Construction of a plasmid for human brain-derived neurotrophic factor and its effect on retinal pigment epithelial cell viability

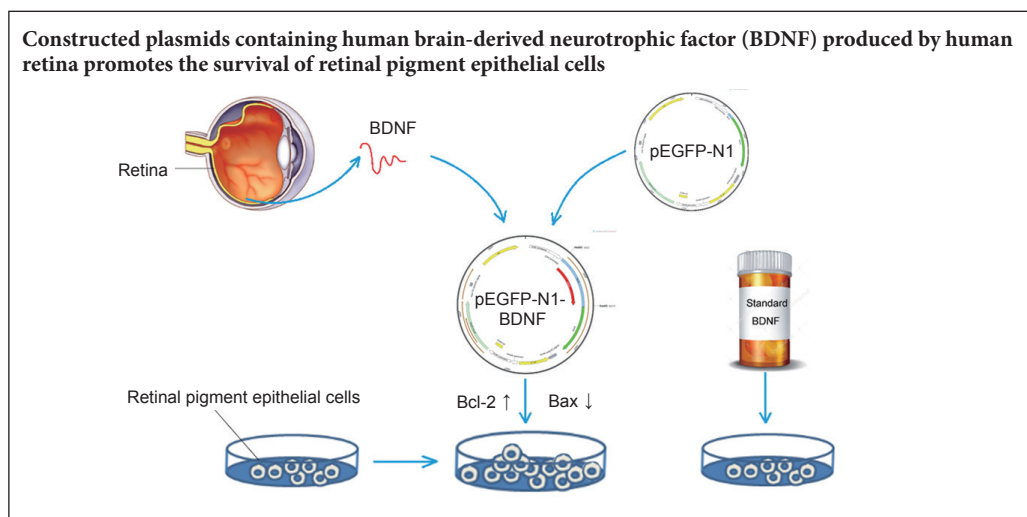Bo-jing Yan, Zhi-zhong Wu, Wei-hua Chong, Gen-lin Li[*]

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Graphical Abstract

Constructed plasmids containing human brain-derived neurotrophic factor (BDNF) produced by human retina promotes the survival of retinal pigment epithelial cells

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Abstract

Several studies have investigated the protective functions of brain-derived neurotrophic factor (BDNF) in retinitis pigmentosa. However, a BDNF-based therapy for retinitis pigmentosa is not yet available. To develop an efficient treatment for fundus disease, an eukaryotic expression plasmid was generated and used to transfect human 293T cells to assess the expression and bioactivity of BDNF on acute retinal pigment epithelial-19 (ARPE-19) cells, a human retinal epithelial cell line. After 96 hours of co-culture in a Transwell chamber, ARPE-19 cells exposed to BDNF secreted by 293T cells were more viable than ARPE-19 cells not exposed to secreted BDNF. Western blot assay showed that Bax levels were downregulated and that Bcl-2 levels were upregulated in human ARPE-19 cells exposed to BDNF. Furthermore, 293T cells transfected with the *BDNF* gene steadily secreted the protein. The powerful anti-apoptotic function of this BDNF may be useful for the treatment of retinitis pigmentosa and other retinal degenerative diseases.

Key Words: nerve regeneration; neurodegenerative disease; brain-derived neurotrophic factor; retinitis pigmentosa; retina; retinal pigment epithelium; biosynthesis; transfection; plasmids; green fluorescent protein; apoptosis; cell survival; neural regeneration

Introduction

Retinitis pigmentosa is a degenerative, inherited disease that results from the progressive degeneration of photoreceptors in the retina (Hartong et al., 2006). Evaluation of retinal function has shown that photoreceptor function begins to diminish many years before the symptoms of night blindness, decreased visual acuity and visual-field scotomas. This progressive photoreceptor degeneration is followed by retinal pigment epithelial and retinal ganglion cell abnormalities.

Retinitis pigmentosa is currently the leading cause of incurable blindness during working life (Haim, 2002). The prevalence in northern China is approximately 1 in 4,000 (You et al., 2013), which is similar to the worldwide rate (Hartong et al., 2006). Hence, effective clinical treatment for retinitis pigmentosa is urgently needed. Over the last few decades, a variety of experimental therapies aimed at repairing

or rescuing impaired vision have been investigated, including gene therapy (Beltran et al., 2012), vitamins, traditional Chinese medicine (Xu and Peng, 2015), retinal transplantation (Reh, 2016), and retinal prosthesis (Xu et al., 2014). Unfortunately, most of these therapies have not proven effective in the clinical setting.

More recent strategies based on neural protection have shown promise for retinitis pigmentosa. Among these, the use of neurotrophic factors has garnered a great deal of attention because of their therapeutic potential (Sahel et al., 2015). To date, the production of neurotrophic factors has been sufficient for laboratory use only. Hence, finding economical ways to synthesize large quantities of neurotrophic factors is key for their clinical application.

Brain-derived neurotrophic factor (BDNF) has the strongest neuroprotective effect on retinal neurocytes among the various neurotrophins, and has the best prospect for treating degenerative fundus diseases (Cuenca et al., 2014). BDNF was discovered in pig brain (Barde et al., 1982) and has been widely used for neural protection (Neumann et al., 2015). Increased expression of BDNF helps preserve retinal function and prevents the apoptosis of photoreceptors and retinal pigment epithelial cells (Azadi et al., 2007), suggesting that BDNF therapy may be a promising method for treating retinitis pigmentosa (Zhang et al., 2009). BDNF is mainly synthesized in cortical areas and is widely expressed in the mammalian brain (Yu and Chen, 2011). However, as a cationic protein, BDNF has difficulty traversing biological membranes, including the blood-retina barrier, which is an obstacle to the use of exogenous BDNF for retinal diseases (Flock et al., 2016). Hence, therapy for retinal diseases mainly relies on BDNF produced by the retinal tissue itself. Receptor expression is another factor influencing the activity of intraocular BDNF. The BDNF receptors (TrkB and p75[NTR]) are regulated temporally, and their expression is restricted to specific subpopulations of retinal neurons (Kolomeyer and Zarbin, 2014). Changes in neurotrophin receptor expression in the degenerating retina are very complex, and may influence the activities of neurotrophins (Llamosas et al., 1997). Thus, BDNF overexpression may be effective for retinal diseases only if the neurotrophin is expressed by the retinal tissue.

