NEURAL REGENERATION RESEARCH
Volume 8, Issue 11, April 2013

www.nrronline.org

doi:10.3969/j.issn.1673-5374.2013.11.009 [http://www.nrronline.org; http://www.sjzsyj.org]
Peng CX, Yin XB, Li MD, He T, Li GL. Construction of a eukaryotic expression plasmid for human retina-derived neurotrophin-3. Neural Regen Res. 2013;8(11):1031-1040.

Construction of a eukaryotic expression plasmid for human retina-derived neurotrophin-3☆

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Abstract

Neurotrophin-3 (NT-3) can promote the repair of central nervous system and retinal damage. In previous reports, NT-3 has been expressed by viral vectors. However, plasmid vectors have a safer profile compared with viral vectors in clinical studies. This study recombined amplified human retinal NT-3 with a eukaryotic expression plasmid containing green fluorescent protein (GFP) to construct an NT-3 expression plasmid, pEGFP-N1-NT-3. The transfection efficiency 48 hours after pEGFP-N1-NT-3 transfection to 293T cells was 50.06 ± 2.78%. Abundant NT-3-GFP was expressed in 293T cells as observed by fluorescence microscopy, suggesting the construct pEGFP-N1-NT-3 effectively expressed and secreted NT-3-GFP. Secretory vesicles containing NT-3-GFP were observed in a constant location in cells by laser scan confocal microscopy, indicating the expression and secretion processes of NT-3 in eukaryotic cells were in accordance with the physical synthesis processes of secreted proteins. Western blot assay showed that pro-NT-3-GFP had a molecular weight of 56 kDa, further confirming NT-3-GFP expression. At 48 hours after transfection, the concentration of NT-3 in culture medium was 22.3 ng/mL, suggesting NT-3 produced by pEGFP-N1-NT-3 was efficiently secreted. This study constructed a human retinal-derived NT-3 eukaryotic expression plasmid that efficiently expressed and secreted NT-3.

Key Words

neural regeneration; gene therapy; biological factor; neurotrophin-3; plasmid; fusion protein; encapsulated cell technology; retinitis pigmentosa; grants-supported paper; neuroregeneration

Research Highlights

(1) Neurotrophin-3 (NT-3) expression plasmid, pEGFP-N1-NT-3, was constructed from recombinant human retinal NT-3 in eukaryotic expression plasmid with green fluorescent protein marker.
(2) Forty-eight hours after pEGFP-N1-NT-3 transfection to 293T cells, transfection efficiency was 46.76% by fluorescence microscopy.
(3) After pEGFP-N1-NT-3 was transfected into 293T cells, NT-3 expression and secretion was similar to natural intracellular environments.
(4) pEGFP-N1-NT-3 expressed and secreted NT-3 efficiently.
(5) NT-3 delivered by encapsulated cell technology might have a controllable, sustained, long-term therapeutic effect in central nervous system and intraocular disease, such as retinitis pigmentosa.

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Received: 2012-12-17
Accepted: 2013-03-26
(N20120813004)

INTRODUCTION

Recently, encapsulated cell technology has been used to study the therapeutic effects of neurotrophic factors for treatment of central nervous system neurodegenerative diseases and retinal diseases such as retinitis pigmentosa[1-4]. Therapy with encapsulated cell technology uses immunoisolated cellular implants that sequester viable cells modified with neurotrophic factor genes surrounded by a semipermeable membrane, which protects them from host rejection. Therefore, encapsulated cell technology can overcome the blood-brain barrier and blood-ocular barriers to continuously deliver neurotrophic factors directly to the region of interest. Moreover, the membrane admits oxygen or required nutrients and releases bioactive cell secretions, but restricts passage of large cytotoxic agents from the host immune system[3]. Hence, encapsulated cell technology facilitates the continuous and long-term control of the administration of protein drugs in the eye and central nervous system and protects implanted cells from the host immune system attack. Furthermore, the implants can be retrieved, providing an additional level of safety[4]. In the eye, ciliary-derived neurotrophic factor is reported to be effective in reducing retinal degeneration in at least 13 animal models of retinitis pigmentosa[5-8]. Currently, ciliary-derived neurotrophic factor delivered by encapsulated cell intraocular implants has also been reported[4, 9-10]. Encapsulated cell implants, NT-501 (Neurotech Pharmaceutical Inc., San Francisco, CA, USA) can produce ciliary-derived neurotrophic factor in the vitreous cavity for at least for 1 year[10]. Presently, a phase II clinical trial using ciliary-derived neurotrophic factor delivered by encapsulated cell technology for geographic atrophy in age-related macular degeneration and retinitis pigmentosa has finished, and a phase III clinical trial for retinitis pigmentosa treatment is ongoing[9-10]. However, the pathogenesis of retinitis pigmentosa is complicated and multiple death signaling mechanisms including cell apoptosis and necrosis are implicated in the death of photoreceptor cells[11-12]. Therefore, it may be unlikely for a single neurotrophic factor to prevent complete photoreceptor cell loss, ciliary-derived neurotrophic factor delivered by encapsulated cell technology may allow long-term rescue of retinal cells. Therefore, based on the encapsulated cell technology, neurotrophic factor vectors or combined vectors containing several neurotrophic factors need to be constructed. However, no such vectors have been reported yet. Human neurotrophin-3 (NT-3) is a 13.6 kDa neuroprotective protein that preferentially binds to the high-affinity TrkC receptor and controls the survival and differentiation of mammalian neurons[13-15]. NT-3 is also an important neurotrophic factor that can repair spinal cord injury[16-17]. In ophthalmology, overexpression of NT-3 stimulates a second wave of dopaminergic amacrine cell proliferation after birth in the mouse retina[18]. Sustained delivery of NT-3 from lens fiber cells in transgenic mice has a neuroprotective effect in degenerative retina[19], providing hope for the use of NT-3 to treat retinitis pigmentosa. Furthermore, new data showed a suitable combination of neurotrophic factors may enhance the neuroprotective efficacy of neurotrophins for cell and tissue injury[20]. Therefore, combination of NT-3 and other neurotrophins delivered by encapsulated cell technology might be more effective for the future treatment of retinitis pigmentosa. Recently, most eukaryotic vectors expressing NT-3 have been viral expression vectors, including adenovirus- associated virus vector and lentiviral vector[21-22].

