Using transient transfected Chinese hamster ovary (CHO) cells by pET28a-LIC-NTF4 in gene therapy

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Abstract

Introduction: Neurotrophins, as a family of proteins, are responsible for induction of the survival, development, and function of neurons. Also, neurotropic factors are growth factors like Neurotrophins that help neurons survive. Moreover, Neurotrophins differentiate between progenitor cells so that neurons are formed. Despite the fact that the majority of mammalian brain neurons are produced prenatally, the capability of growing new neurons from neural stem cells will be preserved by parts of the adult brain. This process is known as neurogenesis. Neurogenesis is stimulated and controlled by neurotrophins.
Materials and methods: The recombinant plasmid transformed to E.coli ´ cell and colonies that contain plasmid were selected by Colony PCR. Enzyme digestion and sequencing were monitored to approve the accuracy of extracted plasmid of the clones.
Results: Plasmid was verified correctly. Based on RT-PCR and western blotting, the transcription of NTF4 gene and the expression of NTF4 protein after transfection were proved.
Conclusion: Plasmid was correctly constructed, CHO Cells were successfully transfected by transfection, and protein could be properly expressed. The results provided a solid foundation for the studies in the area of the transplantation of gene-modified CHO Cells to further spinal cord regeneration.

Keywords: Transfection, Cloning, Molecular, Neurotrophins 4, Genetic Vectors

Introduction

Neurotrophins, as a family of growth factors, are necessary for neurons' survival and maintenance (1). Nerve growth factor, brain-derived neurotropic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4/5 (NTF4) are members of this family. Neurotrophins have various functions that are mediated by binding to high-affinity

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tyrosine kinase receptors and low-affinity p75NTR receptor that have distinct specificities: the TrkA receptor transfers nerve growth factor signals, both BDNF and NTF4 bind to the TrkB receptor, and NT3 interacts mainly with TrkC (2, 3).The expression of the neurotrophin components that signal pathway is not observed only in neural tissues; it designates that neurotrophins have functional roles in other organ systems, as well (4). A cursory perusal of the related literature revels that gene transfer studies have made use of a wide range of mammalian cell types. Recently, it has growingly become significant to introduce transfection reagents that can enable highly efficient DNA uptake into a broad spectrum of cell types (5). Chinese hamster ovary (CHO) cells are cell lines that are derived from the Chinese hamster ovary. Biological and medical research frequently makes use of CHO cells and so do therapeutic companies for commercial production of therapeutic proteins. Introduced in the 1960s, these cells are grown as a cultured monolayer and in their culture medium they need the amino acid proline. Also, studies in the areas of genetics, toxicity screeching, nutrition, and gene expression, specifically those studies that aim at expressing recombinant proteins, use CHO cells(6). Currently, in order to produce recombinant protein therapeutics, the most frequently used mammalian host is CHO cells. The present research was carried out to study the transiently transfect pET28a-LIC-NTF4 in the CHO cells in order to use in gene therapy.

Materials and methods

Sub-Cloning: The carrier vector was primarily transformed in the DH5–α sosh of Escherichia coli strain. After the bacteria were transformed, they were cultured in Luriani berant (L.B) medium inside a shaker incubator. Ampicilin was added to the medium, too. The day after, the proliferated bacteria were prepared to extract plasmid. Through high pure plasmid isolation (Fermntase), the recombinant plasmid was extracted and then subjected to electrophoresis on 0.1% agarose gel. The plasmid was digested via HindIII and NotI enzymes, and the products were separated by electrophoresis with a low-melting-point agarose gel.

PCR: The PCR mix contained 0.5 µg of DNA, 0.1 mM dNTP, 1.5 mM MgCl2, 20 pmole of each specific forward and reverse primers (Forward 5′- GAG CTC ATT CGA CGT CTT CTG CGA-3′ and Reverse 5′- GAA TCC TCA GGA CCG GGT AA CGC-3′), and 1.25 units of Taq DNA polymerase in a final volume of 50µl. PCR was carried out within 30 cycles: denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and elongation at 70°C for 40 s. The PCR product was then subjected to electrophoresis on 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. DraIII and BamHI restriction sites were then added to the 5′ end sequences of the forward and reverse primers, respectively. PCR product was purified, sequenced, and deposited to Gene Bank.

Ligation: After confirmation, they NTF4 were prepared for ligation to expression vector. The gene particle, proliferated by PCR, was clarified via clean up gene kit. The expression vector was cultured simultaneously to proliferate in L.B medium and transformed to linear form making use of Hind III enzyme. The gene particle ligated to the vector using T4 DNA ligase. The ligation product was then incubated at 4 degrees for one night and was then inserted into the process of transformation.