Expression constructs for BDNF from various tissues have previously been generated (Luo et al., 2015); however, human retina-derived BDNF has not yet been developed for gene therapy. In the present study, we constructed a plasmid expression system for BDNF derived from human retina. We then investigated the effects of the expressed BDNF on acute retinal pigment epithelial-19 (ARPE-19) cells. Our findings suggest that BDNF may have clinical potential for the treatment of retinitis pigmentosa.

Materials and Methods

Tissue preparation
Human eyeballs were provided by the eye bank of Tongren Eye Center, Beijing, China. Written informed consent was provided by the anonymous donors. This study was approved by the Institutional Review Board at our university. All experimental protocols were conducted in accordance with the *Declaration of Helsinki*. The eyeballs were maintained in an ice box at 4°C and were received by the lab within 24 hours. The retinas were dissected under a stereomicroscope using microsurgical instruments to diminish the loss of RNA. The use of human retinas in this research was approved by the Ethics Review Committee of Beijing Tongren Hospital in China.

Cell culture
Human 293T cells were provided and cultured by Functional Genomic Research Lab, Tsinghua University, China, in Dulbecco's modified Eagle's medium (DMEM), containing 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA USA) and 1% penicillin-streptomycin in a humidified incubator at 5% CO_2 and 37°C (Thermo Fisher Scientific). The cells were harvested and used in gene transfection protocols with the constructed plasmid vector pEGFP-N1-BDNF.

Human ARPE-19 cells were provided by American Type Culture Collection (Rockville, MD, USA), and subsequently cultured at Functional Genomic Research Lab, Tsinghua University. ARPE-19 cells were seeded on 10-cm plates and cultured in DMEM before the experiments. Upon reaching confluence, the cells were harvested and co-cultured with 293T cells in 6-well Transwell bicameral chambers (Corning Costar, Cambridge, MA, USA).

pEGFP-N1 plasmid vector construction
The Functional Genomic Research Lab, Tsinghua University, provided a vector (pEGFP-N1) carrying a humanized variant of Aequorea victoria green fluorescent protein (enhanced green fluorescent protein; EGFP) under the control of a cytomegalovirus promoter.

TRIzol reagent was used to extract total RNA from fresh retinas (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed to cDNA with a reverse transcription kit (Takara Biotechnology Co., Ltd., Dalian, China) for reverse-transcription polymerase chain reaction (RT-PCR). The PCR products were ligated into the pGEM-T Easy Vector (pGEM-T Easy Vector System I; Promega, Madison, WI, USA). Sequencing and comparison with the GenBank sequence (NM_170733) were performed to confirm the identity of the BDNF cDNA. The human BDNF primer sequences were as follows: forward, 5′-CCC AAG CTT GCC ACC ATG ACC ATC CTT TTC CTT AC-3′; reverse, 5′-CGC GGA TCC CGT CTT CCC CTT TTA ATG GTC A-3′. The BDNF cDNA was inserted between the *Bam*HI and *Hind*III sites in the pEGFP-N1 vector, resulting in a plasmid of approximately 740 bp in length (designated pEGFP-N1-BDNF) (**Figure 1**), which was subsequently extracted with an E.Z.N.A. Endo-Free Plasmid Mini Kit (Omega Bio-Tek, Norcross, GA, USA). The plasmid vectors were evaluated for purity, and concentrations were determined before use. The plasmid concentration was at least 1,000 g/mL, and the $A_{260\ nm/280\ nm}$ ratio was between 1.8 and 2.0, which allowed for high transfection efficiency.

Plasmid delivery *in vitro*

Plasmid-mediated gene transfection was carried out as described previously (Torchilin et al., 2003), with changes made to enhance efficiency. In brief, 293T cells were seeded at 2.5×10^5 cells per well in 6-well plates (Corning Costar, Cambridge, MA, USA). Cells that reached 45–50% confluence after 12 hours of growth in DMEM were serum-starved with 2 mL of Opti-MEM (Gibco-BRL/Invitrogen, Carlsbad, CA, USA). After one hour of culture, the cells were incubated with the liposome-plasmid complexes at 37°C under 6% CO_2 for 6 hours. Next, cells were transfected with Lipofectamine 2000 (Invitrogen) and pEGFP-N1-BDNF/pEGFP-N1 at a concentration of 6.5 μL/mL or 4 μg/mL. The medium was then discarded, and the cells were cultured in normal DMEM medium at 37°C under 6% CO_2 for 24, 48, 72 or 96 hours. Bright field and fluorescence (550 nm; 200×) images were captured for 36 samples at each time point. The transfection efficiencies were estimated separately using the ratio of the positive area or the ratio of the number of positive cells per visual field by using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA) or ImageJ 1.48 V software (National Institutes of Health, Bethesda, MD, USA).

The production of plasmid-derived BDNF in 293T cells 48 hours after transfection was confirmed by confocal scanning microscopy. The pEGFP-N1-BDNF/pEGFP-N1-transfected 293T cells were cultured in 6-well plates for 24, 48, 72 or 96 hours, and staining was performed in triplicate. Briefly, the cells were fixed, and nuclei were stained with Hoechst 33342 (1:5,000; Sigma-Aldrich, St. Louis, MO, USA) for 20 minutes. The cells were washed twice with PBS, and then fixed. A laser scanning confocal microscope (LSM710, Carl Zeiss, Jena, Germany) (600×) was used to detect nuclei stained by Hoechst 33342 or for GFP protein using a 365 nm or 488 nm filter.

