bigger scales in shapes of regular triangle to hexagon with distinct boundary, while the number of cells was significantly less in the two control groups (Figure 5).

DISCUSSION

NGF is the first discovered and best-characterized member of the neurotrophin family [1]. It is produced by and acts upon cells of the visual system, both *in vitro* and *in vivo* and it is able to promote the functional recovery of retinal ganglion cells (RGCs) in an animal model of ocular ischemia and following optic nerve section, to reduce retinal cell damage induced by intraocular hypertension and to delay retinal cell degeneration in rodents with retinitis pigmentosa [2-7]. These effects are mediated by two NGF-receptors, the high-affinity receptor tyrosine kinase (TrkA), and the low-affinity receptor p75 neurotrophin receptor (p75), both located on the surface of NGF-responsive cells. Altered expression of these receptors and/or their ligands can lead to NGF-target cell degeneration [8]. NGF is present in the aqueous humor, increases following ocular injuries, and binds to its specific receptors expressed by the corneal endothelium. It has also been demonstrated that topical NGF eye drops administration promotes corneal healing and exerts anti-inflammatory and immunomodulatory actions on corneal endothelial cells[9-11].

The NGF in the anterior segment played an important role in tissue maintenance and wound healing [12]. High-affinity receptors of NGF are readily expressed on corneal tissues and are able to bind NGF [10-13]. Topical NGF treatment was found to have a profound effect on corneal wound healing while restoring corneal epithelium and improving stromal and endothelial cell function[9,14]. In conclusion, NGF has not only effects of nerve growth and regeneration but also pleiotropic effects on wound healing and tissue reconstruction[15-20].

AAV vectors can efficiently initiate sustained transgene expression *in vivo* and appear to be safe. With the identification of different serotypes and recent progress in the improvement of AAV vectors, such as dual vectors to overcome the limited packaging capacity, self-complementary vectors to increase the level and onset of transgene expression, and capsid modifications to mediate cell specific transduction, it will be possible in the future to design more specific and efficient therapies for use in the gene treatment area [21-23].

In this study, we clone β-NGF gene into AAV vector to construct AAV-β-NGF vector and transfer it to *in vitro* cultured second passage cat corneal endothelial cells. There are three groups: normal CEC group, CEC-AAV control group and CEC-AAV-β-NGF group. Forty-eight hours after transfection, we detected the expression of β-NGF in CEC-AAV-β-NGF group was much higher than the other two control groups. The result of MTT test also showed the proliferation ability was much higher in CEC-AAV-β-NGF

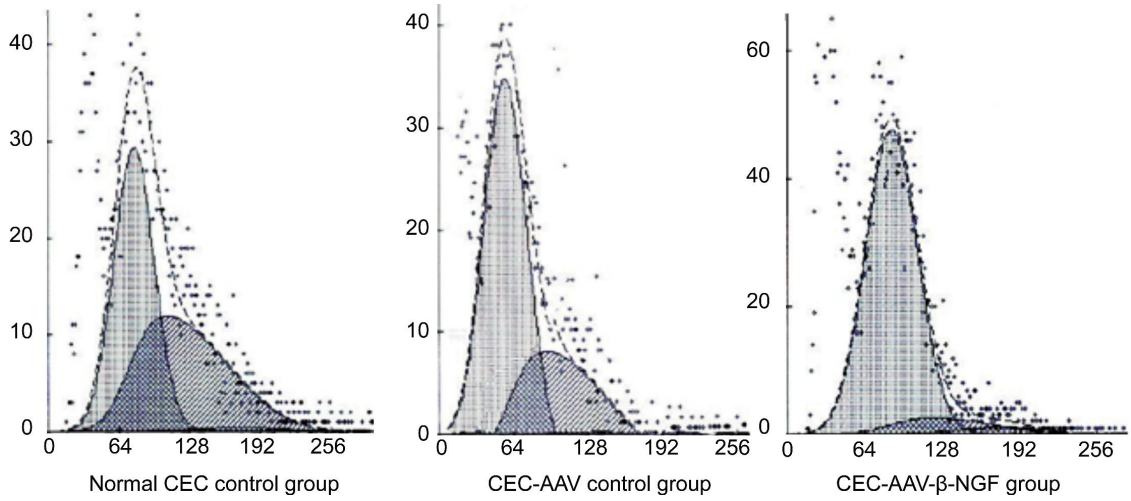

Figure 4 FCM result of percentage of G1 cells 3d after transfection.