Although viral vectors can transfect cells efficiently, they may not be safe for the treatment of human retinal diseases as they can diffuse throughout the body. Thus, the most appropriate NT-3 expression vector for assembling encapsulated cell technology-based NT-3 intraocular delivery system is human retina-derived NT-3 eukaryotic expression plasmid with efficient expression, secretion and transfection rate. Therefore, this study constructed a human retina-derived NT-3 eukaryotic expression plasmid to investigate whether NT-3-green fluorescent protein (GFP) fusion protein could be expressed and secreted normally by eukaryotic cells in a natural environment.

RESULTS

Expression of NT-3-GFP fusion protein in human NT-3-transfected 293T cells

The concentration of total RNA isolated from human retina amplified by reverse transcription (RT)-PCR was 1.31 μg/μL and the ratio of absorbance at 260/280 nm was 1.87. Thus, the quantity and purity of total isolated RNA were sufficient for the following experiments. The concentration of NT-3-cDNA amplified by two rounds of PCR was 40 ng/μL. Its molecular weight was approximately 820 bp equivalent to human NT-3 DNA (813 bp), suggesting the amplified DNA segments were human NT-3 cDNA (Figure 1).

Construction of pEGFP-N1-NT-3 plasmid

A higher concentration of human NT-3 cDNA,

22.3 ng/mL was obtained by TA-Cloning and was used for recombination with the target plasmid pEGFP-N1 (Figure 2). The sequence of human NT-3 cDNA was identical to human NT-3 cDNA from GenBank (NCBI Reference Sequence: NM_001143812.1) except for two point mutations. However, the encoded amino acid sequences were completely identical to the amino acid sequences of human NT-3 DNA by sequencing when compared using Bioedit (Figures 3, 4). These results verified that the amplified DNA segments could correctly encode amino acids of human NT-3, regardless of the two point mutations. The pEGFP-N1-NT-3 plasmid was obtained by recombining human NT-3 cDNA with the linear pEGFP-N1 plasmid.

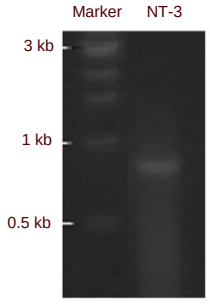

Figure 1 Human neurotrophin-3 (NT-3)-cDNA amplified by reverse transcription (RT)-PCR.

NT-3-cDNA was obtained by two rounds of RT-PCR and detected by electrophoresis. The NT-3-cDNA molecular size was about 813 bp.

NT-3-GFP expression in 293T cells transfected with pEGFP-N1-NT-3

At 24 and 48 hours after transfection with pEGFP-N1 and pEGFP-N1-NT-3, 293T cells expressed GFP and NT-3-GFP fusion proteins, respectively, as detected by fluorescence microscopy (Figure 5). The transfection efficiency of pEGFP-N1-NT-3 at 24 and 48 hours was 17.53 ± 1.27% and 50.06 ± 2.78%, respectively as determined with Image-Pro Plus6.0 image analysis software. At 48 hours after transfection, GFP expression was detected in the cytoplasm and cell nuclei of cells transfected with pEGFP-N1. NT-3-GFP fusion protein expression was detected in many 293T cells transfected with pEGFP-N1-NT-3. However, only some pEGFP-N1-NT-3 transfected cells had NT-3-GFP^{+} vesicles (Figure 6).

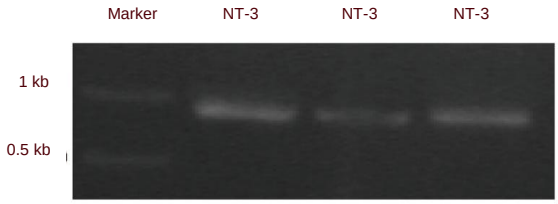

Figure 2 A higher concentration of human neurotrophin-3 (NT-3)-cDNA was obtained by TA-Cloning detected with electrophoresis than that of NT-3-cDNA obtained by PCR.