Detection of BDNF protein by western blot assay

10 ng of purified BDNF was taken as a positive control. Following estimation of protein content with the bicinchoninic acid assay, equal amounts of protein from the pEGFP-N1-BDNF-transfected 293T cells (experimental group), blank plasmid pEGFP-N1-transfected 293T cells (negative control) and ARPE-19 control cells (ARPE-19 control) were processed for western blot assay. At 48 hours after transfection, cells in each group were lysed using the mammalian protein radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific), together with a protease inhibitor cocktail (Roche, Pleasanton, CA, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) was used to separate the protein lysates. Proteins were then transferred onto 0.22-μm nitrocellulose membranes (Hybond-C; Amersham Bioscience, Piscataway, NJ, USA). The membranes were incubated with goat polyclonal antibody against BDNF (1:200; Abcam, Cambridge, MA, USA) overnight in a 4°C refrigerator. A mouse anti-β-actin polyclonal antibody (1:2,000; Sigma-Aldrich, St. Louis, MO, USA) was used as control. Thereafter, the membranes were incubated with rabbit anti-goat IgG (1:5,000; SouthernBiotech, Birmingham, AL, USA) and goat anti-mouse IgG (1:5,000; SouthernBiotech) for 2 hours at room temperature. Signals were visualized using a SuperSignal West Pico chemiluminescence kit (Pierce Biotechnology, Rockford, IL, USA).

BDNF release

At 24, 48, 72 and 96 hours after 293T cells were transfected *in vitro*, the culture supernatants were collected for analysis. BDNF concentrations were quantified with a BDNF enzyme-linked immunosorbent assay (ELISA) kit (Abcam). Six samples for each group at each time point were assessed, and each sample was analyzed in triplicate. An automatic microplate reader was used to read the absorbance at 450 nm (FLUOstar Omega; BMG LABTECH, Offenburg, Germany).

Exposure of ARPE-19 cells to BDNF

A Transwell bicameral chamber (Corning Costar) was used to co-culture the transfected 293T and ARPE-19 cells. The ARPE-19 cells were exposed to released BDNF continuously. Untransfected 293T cells or 293T cells transfected with pEGFP-N1-BDNF or pEGFP-N1 were seeded in the upper chamber of a Transwell plate 12 hours before transfection. ARPE-19 cells were seeded in the lower chamber of an adjacent well 7 hours before transfection of the 293T cells. The upper chamber was transferred to the corresponding adjacent ARPE-19 well at hour 0 after transfection. Purified BDNF (50 ng/mL or the same concentration as released BDNF; Thermo Fisher Scientific) was added to other ARPE-19 wells as a control. ARPE-19 cells were incubated for 24, 48, 72 or 96 hours before harvesting for 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay and western blot assay (**Figure 2**).

Cell survival assay

A cell survival assay was performed with MTT (Solarbio, China) according to the manufacturer's instructions. ARPE-19 cells were placed into 6-well Transwell bicameral chambers and co-cultured with transfected 293T cells for 24, 48, 72 or 96 hours. A total of 0.5 mg/mL MTT was added and incubated for another 4 hours. The medium was then discarded, and 150 μL dimethyl sulfoxide (Sigma-Aldrich) was added and shaken gently for 10 minutes. Absorbance at 490 nm was then read by an automatic microplate reader (FLUOstar Omega; BMG LABTECH). Cell viability was assessed at 24, 48, 72 and 96 hours for released (secreted) BDNF, with standard (purified) BDNF at 50 ng/mL (positive control-50) or at the same concentration as released BDNF (positive control-S), and for control treatment (no BDNF). The absorbance ratios to the blank control were then calculated.

Bcl-2 and Bax protein expression

To observe the effect of BDNF (secreted from 293T-transfected cells) on ARPE-19 cells, the latter were collected at every time point to analyze pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins by western blot assay. Equal quantities of protein (30 μg/mL) were loaded onto the gel.
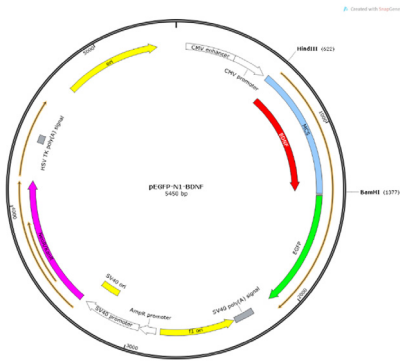
Figure 1 Map of pEGFP-N1-BDNF.
The red arrow represents the human BDNF cDNA. The green curve with an arrow represents EGFP. The BDNF cDNA was inserted between the *Bam*HI site and the *Hind*III site. BDNF: Brain-derived neurotrophic factor; EGFP: enhanced green fluorescent protein.
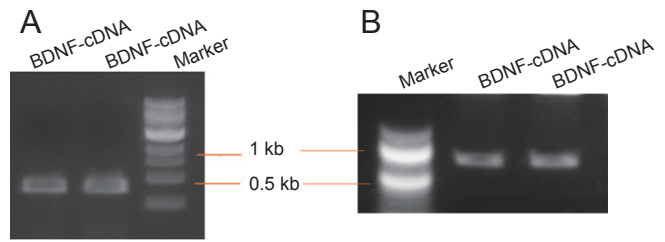

Figure 3 Human BDNF cDNA detected by electrophoresis after RT-PCR and TA cloning.
(A) RT-PCR was used to amplify human BDNF cDNA, and the products were separated by electrophoresis. The left two lanes followed by the marker are BDNF cDNA. The size of the BDNF cDNA was approximately 741 bp. (B) TA-cloning of human BDNF cDNA produced a higher concentration than that obtained with PCR. The right two lanes following the marker are both human BDNF cDNA, indicated by the bars at about 741 bp. RT-PCR: Reverse transcription-polymerase chain reaction; BDNF: brain-derived neurotrophic factor.
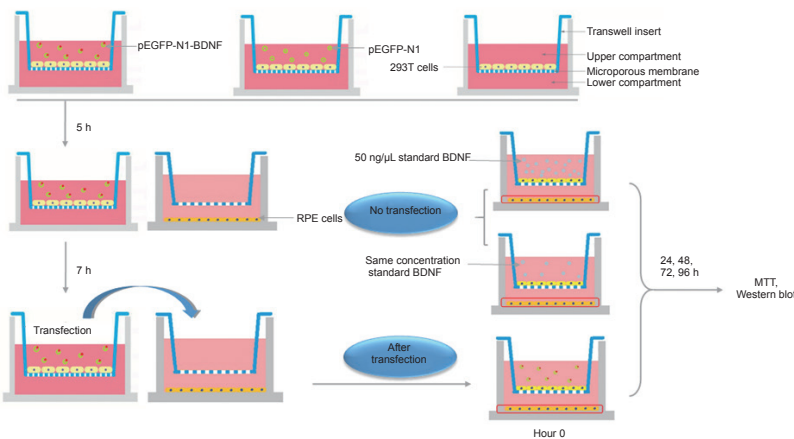