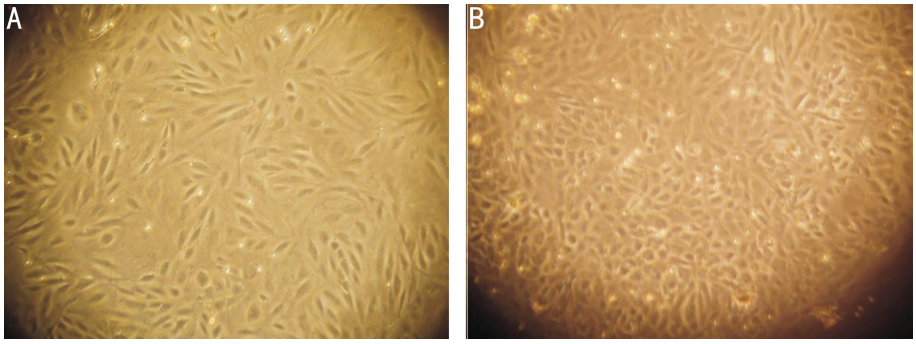

Figure 5 Cells cultured for 48h after AAV-β-NGF transfection (×200) A: Blank control group; B: AAV-β-NGF group.

group than the other two control groups. Twenty-four hours after transfection, CECs in all three groups were passaged by 1:2 and cultured for another 48h followed by FCM test. The result showed percentage of G1 cells in CEC-AAV-β-NGF group is 76.8%, while that is 46.6% in CEC group and 49.8% in CEC-AAV group. All the results above showed that transfection of CEC-AAV-β-NGF could prominently promote the proliferation of *in vitro* cultured cat CECs and AAV has no toxicity in the transfection.

Herein, we report the potential of *β-NGF* gene therapy as a promising approach to promote corneal endothelial cells survival and proliferation thus provide further insight into the mechanisms of corneal endothelium blind treatment.

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Conflicts of Interest: Luo WJ, None; **Liu M,** None; **Zhao GQ,** None; **Wang CF,** None; **Hu LT,** None; **Liu XP,** None.

REFERENCES

1 Levi-Montalcini R. The nerve growth factor 35 years later. *Science* 1987;237(4819):1154-1162.

2 Rocco ML, Balzamino BO, Petrocchi Passeri P, Micera A, Aloe L. Effect of purified murine NGF on isolated photoreceptors of a rodent developing retinitis pigmentosa. *PLoS One* 2015;10(4):e0124810.

3 Oku H, Ikeda T, Honma Y, Sotozono C, Nishida K, Nakamura Y, Kida T, Kinoshita S. Gene expression of neurotrophins and their high-affinity Trk receptors in cultured human Müller cells. *Ophthalmic Res* 2002;34(1):38-42.

4 Siliprandi R, Canella R, Carmignoto G. Nerve growth factor promotes functional recovery of retinal ganglion cells after ischemia. *Invest Ophthalmol Vis Sci* 1993;34(12):3232-3245.

5 Carmignoto G, Maffei L, Candeo P, Canella R, Comelli C. Effect of NGF on the survival of rat retinal ganglion cells following optic nerve section. *J Neurosci* 1989;9(4):1263-1272.

6 Lambiase A, Centofanti M, Micera A, Manni GL, Mattei E, De Gregorio A, de Feo G, Bucci MG, Aloe L. Nerve growth factor (NGF) reduces and NGF antibody exacerbates retinal damage induced in rabbit by experimental ocular hypertension. *Graefe's Arch Clin Exp Ophthalmol* 1997;235(12):780-785.

7 Lenzi L, Coassin M, Lambiase A, Bonini S, Amendola T, Aloe L. Effect of exogenous administration of nerve growth factor in the retina of rats with inherited retinitis pigmentosa. *Vision Res* 2005;45(12):1491-1500.