Colony PCR: In order to verify that the gene entered the destination vector, the bacteria were cultured on the plates containing antibiotic. The proliferated colonies entered the colony PCR stage. The proliferated colonies on the antibiotic-containing plates were picked via a sampler and were inserted into a microtube along with revers and forward primers and

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Taq DNA polymerase and were then put inside the thermocycler machine. The PCR product ran on agarose gel and those positive samples which produced a distinct band were transferred to L.B. medium-containing antibiotic. The product was purified via highly pure plasmid isolation kit the same day. Two simultaneous enzymes that had a common buffer were used to analyze the recombinant plasmid. The site of action for these enzymes was located at the two sides of the inserted particle on the plasmid. The samples were incubated for one hour at 37 degrees and all were ran on the gel.

Digestion and sequencing: As the final confirmation of recombinant plasmid following the extraction via kit, those plasmids showing a correct pattern in enzymal analysis were sent to Seq Lab Company in China for sequencing procedure making use of the universal primers. The results of the sequencing were assessed using Blast program (www.ncbi.nlm.nih.gov/blast) to be matched with the genetic bank genes.

Transfection: CHO cells were cultured in Ham s F10 (Gibco-BRL,Scotland) that were supplemented with 10% FCS (Gibco-BRL,Scotland) , 100 u/ml penicillin and 100 µg/ml streptomycin. Then, in order to import gene into the cells, the transfection method was used. The designed gene was imported into the plasmid and then the plasmid was transferred to the CHO. The transfection was carried out in 6 chambered petries in which the cells were cultured between 24–48 hours ago. During the transfection, the cells population was about 2.5 8 105 in each petri. A standard kit was used for liposomes to perform the transfection. The authors used lipofectamin ragin. One microgram of plasmid DNA was added to 350 microgram of a serum-free medium and was left in room temperature for 30 minutes. At the same time, 10 micro grams of lipofectamin was added to 350 micrograms of medium and was left for 30 minutes in room temperature. Then, these two tubes were mixed up and the product was incubated at 37 degrees for 1 hour. Then, the content of microtube was thrown on the cells. The cells were then incubated for 4 hours after which medium and serum were added to the cells and were incubated for 48 hours. The medium of cells was exchanged one day after transfection. In the present study, SDS-page and Western Blotting tests were utelized in order to trace the recombinant protein in eukaryotic systems.

RT-PCR: The expression of NTF4 in transfected and non-transfected CHO groups was examined via RT-PCR. In order to extract total RNA, RNA kit (Invitrogen) was used. The extracted RNA was then treated by DNaseI (Invitrogen) to obtain purified RNA. Next, the purified RNA was analyzed via spectroscopy and agarose gel electrophoresis. Next, according to the manufacturer's instruction, 1000 ng extracted RNA was used to synthesize 20 µl first strand cDNA (Fermentas) of cDNA (500 ng) was used for PCR (Master Mix, 2×, Fermentas) using a thermo cycler (Bio RAD) for 35 cycles. As a negative control, cDNA was omitted from the reaction. The product size of PCR was analyzed by 2% agarose gel electrophoresis.

Results

NTF4 sub-cloning: The Sequencing data revealed the proNTF4 gene has precisely subcloned into the pET28a-LIC- NTF4 to construct pET28a-LIC- NTF4 vector with no mutation. Also, sequencing data analyses of the inserted proNTF4 in the expression vector confirmed the validity of the sequence of NTF4 gene as published by Uniprot and GenBank bioinformatics databases (Fig. 1, 2, 3).