The bars at about 813 bp were human NT-3-cDNA.

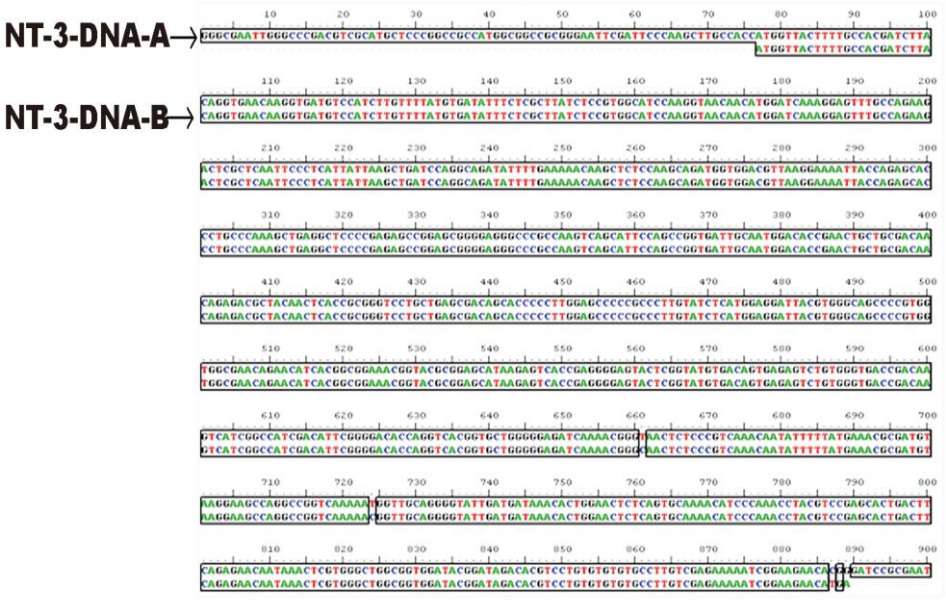

Figure 3 Sequences of amplified neurotrophin-3 (NT-3) DNA-A compared with NT-3 DNA-B from GenBank.

The sequences of NT-3-DNA-A amplified were identical to the sequences of NT-3-DNA-B from GenBank except for two point mutations at 660 bp and 720 bp and without stop codons as compared with Bioedit Microsoft.

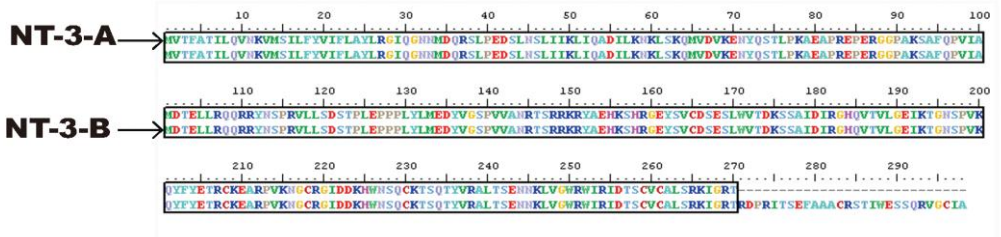

Figure 4 Amino acids sequences of neurotrophin-3 (NT-3)-A coded by amplified NT-3-DNA-A compared with the amino acid sequences of NT-3-B from GenBank.

The amino acids sequences of NT-3-A were identical to NT-3-B compared using Bioedit Microsoft.

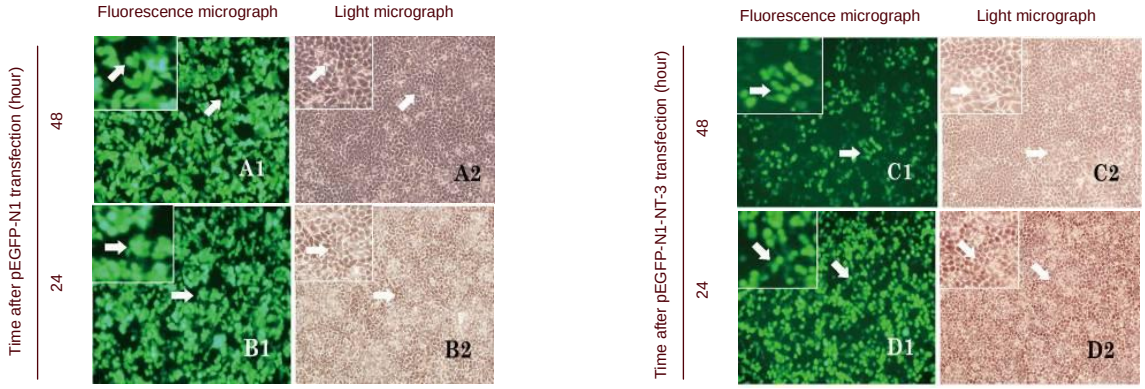

Figure 5 Micrographs of 293T cells transfected with pEGFP-N1 and pEGFP-N1-NT-3 (× 80).