8 Meakin SO, Shooter EM. The nerve growth factor family of receptors. *Trends in Neurosciences* 1992;15(9):323-331.

9 Lambiase A, Rama P, Bonini S, Caprioglio G, Aloe L. Topical treatment with nerve growth factor for corneal neurotrophic ulcers. *N Engl J Med* 1998;338(17):1174-1180.

10 Lambiase A, Manni L, Bonini S, Rama P, Micera A, Aloe L. Nerve growth factor promotes corneal healing: structural, biochemical, and molecular analyses of rat and human corneas. *Invest Ophthalmol Vis Sci* 2000;41(5):1063-1069.

11 Cellini M, Bendo E, Bravetti GO, Campos EC. The use of nerve growth factor in surgical wound healing of the cornea. *Ophthalmic Res* 2006;38(4):177-181.

12 Klenkler B, Sheardown H. Growth factors in the anterior segment: role in tissue maintenance, wound healing and ocular pathology. *Exp Eye Res* 2004;79(5):677-688.

13 Micera A, Lambiase A, Puxeddu I, Aloe L, Stampachiacchiere B, Levi-Schaffer F, Bonini S, Bonini S. Nerve growth factor effect on human primary fibroblastic-keratocytes: possible mechanism during corneal healing. *Exp Eye Res* 2006;83(4):747-757.

14 You L, Kruse FE, Völcker HE. Neurotrophic factors in the human cornea. *Invest Ophthalmol Vis Sci* 2000;41(3):692-702.

15 Fink DM, Connor AL, Kelley PM, Steele MM, Hollingsworth MA, Tempero RM. Nerve growth factor regulates neurolymphatic remodeling during corneal inflammation and resolution. *PLoS One* 2014;9(11):e112737.

16 Sornelli F, Lambiase A, Mantelli F, Aloe L. NGF and NGF-receptor expression of cultured immortalized human corneal endothelial cells. *Mol Vis* 2010;16:1439-1447.

17 Chen L, Wei RH, Tan DT, Beuerman RW, Li W, Zhao S. Nerve growth factor expression and nerve regeneration in monkey corneas after LASIK. *J Refract Surg* 2014;30(2):134-139.

18 Sarkar J, Chaudhary S, Jassim SH, Ozturk O, Chamon W, Ganesh B, Tibrewal S, Gandhi S, Byun YS, Hallak J, Mahmud DL, Mahmud N, Rondelli D, Jain S.CD11b+GR1+ myeloid cells secrete NGF and promote trigeminal ganglion neurite growth: implications for corneal nerve regeneration. *Invest Ophthalmol Vis Sci* 2013;54(9):5920-5936.

19 Blanco-Mezquita T, Martinez-Garcia C, Proença R, Zieske JD, Bonini S, Lambiase A, Merayo-Lloves J. Nerve growth factor promotes corneal epithelial migration by enhancing expression of matrix metalloprotease-9. *Invest Ophthalmol Vis Sci* 2013;54(6):3880-3890.

20 Hong J, Qian T, Le Q, Sun X, Wu J, Chen J, Yu X, Xu J. NGF promotes cell cycle progression by regulating D-type cyclins via PI3K/Akt and MAPK/Erk activation in human corneal epithelial cells. *Mol Vis* 2012;18:758-764.

21 Li C, Bowles DE, van Dyke T, Samulski RJ. Adeno-associated virus vectors: potential applications for cancer gene therapy. *Cancer Gene Ther* 2005;12(12):913-925.

22 Sondergaard PC, Griffin DA, Pozsgai ER, Johnson RW, Grose WE, Heller KN, Shontz KM, Montgomery CL, Liu J, Clark KR, Sahenk Z, Mendell JR, Rodino-Klapac LR. AAV.Dysferlin Overlap Vectors Restore Function in Dysferlinopathy Animal Models. *Ann Clin Transl Neurol* 2015;2(3):256-270.

23 Rossmiller BP, Ryals RC, Lewin AS. Gene therapy to rescue retinal degeneration caused by mutations in rhodopsin. *Methods Mol Biol* 2015;1271:391-410.