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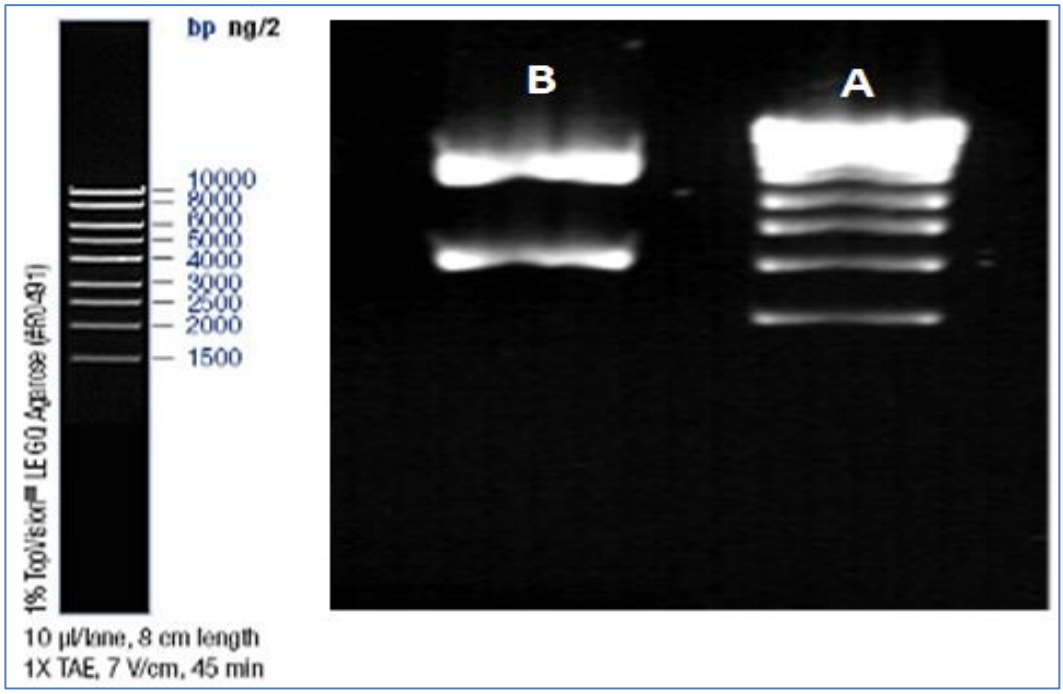


Figure 1. Purification of plasmid pET28a-LIC- NTF4. A, ladder and B, plasmid pET28a-LIC-NTF4 after digestion

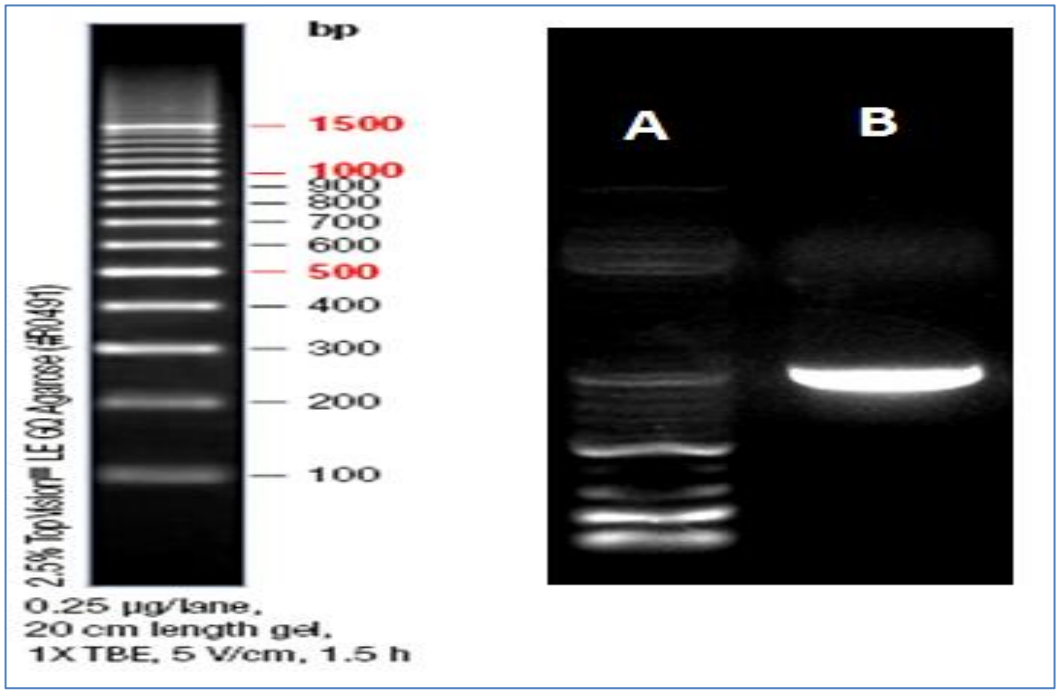


Figure 2. PCR amplification at different temperatures and optimized temperature of 70Cº. Lane A, ladder 100bp and Lane B, PCR product