(A1, A2) 293T cells transfected with pEGFP-N1 at 24 hours after transfection. (A1) Fluorescence micrograph with GFP expression (white arrow) in most cells and (A2) light micrograph with the same visual field as A1. (B1, B2) 293T cells transfected with pEGFP-N1 at 48 hours after transfection. (B1) Fluorescence micrograph with GFP expression (white arrow) more than 24 hours after transfection and (B2) light micrograph with the same visual field as B1.

(C1, C2) 293T cells transfected with pEGFP-N1-NT-3 at 24 hours after transfection. (C1) Fluorescence micrograph with NT-3-GFP fusion protein expression (white arrow) in most cells and (C2) light micrograph with the same visual field as C1. (D1, D2) 293T cells transfected with pEGFP-N1-NT-3 at 48 hours after transfection. (D1) Fluorescence micrograph with NT-3-GFP fusion protein expression (white arrow) more than 24 hours after transfection and (D2) light micrograph with the same visual field as D1. NT-3: Neurotrophin-3; GFP: green fluorescent protein.

Western blot assay showed that at 48 hours after transfection, GFP expressed by 239T cells transfected with pEGFP-N1 and pEGFP-N1-NT-3 was greater than in non-transfected cells. 293T cells transfected with pEGFP-N1-NT-3 expressed pro-NT-3-GFP fusion proteins (Figure 7).

293T cells transfected with pEGFP-N1-NT-3 secreted NT-3

293T cells transfected with blank plasmid secreted 0.33 ng/mL human NT-3 factor in the culture medium as measured by enzyme linked immunosorbent assay. In contrast, 293T cells transfected with pEGFP-N1-NT-3 secreted 22.3 ng/mL human NT-3 (Figure 8).

DISCUSSION

NT-3 is a member of the neurotrophin family, which controls survival and differentiation of mammalian neurons[23-25]. This protein is strongly associated with both neural growth factor and brain-derived neurotrophic factor and may be involved in the maintenance of the adult nervous system. It may also affect the development of neurons in embryos when expressed in embryonic placenta. NT-3 deficient mice generated by gene targeting displayed severe movement defects in limbs[26]. Recent research showed that linear ordered collagen scaffolds loaded with NT-3 promoted axonal regeneration and partial function recovery after complete spinal cord transection[27-28]. Furthermore, NT-3 is essential for maintaining the normal functions of peripheral sensory neurons[29]. Previous studies indicated that NT-3 provides essential trophic support to auditory neurons and that injury to secreting NT-3 cells in the inner ear is followed by irreversible degeneration of spiral ganglion neurons and subsequent impaired hearing or deafness[30].

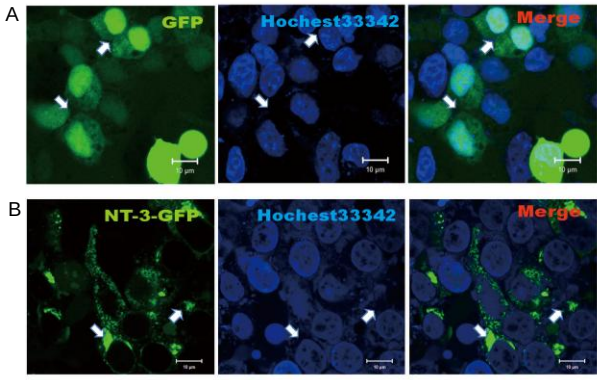

Figure 6 GFP and NT-3-GFP were expressed in 293T cells after transfection (laser confocal scanning microscopy).

The GFP distribution in 293T cells was determined by laser confocal scanning microscopy. Hoechst 3342 staining was blue when excited by light with 405 nm wave length. GFP was green when excited by light with 514 nm wave length.

(A) Image of cells transfected with control pEGFP-N1 showed non-secreted GFP was expressed in both nuclei and cytoplasm, without appearance of secreting vesicles (marked with white arrows).

(B) Cells transfected with pEGFP-N1-NT-3 showed NT-3-GFP fusion proteins had disappeared in the nuclei, and strong fluorescence staining (marked with white arrows) accumulated in secreting vesicles in the cytoplasm. NT-3: Neurotrophin-3; GFP: green fluorescent protein.

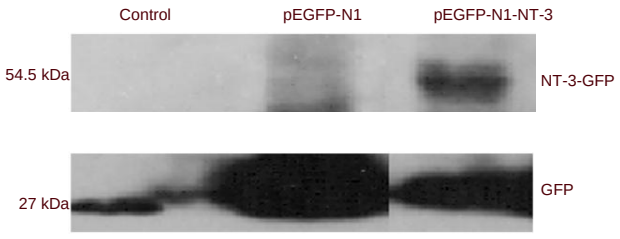

Figure 7 The expression of GFP and NT-3-GFP fusion protein in 293T cells assayed by western blot assay 48 hours after transfection.

The control showed non-specific staining at about 27 kDa. 293T cells transfected with pEGFP-N1 showed GFP expression at 27 kDa without the NT-3-GFP fusion protein. pEGFP-N1-NT-3 (293T cells transfected with pEGFP-N1-NT-3) showed GFP size of 27 kDa and pro-NT-3-GFP fusion protein showed a protein of about 54.5 kDa (proNT-3: 27.5 kDa + GFP: 27 kDa = 54.5 kDa). NT-3: Neurotrophin-3; GFP: green fluorescent protein.