Figure 2 Flow chart of transfected 293T cells co-cultured with ARPE-19 cells using the Transwell double chamber system.
Untransfected 293T cells and cells transfected with pEGFP-N1-BDNF or pEGFP-N1 were seeded in the upper chamber of a Transwell plate 12 hours before transfection. ARPE-19 cells were seeded in the lower chamber of an adjacent well 7 hours before transfection of 293T cells. The upper chamber was then transferred to the corresponding ARPE-19 well at hour 0 after transfection. Purified standard BDNF (50 ng/mL or the same concentration as BDNF in the medium) was administered to other ARPE-19 wells as a control. ARPE-19 cells were incubated for 24, 48, 72 or 96 hours before harvesting, for the MTT and western blot assays. ARPE-19: Acute retinal pigment epithelial-19; BDNF: brain-derived neurotrophic factor; EGFP: enhanced green fluorescent protein.
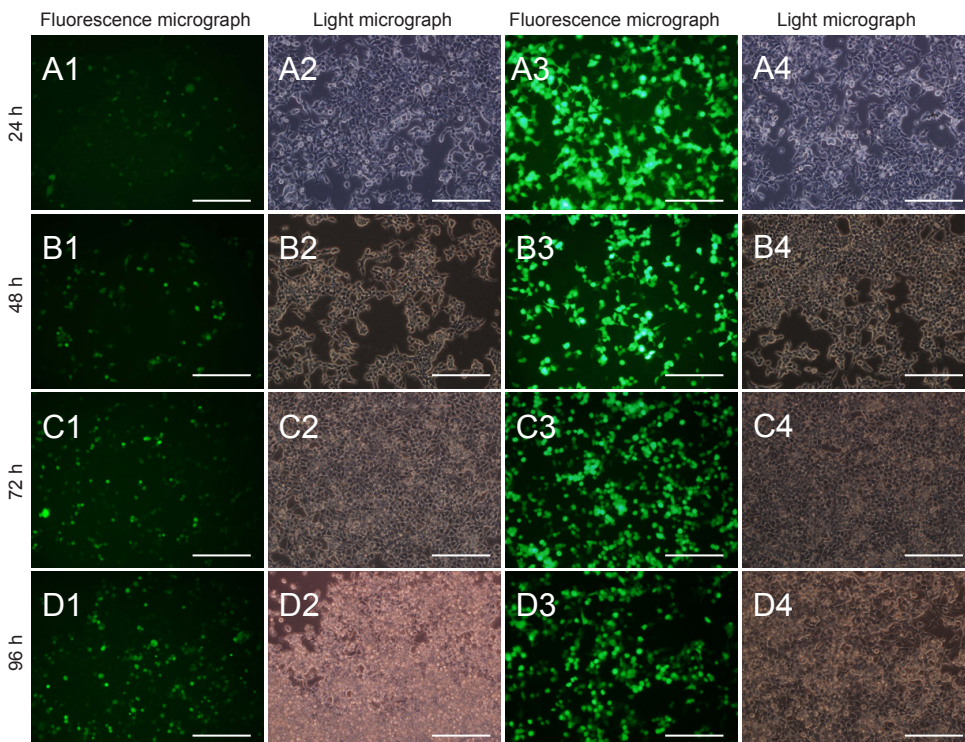

Figure 5 Micrographs of 293T cells transfected with pEGFP-N1 or pEGFP-N1-BDNF after transfection at the various time points by fluorescence microscopy (× 200).
(A1, A2, B1, B2, C1, C2, D1 and D2): 293T cells transfected with pEGFP-N1-BDNF 24–96 hours (h) after transfection under fluorescence and light microscopes. A2, B2, C2, D2 are light micrographs of the same visual field as A1, B1, C1, D1, respectively. With increasing time after transfection, EGFP signals (green) increased, suggesting increased expression of EGFP-BDNF protein. (A3, A4, B3, B4, C3, C4, D3 and D4) 293T cells transfected with pEGFP-N1 24–96 h after transfection under fluorescence and light microscopes. A4, B4, C4, D4 are light micrographs of the same visual fields as A3, B3, C3, D3, respectively. With increasing time after transfection, EGFP signals increased, suggesting increased expression of EGFP protein. Scale bars: 250 μm. EGFP: Enhanced green fluorescent protein; BDNF: brain-derived neurotrophic factor.
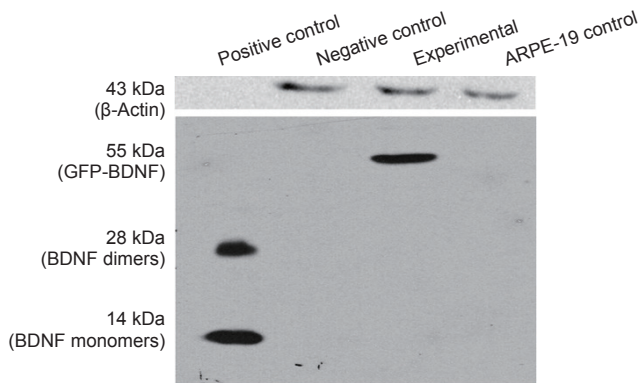
Figure 8 Expression of standard purified BDNF (positive control) and BDNF-GFP fusion protein (experimental group) in 293T cells, assessed by western blot assay 48 hours after transfection.
(A) Beta-actin control showing a 43 kDa band, except for the positive control group. (B) Positive control group showing the location of monomers and dimers of BDNF at 14 kDa and 28 kDa, respectively. 293T cells transfected with pEGFP-N1 (negative control) and ARPE-19 cells (ARPE-19 control) showed no signal at the corresponding locations. The BDNF group showed a band at 55 kDa, suggesting the production of fusion protein, GFP-BDNF (BDNF: 28 kDa + GFP: 27 kDa = 55 kDa), after transfection with pEGFP-N1-BDNF. BDNF: Brain-derived neurotrophic factor; EGFP: enhanced green fluorescent protein; ARPE-19: acute retinal pigment epithelial-19.
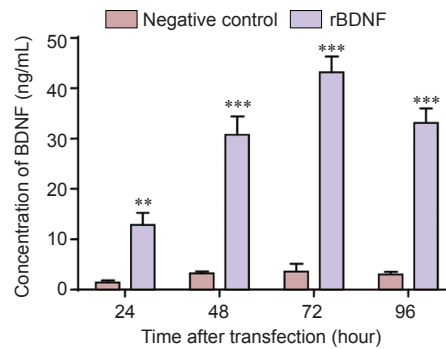