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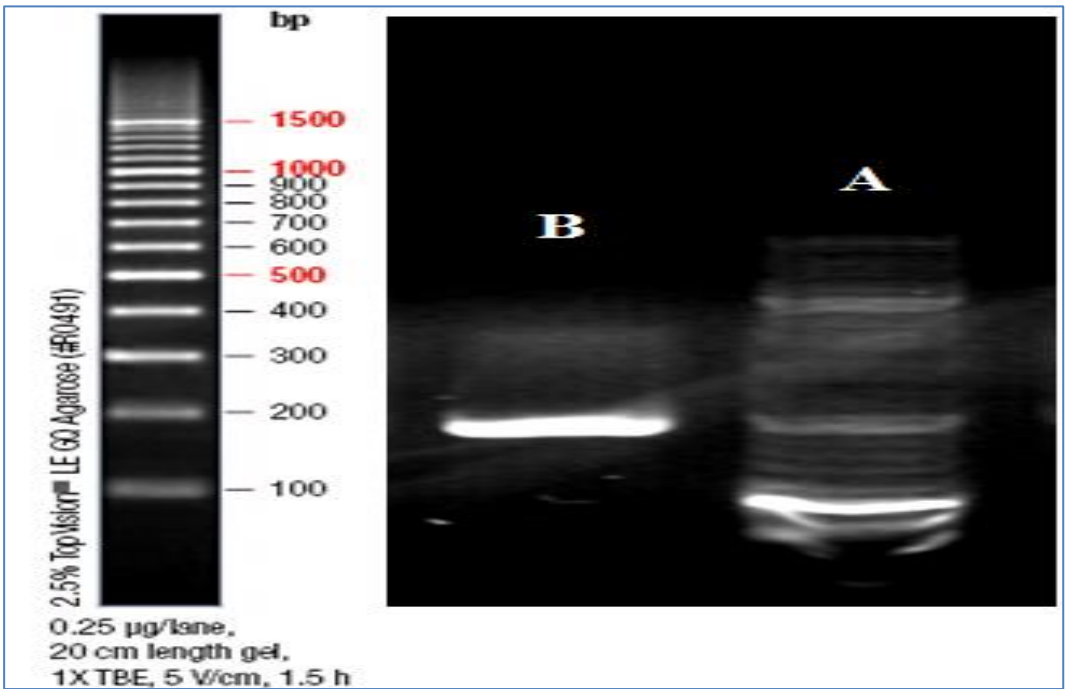


Figure 3. Clooney-PCR on plasmid recombinant. Lane A, ladder 100bp and lane B, PCR product

Enzyme digestion of pET28a/NTF4: The cloned recombined plasmid was named pET28a/NTF4. Theoretically, by restricting enzyme EcoR I and BamH I, PET28A/NTF4 must have two fragments. According to the results, it was shown that the size of the recombinant plasmid and restriction fragments corresponded with the theoretical value, meaning that NTF4 was successfully combined (Fig.4).

Stable transfection of CHO Cells with expression vector: When Kanamycin was added to the medium at optimum concentration, the individual antibiotic-resistant CHO colonies appeared. The cells were then subjected to further analysis.