NT-3 also plays an important role in the eye. It affects the development of neonatal retinal cells, but also offers neuroprotection for degenerative retinal cells. A study demonstrated that overexpression of NT-3 in the mouse eye accelerated laminar refinement of retinal ganglion cells before eye opening in mice.

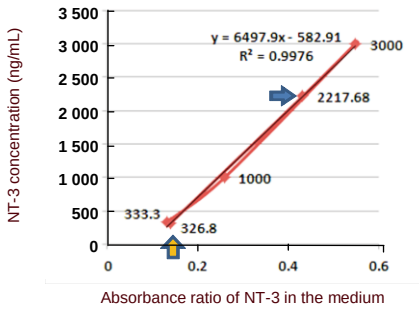

Figure 8 Concentration of human NT-3 factor in cell medium assayed by enzyme linked immunosorbent assay.

The yellow arrow highlights control 293T cells. The concentration of human NT-3 factor in the medium was 333.3 pg/mL (0.33 ng/mL). The blue arrow represents medium of 293T cells transfected with pEGFP-N1-NT-3 diluted 10 times, with a value of 2 230 pg/mL (2.23 ng/mL). Thus, the concentration of human NT-3 factor in the medium was 22 300 pg/mL (22.3 ng/mL). NT-3: Neurotrophin-3; GFP: green fluorescent protein.

Furthermore, NT-3 overexpression increased the number of dendritic branches, but preferentially reduced dendritic elongation in retinal ganglion cells[31]. Another study showed that overexpression of NT-3 upregulated the number of dopaminergic amacrine cells and led to a consequent increase in the density of dopaminergic amacrine cell dendrites in mice. The study further investigated the effect of NT-3 overexpression on retinal apoptosis and mitosis during development and found that NT-3 did not affect the wave of retinal cell apoptosis that normally occurs during the first 2 weeks after birth. Overexpression of NT-3 promoted additional mitosis of dopaminergic amacrine cells at postnatal day 4, but did not affect cell mitosis before birth, the peak period of amacrine cell genesis in wild-type mouse retinas[32]. Most importantly, NT-3 is a crucial neurotrophic factor and has neuroprotective effects on degenerative retinal cells. Previous studies showed that the sustained delivery of NT-3 in the mouse eye by transgenic techniques can offer neuroprotection to photoreceptors subjected to either constant light or several different inherited retinal degeneration disorders[19]. However, neurodegenerative diseases of the central nervous system and retina are chronic disorders, often lasting several decades, thus requiring long-term treatment with NT-3.

Encapsulated cell technology can provide an efficient method for the long-term delivery of NT-3 to the central nervous system or eye. Furthermore, the delivery method of encapsulated cell technology containing ciliary-derived neurotrophic factor has been verified for use in human eyes[9]. Construction of an NT-3 eukaryotic

expression vector, the transfection of host cells with this vector and the following screening of host cell lines that can sustain and stably express NT-3, is crucial to the development of encapsulated cell technology that continuously delivers NT-3 to the central nervous system or eye. Viral expression vectors and plasmid expression vectors have been widely used in recent studies[21-22]. In animals and in vitro studies, NT-3 adenoviral, lentiviral and retroviral expression vectors have been used because of their high transfection efficiencies to host cells. Nevertheless, compared with plasmid expression vectors, viral expression vectors are complicated to assemble. More importantly, they may diffuse throughout the body and induce immune reactions, even causing death[31]. Thus, these disadvantages make them difficult to use in the clinic. Because the main purpose of assembling of encapsulated cell technology containing NT-3 is to perform clinic studies in the future, we constructed an NT-3 plasmid expression vector. Therefore, pEGFP-N1 as a plasmid expression vector may be safer than viral vectors, although it is limited by low transfection efficiency. The use of high efficient transfection reagents such as Vigofect and Lipofectamine for specific cell lines and electroporation can increase the low transfection efficiency of plasmid vectors[34]. Using the transfection reagent Vigofect, the transfection efficiency of pEGFP-N1-NT-3-transfected 293T cells was 50.06 ± 2.78%, which was high enough to screen cells expressing NT-3-GFP. The concentration of secreted NT-3 from transiently infected cells (50.06 ± 2.78% of total cells) was 22.3 ng/mL. In cells with stable transfection (100%), the concentration of NT-3 was about 47.7 ng/mL. This is the first report regarding the amount of secreted recombinant NT-3 by an expression vector. A previous study showed a lentiviral vector secreted 41.40 ± 1.32 ng/mL recombinant rat BDNF-GFP as assayed by enzyme linked immunosorbent assay[35]. This indicated that human NT-3 expression plasmid could express and secrete recombinant protein at similar levels to viral expression vectors, whilst avoiding immune reactions and toxicity.