Figure 9 BDNF release profiles of transfected 293T cells detected by ELISA.
pEGFP-N1-BDNF-transfected 293T cells released BDNF persistently, and the concentration was significantly higher than in the negative control group. At 24–72 hours, the secretion of BDNF rose gradually and reached a peak at 72 hours, which was 12-fold more than in the control group (***$P < 0.001$, mean ± SEM, $n = 18$, Student's t-test). Thereafter, the secretion rate of BDNF slightly slowed, but was still higher compared with the control group. The pEGFP-N1-transfected 293T cells (negative control group) maintained a low level of release (**$P < 0.01$, ***$P < 0.001$, mean ± SEM, $n = 18$, Student's t-test). rBDNF: Released BDNF; BDNF: brain-derived neurotrophic factor; EGFP: enhanced green fluorescent protein.

The following groups were evaluated: transfected 293T cells (releasing BDNF) co-cultured with ARPE-19 cells, ARPE-19 cells cultured alone as a blank control, pEGFP-N1 plasmid-transfected 293T cells co-cultured with ARPE-19 cells as a negative control, purified BDNF at the same concentration as released BDNF (positive control-S) or at 50 ng/mL (positive control-50). Experiments were performed in each group at the same time point. Each protein sample was run on two electrophoresis gels in an experiment, and analyzed in triplicate by the software. The data were derived from six independent experiments for the same condition. We compared the ratio of Bax/Bcl-2 to β-actin to assess the survival and growth status of each group, which reflected the effect of BDNF on the ARPE-19 cells.

After electrophoresis on 12% gels, proteins were transferred to nitrocellulose membranes. Primary polyclonal antibodies against β-actin (mouse, 1:2,000; Sigma-Aldrich), Bcl-2 (rabbit, 1:1,000; Cell Signaling, Danvers, MA, USA) and Bax (rabbit, 1:1,000; Cell Signaling) were used to probe the membranes overnight at 4°C. The membranes were then treated for 2 hours with secondary antibodies—goat anti-rabbit IgG (1:5,000; SouthernBiotech) or goat anti-mouse IgG (1:5,000; SouthernBiotech)—at room temperature. A SuperSignal West Pico chemiluminescence kit was used to detect the proteins (Pierce Biotechnology, Rockford, IL, USA). After exposure, the film was scanned and analyzed using Image J v1.48 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis
Data, expressed as the mean ± SEM, were analyzed with SPSS 20.0 software (IBM Corporation, Armonk, NY, USA). ELISA data were analyzed by Student's t-test, while MTT and western blot data were analyzed by one-way

analysis of variance with *post-hoc* Tamhane 2 test. $P < 0.05$ was considered statistically significant.

Results
Construction of the pEGFP-N1-BDNF plasmid
Electrophoresis was used to detect the BDNF cDNA amplified by PCR (**Figure 3**). The size of the cDNA was approximately 740 bp, similar to that of the human BDNF cDNA (741 bp). TA cloning yielded higher concentrations of the human BDNF cDNA (**Figure 3**), whose sequence was completely identical to the human GenBank sequence (NM_170733; compared with Vector NTI v11.5.1 software) (**Figure 4**). The pEGFP-N1-BDNF plasmid was then successfully constructed by recombining the human BDNF cDNA with the linear pEGFP-N1 plasmid.

BDNF-GFP expression in 293T cells transfected with pEGFP-N1-BDNF
At 24, 48, 72 and 96 hours after transfection, the transfection efficiency was calculated for the different time points (**Figure 5**). The transfection efficiency rose gradually, although the efficiency measured using the area was higher than that calculated by counting the number of positive cells (**Figure 6**). To localize BDNF in transfected 293T cells and to examine secretion, the BDNF-GFP fusion protein was detected by confocal laser scanning microscopy. In the early stage (24 hours) after transfection, a few GFP puncta (vesicles) were detected around the nuclei of cells. In the mid stage of transfection, more GFP-positive puncta were present mainly around the nuclei of cells, with some fluorescence detectable in the cytoplasm. In the late stage, GFP was distributed uniformly in the cytoplasm, with additional puncta near the nuclei. BDNF-GFP fusion protein expression was detected in many 293T
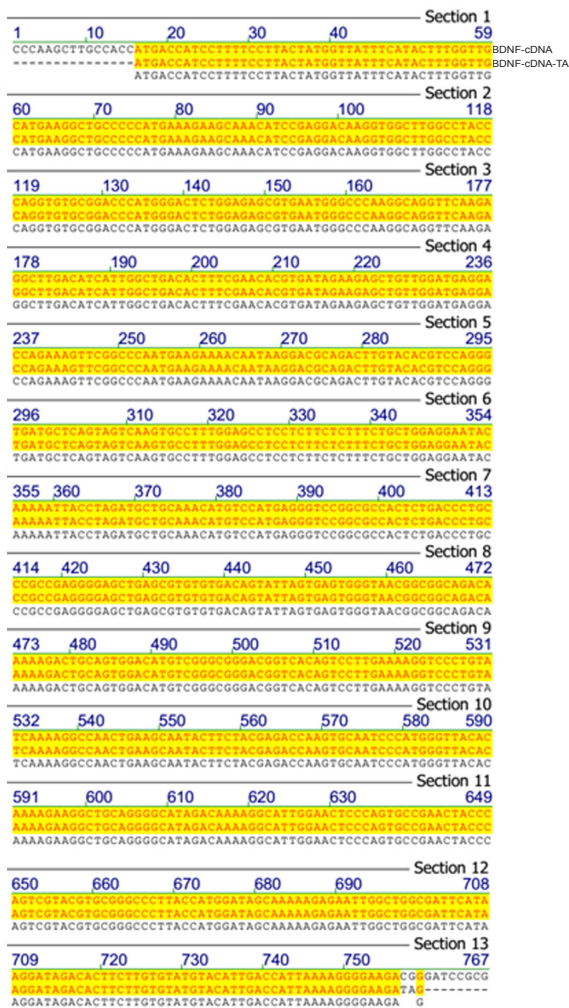
Figure 4 Amplified BDNF cDNA sequence compared with the sequence from GenBank.