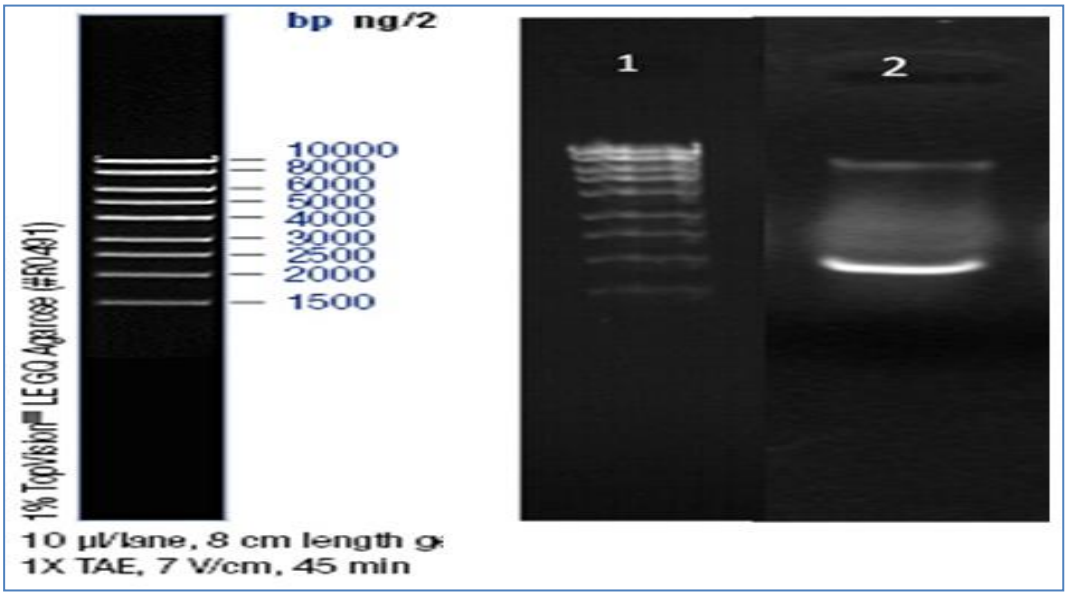


Figure 4. Enzymatic digestion of recombinant plasmid via SmaI enzyme. Lane 1, Marker 100bp and lane 2, plasmid

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SDS-PAGE and Western blot results: SDS-PAGE analyses of the stable transfected CHO Cells led to the detection of NTF4 expression in the lysate. Additionally, semi-quantitative Western-blot data analysis showed that the NTF4 synthesis and secretion in the transfected CHO Cells (Fig. 5).

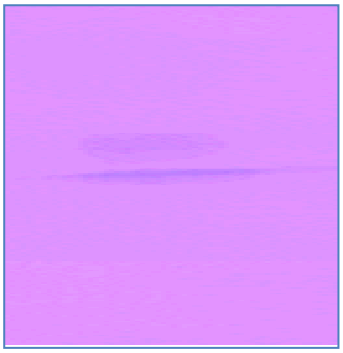


Figure 5. Western blotting of NTF4 Gene**.**

Discussion In the present study, the recombinant vector containing fusion gene NTF4 was successfully constructed via molecular biology techniques. The findings pave the way and provide the basis for further research in the field of Surviving for gene therapy. NTF4 is a type of neuropoietic cytokine. Survival and differentiation of several cell types such as sensory, sympathetic, and motor neurons are induced via NTF4 (1,2,3). It is also used as a protein in the pathogenesis of regenerative diseases (4,5). Yet, contrary to the mice that lack NTF4, those that have a homozygous null mutation in the gene that encode the NTF4Rα chain die perinatally and present severe motor neuron deficits (3). This data suggests that there exists a second NTF4R ligand that has a grave role in development of the neonatal nervous system (6, 7). So far, several researchers have studied neuron growth factors and protective agents (8). Multiple experimental and animal models have proven that neuronal growth factors have protective effects on diseases related to central nervous system like strokes, traumatic lesions, and neurodegenerative problems (9) and some of these neuronal growth factors have already been implemented in certain clinical applications, e.g. Neurotrophins (10). Some neuronal growth factors such as Neurotrophins (NT) have already clinical applications (11). Neurotrophins are among groups of proteins whose responsibility includes development, surveillance, and death of various communities of dopaminergic, sensory, and motor neurons (12,13). It has been shown that these factors have trophic effect on nervous system growth, however how the protective effects of these factors work exactly has remained unknown. One of the members of neurotrophic factors family is NTF4 (14,15). The NTF4 gene is expressed in glial cells of central and peripheral nervous system. It has roles in expressing gene and watching over and developing different types of neurons (16). NTF4 concentration increases prior to birth and if NTF4 gene is depleted or mutated, many nervous system diseases, such as Huntington will be inevitable (17). The NTF4 gene is also considered in breast cancer, and leads to surveillance and growth of neoplastic cells (18).According to the recent studies, NTF4 has a protective autocrine effect on CNS injured neurons (19). The results obtained from immunohistochemical studies have show NTF4 protein can be expressed via transplanted cells transfected by NTF4 gene. Hoane et al, made an attempt to increase the neurotrophic efficacy of NTF4 gene and reached promising results (20). They first differentiated rat emberional stem cells to neural and glial progenitors and then by cloned and transfected NTF4 gene to this cells and then injected them into the injured site of the animal via CCI technique. It is reported that by transplanting this differentiated stem cells to the injured site, sensory and motor behaviors of rats could improve within 2-4 weeks and the injury extender reduced significantly after 40 days (21). Gene cloning and transfecting is integral in biological and developmental researches

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as well as diagnosis, prevention, and treatment of neurogenic diseases (22).

Conclusion

The results of the present study prove Transfection Reagent are easy to use and give higher transfection efficiencies for CHO cells. This activated reagent potential to deliver NTF4 into CHO cells. Additionally, the result showed that the recombinant vector had the ability for transfection in CHO cells and thus this vector can be utilized in gene therapy.

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