We selected the pEGFP-N1 plasmid as an expression vector because it is a eukaryotic expression vector and provided the potential to produce soluble, correctly folded recombinant protein that had undergone all necessary post-translational modifications required for functionality. Equally important, pEGFP-N1 contains a gene encoding enhanced green fluorescent tag protein, which allows the tracking of target proteins. Compared with animal-derived NT-3 cDNA, human retina-derived NT-3 is similar to human neuron tissue-derived NT-3 and thus can be used in clinical trials for treatment of retinitis pigmentosa. Thus, we constructed a human-derived NT-3 plasmid.

In this study, total RNA containing human NT-3 was isolated from human retinal tissue by a mono-phasic solution of phenol and guanidine isothiocyanate. This result is consistent with other investigators' reports that NT-3 is expressed partly in human retinal tissue as mRNA and protein[33]. The low expression of NT-3 in the retina was sufficient for our experiments. However, two rounds of PCR increased the concentration of NT-3 cDNA. For recombination to the target plasmid pEGFP-N1, we used TA-Cloning vector, which is an efficient plasmid vector for amplifying large numbers of PCR fragments in *E. coli* [21] and obtained enough insert DNA fragments to ligate with the target linear pEGFP-N1. Sequencing and comparison using Bioedit Microsoft demonstrated that the inserted DNA fragments could correctly express human NT-3 protein.

In this study, we inserted Kozak sequence (GCC ACC) in the front of initial code ATG that enhanced DNA transcription in eukaryotic cells[36]. The efficient expression of NT-3-GFP fusion protein may be attributable to the Kozak sequence. Furthermore, at later stages of expression, secretory vesicles containing NT-3-GFP fusion protein in cells transfected with pEGFP-N1-NT-3 were detected by laser confocal scanning microscopy, but not in cells transfected with control pEGFP-N1, which expressed non-secreting protein GFP. This verified that the processes of NT-3-GFP fusion protein expression and secretion by pEGFP-N1-NT-3 in eukaryotic cells were almost identical to that of the natural environment of eukaryotic cells.

In conclusion, the eukaryotic expression plasmid containing human retina-derived NT-3 efficiently transfected cells to produce NT-3 gene modified cells that could be used to develop NT-3 transgenic mice and study the neuroprotective effects of NT-3 in retinal cells and central nervous system neuron cells. This expression vector may also be useful for encapsulated cell technology-based delivery system of NT-3 and other neuroprotective factors for retinitis pigmentosa treatment in the future. Compared with previous NT-3 eukaryotic expression vectors, the human-derived NT-3 expression plasmid constructed in this study may be more compatible with human neural tissues than animal-derived NT-3 proteins. Moreover, this plasmid expression vector may be safer and more practical for use in humans than viral vectors. The neuroprotective

effects of NT-3 for the treatment of degenerative central nervous system cells and retinal cells and its support of the development of the embryonic and neonatal neural system have been confirmed, but their mechanisms at the molecular and cellular levels are unclear. Thus, the construction of a human NT-3 expression plasmid would help future studies. Thus, the effect of NT-3 secreted by pEGFP-N1-NT-3 on retinal cells requires further investigation.

MATERIALS AND METHODS

Design

The experiment was a controlled, *in vitro* gene experiment.

Time and setting

The experiment was performed at the Laboratory for Functional Genomic Research, School of Life Sciences, Tsinghua University, China between September 2011 and May 2012.

Materials

Retinal tissue was obtained in the Eye Bank of Tongren Eye Center, Beijing, China. The donor was a 29-year-old male, who died in a road accident. He did not have a history of eye diseases. Moreover, the retina did not undergo pathological changes after flattening. This research was performed in accordance with the *Declaration of Helsinki*.

Methods

Isolation of total RNA of retinal tissue

Total RNA was isolated from retinal tissue by Trizol reagent (Invitrogen), a mono-phasic solution of phenol and guanidine isothiocyanate[37]. The fresh retinal tissue and part of the iris tissue weighing approximately 100 mg was isolated from the donor, frozen in liquid nitrogen, grinded in pre-chilled RNase-free mortars with liquid nitrogen, and then the powder was transferred to a 2 mL RNase-free Eppendorf tube. A volume of 1 mL of Trizol reagent was added into the sample (1 mL Trizol reagent can be used to isolate RNA from 100 mg tissues or 10^7 cells according to the user's manual from Invitrogen) and mixed. The homogenized sample was incubated at room temperature to permit the complete dissociation nucleoprotein complexes. A volume of 200 μL chloroform per 1 mL Trizol reagent was added and the tube was shaken vigorously by hand for 15 seconds at room temperature for 2 to 3 minutes. The sample was centrifuged at 10 957 × *g* for 15 minutes at 4°C and the mixture was separated into an upper colorless aqueous phase and a lower red phenol-chloroform phase. The aqueous phase that contained the RNA was transferred to a fresh RNase-free tube. RNA was precipitated in the aqueous phase by mixing 500 μL of isopropyl alcohol at room temperature for 10 minutes. The specimens were centrifuged at 12 000 r/min for 10 minutes at 4°C. After removal of the supernatant, the specimen was kept as a pellet and washed once with 1 mL 75% alcohol and centrifuged at 7 500 r/min for 5 minutes at 4°C. The RNA pellet was dried and dissolved with 50 μL RNase-free water (0.1% diethyl pyrocarbonate treated).