The amplified sequence was identical to the GenBank sequence, without stop codons (compared with Vector NT I software). BDNF: Brain-derived neurotrophic factor.
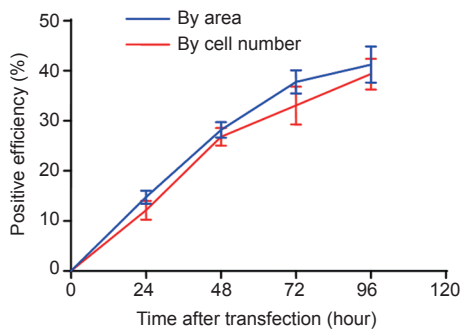

Figure 6 Efficiency of 293T cells transfected with pEGFP-N1-BDNF at different time points after transfection.

We calculated efficiency with Image-Pro Plus 6.0 or ImageJ v1.48 software using positive area or positive cell number. A total of 36 samples were separately counted at 24, 48, 72 and 96 hours. The two methods provided the same results; *i.e.*, a gradual rise in efficiency from the beginning to 96 hours, although the efficiency measured by area was higher than that measured by positive cell number. BDNF: Brain-derived neurotrophic factor; EGFP: enhanced green fluorescent protein.
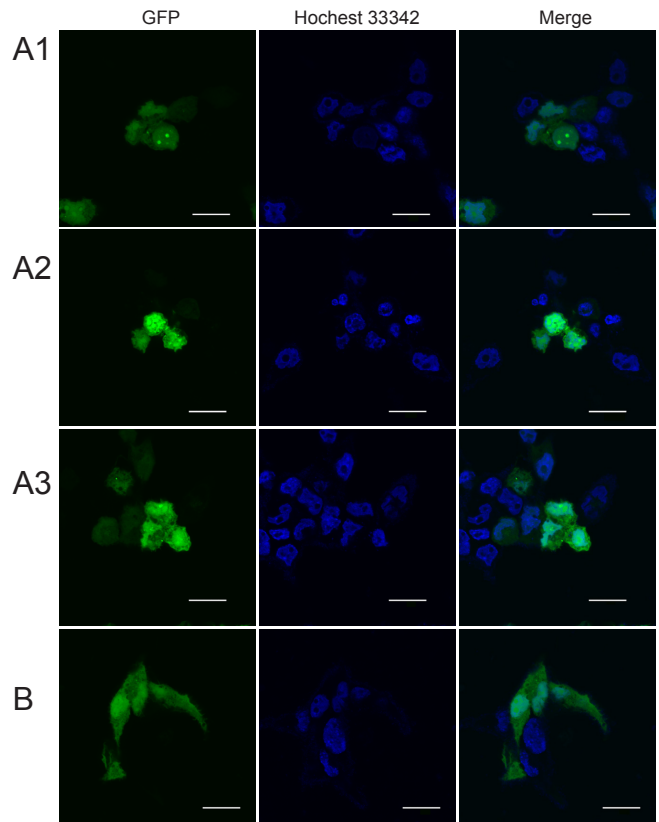

Figure 7 BDNF-GFP or GFP expression in 293T cells after transfection by confocal laser scanning microscopy.

GFP distribution in 293T cells was determined by confocal laser scanning microscopy (× 600). Hoechst 3342 staining was blue when excited by a 405 nm light. GFP was green when excited by a 514 nm light. (A1) Image of cells transfected with pEGFP-N1-BDNF in the early stage (24 hours after transfection). The micrograph shows a few vesicles expressing GFP around the nuclei of cells (marked with white arrows). (A2) Image of cells transfected with pEGFP-N1-BDNF in the middle stage (48 hours after transfection). The micrograph shows more GFP vesicles mainly around the nuclei of cells, with some GFP signals in the cytoplasm. (A3) Image of cells transfected with pEGFP-N1-BDNF in the late stage (72 hours after transfection). GFP is distributed uniformly in the cytoplasm, with some vesicles near the nuclei. (B) Cells transfected with control pEGFP-N1 in the mid stage, showing GFP distributed uniformly in both nuclei and the cytoplasm from the early stage to the end stage without vesicles. Scale bar: 20 μm. BDNF: Brain-derived neurotrophic factor; EGFP: enhanced green fluorescent protein; GFP: green fluorescent protein.

cells transfected with pEGFP-N1-BDNF, in comparison to cells transfected with pEGFP-N1, in which GFP was uniformly distributed in both nuclei and the cytoplasm from an early stage to the late stage without the presence of vesicles (**Figure 7**).

Western blot assay showed that after 48 hours of transfection, 293T cells transfected with pEGFP-N1-BDNF expressed the BDNF-GFP fusion protein (55 kDa). The two control groups had no detectable staining, suggesting that pEGFP-N1-BDNF-transfected 293T cells synthesized the recombinant BDNF-GFP (**Figure 8**).

BDNF concentrations in the culture media in 6-well Transwell plates were determined by ELISA. At 24, 48, 72 and 96 hours, the transfected 293T cells secreted BDNF at average concentrations of 12.81 ± 2.42, 30.73 ± 3.68, 43.15
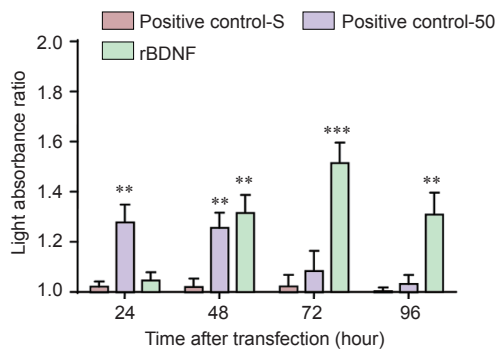
Figure 10 Influence of released BDNF on ARPE-19 cell survival, assessed by MTT assay.

At 0 hours, standard BDNF was administered at the same concentration as in the BDNF control (positive control-S) group or the 50 ng/mL BDNF (positive control-50) group. We set the blank control, with nothing added to the cultured ARPE-19 cells, as a reference. The absorption ratio (to the blank control) in the positive control-S group was nearly 1, suggesting that cell survival in the positive control-S group was similar to that in the blank control. At 24–48 hours, cell survival in the positive control-50 group was substantially higher. This difference disappeared at 72 hours. However, the released BDNF influenced ARPE-19 cells significantly after 48 hours, when the concentration of released BDNF reached 33 ng/mL (**$P < 0.01$, ***$P < 0.001$, mean ± SEM, $n = 18$, one-way analysis of variance with *post-hoc* Tamhane 2 test). MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide. BDNF: brain-derived neurotrophic factor; rBDNF: released BDNF; ARPE-19: acute retinal pigment epithelial-19.
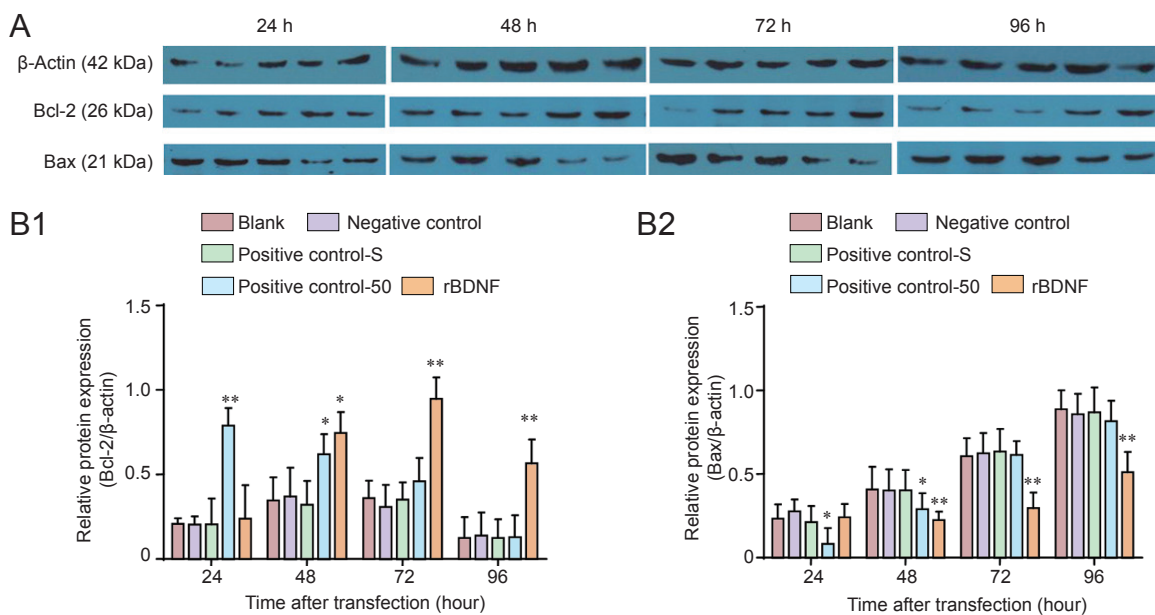

Figure 11 Influence of released BDNF on Bcl-2/Bax in ARPE-19 cells at different time points after transfection.

At 24, 48, 72 and 96 hours, ARPE-19 cells were lysed, and β-actin, Bcl-2 and Bax proteins were detected by western blot assay. (A) Western blots of the three proteins at four different time points. Each panel included five groups: From left to right, blank control, negative control, positive control-S (same concentration as rBDNF control), positive control-50 (50 ng/μl control) and rBDNF. (B1-B2) Protein levels of Bcl-2 and Bax in each group were measured with Image J software. B1 represents the ratios of Bcl-2 to β-actin in the different groups. At 24–48 hours, Bcl-2 was significantly higher in the positive control-50 group than in the negative control group. Bcl-2 in the rBDNF group began to increase at 48 hours and reached a peak at 72 hours, followed by a slight decrease at 96 hours. Bcl-2 in the rBDNF group was significantly higher than in the control groups at 48–96 hours. B2 represents the gray value ratios of Bax to β-actin in the different groups. At 24–48 hours, Bax was significantly lower in the positive control-50 group than in the negative control group, corresponding to the Bcl-2 changes. In the rBDNF group, Bax was lower than in the negative control group at 48–72 hours (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, mean ± SEM, $n = 12$, one-way analysis of variance with *post-hoc* Tamhane 2 test). h: Hours; BDNF: brain-derived neurotrophic factor; rBDNF: released BDNF; ARPE-19: acute retinal pigment epithelial-19.

± 3.15 and 33.12 ± 2.88 ng/mL, respectively. The pEG-FP-N1-transfected 293T cells also produced BDNF protein (2.78 ± 0.73 ng/mL on average). At the peak of secretion (72 hours), BDNF concentration in the pEGFP-N1-BDNF group was 12-fold ($P < 0.001$) greater than that in the blank plasmid-transfected group (**Figure 9**).

Effects of the recombinant BDNF on ARPE-19 cells

MTT data are summarized in **Figure 10** and show that the same concentration of purified BDNF (positive control-S) as released BDNF had no significant effect over the four time points, while 50 ng/mL of purified BDNF (positive control-50) significantly promoted survival ($P < 0.01$). The maximum

survival rate was 133.51 ± 6.10% at 48 hours. By comparison, released BDNF significantly increased ($P < 0.01$) ARPE-19 cell survival after 48 hours, with a maximum survival of 151.47 ± 8.10% at 72 hours, with a slight decrease at 96 hours. The culture medium of cells treated with purified BDNF was kept to examine concentration changes at the end of each time point. BDNF concentrations were all below 4 ng/mL, and were similar to the negative control group, suggesting that purified BDNF protein *in vitro* has a short half-life.