Amplified cDNA of human NT-3 by RT-PCR

The complete cDNA sequence of human NT-3 without stop codon was amplified by RT-PCR technique using 5'-primer (5'-CCC AAG CTT GCC ACC ATG GTT ACT TTT GCC ACG ATC-3') (synthesized by Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China) which introduced an *Hin*d III cleavage site and Kozak sequence (GCC ACC)[33] at the C terminus, and 3'-primer (5'-CGC GGA TCC CGT GTT CTT CCG ATT TTT CTC GA-3') (synthesized by Sangon Biotech) which introduced a *Bam*H I cleavage site and removing stop codon at the N terminus. The RT-PCR amplification used avian myeloblastosis virus reverse transcriptase and Taq HS DNA polymerase in buffer (RNA PCR Kit, Code: DRR019A; Takara Biotechnology (Dalian) Co., Ltd., Dalian, Liaoning Province, China) and the annealing temperature was setup at 55°C. RT-PCR product (6 μL) was used for agarose electrophoresis. A high concentration of human NT-3 cDNA was obtained by a second cycle of PCR with the first RT-PCR product as a template.

Construction of a plasmid expressing human NT-3-GFP fusion protein

The cDNA of human NT-3 was purified using purification of PCR product kits (code: DR02; Biomed, Beijing, China) and its concentration was measured by agarose electrophoresis. The cDNA concentration of human NT-3 was improved by TA-Cloning[30]. Purified human NT-3 cDNA (40 ng/μL) was recombined with T-vector pGEM®-T easy vectors (50 ng/μL) (code: A137A; Promega, Madison, WI, USA) according to the molar ratio of vector to inserted DNA fragment 1:12 by T4 DNA ligase (code: M180A; Promega) at 16°C overnight. Recombinant pGEM®-T easy vectors were used to transform competent *E. coli*. by heat shock transformation and the positive clone by ampicillin and blue/white screening. Further identification was

performed by enzyme digestion analysis, and the positive clone with insertion of human NT-3 cDNA was sent to Invitrogen for sequencing. The identified DNA sequences were compared with human NT-3 cDNA sequences from GenBank in the National Center for Biotechnology Information by Bioedit Microsoft (Ibis Biosciences, Carlsbad, CA, USA). A clone with an identical amino acid sequence to human NT-3 was selected and used to obtain high concentrations of cDNA of human NT-3 by enzyme digestion. Human NT-3 cDNA was obtained by subcloning and was recombined with the target expression plasmid pEGFP-N1 using T4 DNA Ligase (code: M0202V; Biolabs Company) and heat shock transformation to construct a plasmid expressing human NT-3-GFP fusion protein, pEGFP-N1-NT-3 (Figure 9).

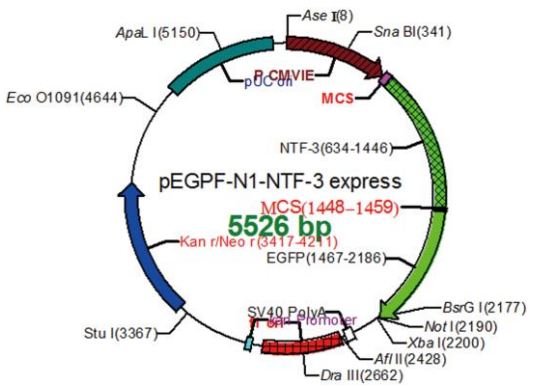

Figure 9 Map of pEGFP-N1-NT-3.

The green arrow with grid lines represents human NT-3-cDNA. Green curve with arrow represents GFP DNA. NT-3: Neurotrophin-3; GFP: green fluorescent protein.

Transfection of 293T cells with recombinant human NT-3 plasmid

Vigofect (Ca#T001; Vigorous Co., Ltd., Bangkok, Thailand) was used to transfect 293T cells with the plasmids pEGFP-N1 and pEGFP-N1-NT-3. Before 24 hours of transfection, 293T cells (ATCC) were plated in five wells of a 6-well plate in 2 mL complete medium (Dulbecco's modified Eagle's medium, DMEM) at a density of 1–2 × 10^5/mL and the medium was completely changed before 1 hour of transfection. A volume of 5 μg of plasmid and 2 μL of Vigofect were diluted, respectively, with 100 μL of blank medium (DMEM) per well and incubated for 5 minutes.

Transfection efficiency was determined by fluorescence microscopy at 24 hours at room temperature. They were mixed completely and incubated for 15 minutes at room temperature and the mixture with pEGFP-N1 was placed into two wells as a blank plasmid control. The mixture with pEGFP-N1-NT-3 was placed into two wells and served as subject wells, with one well containing 293T cells as a control. After 6 hours of transfection, the medium was replaced with complete DMEM and continuously maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Fluorescence microscopy for transfection efficiency of 293T cells with recombinant human NT-3 plasmid

Transfection efficiency was determined by fluorescence microscopy at 24 and 48 hours: three images of three visual fields without overlap per well were assessed by fluorescence microscopy (Olympus, 1 × 71). Cells expressing GFP or NT-3-GFP fusion protein were positively transfected and the transfection efficiencies were estimated by the ratio of the number of GFP-positive cells to the total number of cells. Transfection efficiencies were determined with Image-Pro Plus6.0 image analysis program (Media Cybernetics, Rockville, MD, USA).