Bcl-2 and Bax proteins in ARPE-19 cells were analyzed by western blot assay (**Figure 11**). ARPE-19 cells in the released BDNF group were influenced by the continued release of BDNF after 48 hours. Bcl-2 protein in the released

BDNF group exceeded that in the control group ($P < 0.05$, $P < 0.01$). Bax protein in the released BDNF group was lower than in the control groups at the same time points ($P < 0.01$), suggesting a reciprocal relationship between these two proteins. At 72 hours, Bcl-2 protein expression reached a peak ($P < 0.01$), likely accounting for the change in survival identified with the MTT assay. In the positive control-50 group, Bcl-2 protein was higher ($P < 0.05$, $P < 0.01$) than in the control groups at 24–48 hours, and Bax protein was correspondingly lower. At later time points, the levels of these two proteins in the positive control-50 group were not substantially different from the other control groups.

Discussion

Photoreceptor cells in the retina are thought to express the retinitis pigmentosa genes (Phelan and Bok, 2000). Gene mutations can lead to defects in the structure of photoreceptors as well as altered function (Daiger et al., 2013). Retinal pigment epithelial cells play an important role in the survival of photoreceptors. Therefore, therapeutic strategies that protect and promote the survival of retinal pigment epithelial cells may control the progression of retinitis pigmentosa.

BDNF has been shown to have neuroprotective functions (Neumann et al., 2015) and promote neural regeneration (Zheng et al., 2016). Increased expression of BDNF can preserve retinal function and prevent apoptosis of photoreceptors and retinal pigment epithelial cells (Azadi et al., 2007). In the retina, neurotrophic factors, such as BDNF, are secreted by retinal pigment epithelial cells (Ming et al., 2009). The retinal neuroepithelium has been shown to be a site where BDNF-induced activation of the TrkB pathway occurs, which promotes the differentiation and survival of the retinal pigment epithelium (Liu et al., 1997).

Finding an efficient and effective method of targeted neurotrophic factor synthesis is important for their therapeutic use, especially for retinitis pigmentosa. Several methods have been developed to synthesize neurotrophic factors. Due to the low production rate, innovative ways based on molecular biology and genetics have been developed. Gene-based biosynthesis of neurotrophic factors has attracted much attention. The construction of BDNF by genetic engineering from other tissues has been reported (Luo et al., 2015); however, human retina-derived BDNF has not yet been cloned. Thus, we demonstrate the first successful production of retinal BDNF by genetic engineering. Similar to other neurotrophic factors, the half-life of BDNF protein is short *in vitro*. To realize the stable and long-term expression of intraocular BDNF, we produced a BDNF plasmid for clinical application (Chen et al., 2007; Song et al., 2015). To develop a safe and effective expression vector, we chose a plasmid construct instead of a viral vector to avoid possible adverse events associated with clinical viral vector use (Thomas et al., 2003). The BDNF-GFP chimeric protein was previously shown to be secreted from neurons in a manner similar to normal endogenous secreted proteins (Haubensak et al., 1998). The protein is synthesized and modified by the rough endoplasmic reticulum, is then transferred to the Golgi apparatus for further processing, and is subsequently packaged in secretory vesicles and released from the cells.

At 24–72 hours, the transfected cells were in a relatively rapid growth phase, and as a result, BDNF protein increased gradually and reached a peak at 72 hours. The recombinant BDNF has a longer half-life than normal BDNF. With the accumulation of metabolites and toxins in the culture medium, the growth of ARPE-19 cells subsided, followed by a decrease in BDNF secretion. Future studies should focus on prolonging the secretion of the protein.

Although the concentration of released BDNF in the rBDNF group was less than 50 ng/mL, the anti-apoptotic activity towards ARPE-19 cells exceeded that of the positive control-50 group. This may have been because the recombinant BDNF had greater activity towards human ARPE-19 cells than the purified (standard) BDNF. Additionally, the synthesized BDNF was released continuously into the medium, and therefore, it had the opportunity and time to protect ARPE-19 cells.

Bax and Bcl-2 play important roles in ARPE-19 cell survival and death (Mukherjee et al., 2007). BDNF can prevent cells from undergoing apoptosis by downregulating Bax and upregulating Bcl-2 (Schabitz et al., 2000). The ratios of Bax and Bcl-2 to β-actin likely underlie the differences in ARPE-19 cell survival. At 24–48 hours, Bcl-2 protein expression increased substantially, while Bax protein expression decreased, in the positive control-50 group, coincident with the increase in the growth of ARPE-19 cells. In the cells exposed to released (secreted) BDNF, Bcl-2 protein expression increased from 48 hours and reached a peak at 72 hours. Bax protein expression decreased from 48 hours and was lower than that in the control group until 96 hours. The reciprocal changes in Bax and Bcl-2 likely account for the anti-apoptotic action of BDNF on the ARPE-19 cells. The positive control-S group was not different from the blank control or negative control group, suggesting that the purified BDNF was not at a high enough concentration to exert an anti-apoptotic effect.

Retinitis pigmentosa is an inherited retinal disease with a protracted disease course. Therefore, neurotrophic factor therapy requires sustained and stable intraocular production and release. In the present study, we found that transient transfection with a BDNF plasmid vector can result in sustained BDNF release. At present, the biotechnology industry has been focused on the development of sustained-release products to permit the stable and long-term release of factors in the target tissue. The Neurotech company has made ciliary neurotrophic factor into a slow release formulation by encapsulation (named NT-501). This product has entered the phase II clinical trial stage (Kauper et al., 2013). Our group is also looking for novel sustained-release material for intraocular BDNF therapy. Recently, a novel sustained-release material, polylactic-co-glycolic acid (PLGA), has been used in therapy for retinal degeneration in rats (Seiler et al., 2008). PLGA is easily produced and is non-toxic to humans, and it was approved by the Food and Drug Administration. We hope to develop a sustained-release product using PLGA to allow for the stable and long-term intraocular release of BDNF. Another group of our team is trying to fuse BDNF and PLGA together and inject the mixture to vitreous cavity of rabbits. Recently, an

experiment using intravitreal injection of GDNF-secreting cells in a rat model proved to exert a neuroprotective effect for at least three months, which gave us confidence to fuse BDNF-secreting cells and sustained-release material (Gregory-Evans et al., 2009). We believe that cheap and safe sustained-release BDNF has therapeutic potential for retinitis pigmentosa and other retinal diseases.

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