Hoechst 33342 staining of NT-3-GFP fusion protein expression in 293T cells with recombinant human NT-3 plasmid

At 48 hours after transfection, the transfected cells and control cells were fixed and cell nuclei stained with Hoechst 33342. The medium was discarded and cells were washed once with PBS. Cells were fixed in 1 mL of 4% paraformaldehyde/PBS on ice for 20 minutes. After removal of the supernatant and two washes with PBS, cells were stained with Hoechst 33342 (1:5 000) for 10–15 minutes. After three washes with PBS, the fixed cells were treated with glycerol. The expression of NT-3-GFP fusion protein was observed by laser confocal scanning microscopy (LSM710, Carl Zeiss, Jena, Germany).

Detection of NT-3 expression in cells by western blot assay

Whole cell lysates of non-transfected, pEGFP-N1-transfected, and pEGFP-N1-NT-3-transfected 293T cells were analyzed at 48 hours post transfection. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 16 μL of cell lysates were mixed with 5 × sodium dodecyl sulfate sample buffer, heated for 5 minutes at 95°C and separated on 12% sodium dodecyl sulfate-polyacrylamide gels. After electrophoresis, the proteins were transferred onto a 0.45-μm pore size nitrocellulose membrane (Hybond™-C; Amersham Bioscience, Piscataway, NJ, USA) using the semidry blotting technique, followed by Ponceau staining to confirm

transfer. The specimens were blocked with 10% milk Tris-buffered saline/Tween-20 for 1 hour on a shaking bed (< 150 r/min), and incubated with rabbit anti-GFP polyclonal antibody (1:2 000; Proteintech Group, Chicago, IL, USA) at 4°C, overnight on a shaking bed (< 150 r/min). This was followed by three washes with Tris-buffered saline/Tween-20. The specimens were then incubated in goat anti-rabbit IgG (H + L) conjugated to horseradish peroxidase (1:5 000; YTHX Biotechnology, Beijing, China/ZSGB-BIO, Beijing, China) at room temperature for 1 hour on a shaking bed (< 150 r/min) followed by three washes with Tris-buffered saline/Tween-20. Protein bands were visualized by chemiluminescence (Kodak Medical X-ray Processor, Carestream Health, Rochester, NY, USA).

NT-3 expression detected by enzyme linked immunosorbent assay

Cell culture medium was measured for GFP expression and NT-3-GFP fusion protein expression using medium of GFP-positive cells as a control sample and NT-3-GFP fusion protein-positive cells as the experiment samples. Cells were collected at 48 hours after transfection and centrifuged at 25 000 r/min at 4°C for 12 minutes. The supernatant was placed in an Eppendorf tube and diluted 10-fold with assay dilution (Abcam Company). All standards were prepared in accordance with the manufacturer's protocol and 100 μL of each standard and sample were added into appropriate wells for 2.5 hours at room temperature with gentle shaking. After the solution was discarded, the specimens were washed four times with 1 × wash solution (Abcam Company) and incubated with 100 μL of 1 × prepared biotinylated antibody (Abcam Company) for 1 hour at room temperature with gentle shaking. Following removal of the solution and washing, the specimens were incubated with 100 μL of 1 × prepared streptavidin solution (Abcam Company) for 45 minutes at room temperature with gentle shaking. Following removal of the solution and washing, the specimens were incubated with 100 μL of TMB one-step substrate reagent (Abcam Company) for 30 minutes at room temperature in the dark with gentle shaking, with 50 μL of stop solution (Abcam Company). Absorbance values were measured at 450 nm on a microplate reader (Bio-Rad Model 680; Bio-Rad Laboratories, Hercules, CA, USA).

Acknowledgments: We would like to thank Functional Genomic Research, Tsinghua University, for the kind gift of the 293T cell line, pEGFP-N1 plasmid, transfection reagent Vigofect and GFP antibodies. We sincerely thank all the members of this laboratory, especially the leader, Professor Shuping Zhang, for their help and advice with construction of pEGFP-N1-NT-3.

Funding: This study was supported by the National Natural Science Foundation of China, No. 30973262.

Author contributions: Chunxia Peng performed the majority of the experiments, wrote the manuscript, provided data, and performed data analysis. Xiaobei Yin conducted part of the experiments. Mengda Li and Ting He participated in the study instruction and analysis. Genlin Li was the study designer, the paper validator, and the fund header. All authors have read and approved the final version of the manuscript.

Conflicts of interest: None declared.

Ethical approval: The application of human retinal tissue in this research was approved by Eye Bank of Tongren Eye Center, Beijing, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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(Reviewed by Croxfor L, Norman R, Zhong XF, Luo WJ)
(Edited by Yu J, Qiu Y, Li CH, Song LP)