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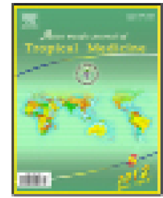

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Establishment and expression of recombinant human glial cell line-derived neurotrophic factor and TNF α receptor in human neural stem cells

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ABSTRACT

Objective: To investigate the interference and expression of human glial cell line-derived neurotrophic factor (hGDNF) and soluble TNF alpha (sTNFR Ⅰ) receptor genes in neural stem cells and to evaluate the roles of these proteins in the genetic treatment of spinal cord injury. **Methods:** Full-length of GDNF cDNA (558 bp) and sTNFR Ⅰ cDNA (504 bp) were inserted into the early 1 region of adenovirus genomic DNA respectively and were immediated by the human cytomegalovirus (gene promoter/enhancer). These adenoviruses were propagated in HEK293 cells via homologous recombination for 7-10 days in vivo, then they were used to infect human neural stem cells. The infection and expression of gene were tested under immunofluorescence, ELISA and Western-blot after 48 hours. **Results:** Almost all the cultured cells showed the nestin immunofluorescence positive staining, which was the characteristics of neural stem cell. A great quantity of EGFP and RFP were observed in neural stem cells, which indicated the expression of GDNF and sTNFR Ⅰ. After transfection of GDNF and sTNFR Ⅰ genes, many neural stem cells show GFAP and tubulin immunofluorescence positive staining, which meant that most neural stem cells differentiated into neuron at that condition. **Conclusions:** The infective efficiency of adenovirus is greatly acceptable to neural stem cell, thus adenovirus provide a useful vector for exogenous GDNF and sTNFR Ⅰ genes expressing in neural stem cells, which is useful for differentiation of neural stem cell.

1. Introduction

For the past few years, combination of neural stem cells (NSCs) transplant and gene therapy has been one of the hotspots in the study of spinal cord injury treatment. After the injury of spinal cord, massive nervous structures will be destroyed because of primary injury and secondary inflammatory changes. And formation of glial scar contributes further to the damage of stem cell niche for nerve regeneration, leading to the regenerative difficulty of nerves. Hence, ideal clinical therapy for spinal cord injury is yet to be developed at present. It has been found that after the transplant few neural stem cells will differentiate into neuronic and most of the cells will differentiate into neurogliocyte, which contribute nearly no repairing effect to spinal cord injury[1]. While, if gene therapy is employed in addition to neural stem cell transplant to modify the transplanted cells and decrease the formation of glial scar, inducing its differentiation toward neurogliocyte and optimizing the regeneration conditions, it is hopeful to achieve better effect in the treatment of spinal cord injury.

Therefore, the present study is aimed to modify the genes related to the above mentioned two aspects. It has been shown in past studies that inflammation reaction and formation of glial scar can be effectively inhibited by blocking the action of TNF α using soluble TNF alpha (sTNFR Ⅰ) receptor receptor[2,3], and neurotrophic factor human glial cell line-derived neurotrophic factor (hGDNF) can induce the differentiation of neural stem cells towards neurons, overcoming the draw backs of differentiation towards multidirectional astroglia by neural stem cells transplant treatment alone[4-6]. Hence, the present study is aimed at construct adenovirus vectors that could be used in the expression of TNF α Ⅰ receptors and GDNF and transfect neural stem cells, facilitating further study in the treatment of spinal cord injury with combination of stem

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cell transplant and gene therapy.

2. Materials and methods

2.1. Separation and cultivation of neural stem cells

The aborted twelve-week human embryo was employed in the study (Informed consent was obtained from Ethical Committee of Shenzen Guanlan People's Hospital and the pregnant woman). After being soaked in 75% ethyl alcohol, the pallium tissue of the embryo was separated immediately under aseptic condition and washed in 4 ℃ D-Hank's solution for three times. The tissue was cut up, added into DMEM/F12, blown and hit gentlely, and then screened by 200-μm cell strainer. The screened tissue was then inoculated into culture flakes at 5×10^5 per flake and incubated in 5% CO_2, at 37 ℃. The constituents of culture medium are DMEM/F12 (1 : 1), supplementary culture medium B27 (all purchased form Gibco Company), 20 ng/mL epidermal growth factor (EGF) and 20 ng/mL bFGF (all purchased from Pepro Tech Company). The culture solution was replaced every three days, and passage of the cells was performed every 7-10 days. The method utilized for cell passage was mechanical isolation. The neurospheres were cut into pieces using dedicated cutting equipment, blown and hit to make a single-cell suspension and then inoculated into new culture flakes after counting.

2.2. Construction and identification of the recombinant adenovirus vector

2.2.1. Construction and identification of human GDNF recombinant adenovirus vector

The methods for the construction and identification of human GDNF recombinant adenovirus vector were present in the previous published paper of our research group. The pLNCX2/GDNF plasmid (provided by Institute for Neuroscience Research, Capital Medical University) was double digested by *Bgl* Ⅱ and *Hind* Ⅲ enzymes. After identified by agarose electrophoresis, the sequence was detected (Beijin AuGCT Biology Co., Ltd.), and compared with human GDNF (hGDNF) sequence in Genebank. And *Bgl* Ⅱ and *Hind* Ⅲ enzymes were used to digest pLNCX2/GDNF plasmid and pAdtrack-CMV plasmid respectively after the identification. The needed fragments were screened by determining the molecular weight in agarose electrophoresis and then recombined with T4 ligase. The ligased products were used for the transformation of DH5 α competent cells. And then the needed cells were cloned and double digested by *Bgl* Ⅱ and *Hind* Ⅲ enzymes to determine the positive clone.

pAdtrack-CMV plasmid (named asp Adtrack-CMV-hGDNF plasmid) containing hGDNF were screened. 2 μL Adeasy-1 plasmid and 5 μL (200 ng) Adtrack-CMV- hGDNF plasmid were transformed into BJ5183 competent cells at the same time to obtain recombinant (named as pAdeasy-hGDNF plasmid). The transformation condition, methods and identification could be referred to published paper[7].

2.2.2. Construction and identification of soluble human TNF α receptor recombinant adenovirus vector

RT-PCR method was used in the extraction of total RNA from U937 cells, and extracellular fragments (275-772 bp) TNFR type Ⅰ were amplified using the RNA as template. The primers for amplification are: P1 (5'-CAGGATCCGATAGTGTGT2GTCCCCAAG-3') and P2 (5'-CCCAAGCTTCTCAGTGCC2CTTAACATTC-3'). The amplification was conducted in a reaction system of 50 μL volume: reverse transcription were performed at 48 ℃ for 45 min, then the obtained products were denatured for 2 min at 90 ℃. Thirty PCR recycles were then performed (denatured for 30 s at 94 ℃, cooled for 1 min at 60 ℃, extended for 1 min at 68 ℃), and the fragment were extended for 7 min at 68 ℃. The obtained PCR amplified fragments were identified by 2% agarose gel electrophoresis containing EB.

The cDNA of targeted fragment and pET28a vector were double digested by *Bgl* Ⅱ and *Hind* Ⅲ enzymes, respectively, recombined. The recombinant products were used for the transformation of DH5 α competent cells.

2.3. Packaging of the corresponding adenovirus vector

2.3.1. Packaging of the adenovirus vector

The above mentioned two kinds of adenovirus were packed using similar methods. The kidney cells of human embryo HEK293A were inoculated in 25 mL cultural flakes at 1×10^5 per flake. When the density of cells reached to 50%-70%, the linearized pAdeasy-hGDNF plasmid and pAdeasy-sTNFR Ⅰ plasmid by Pac Ⅰ enzyme were transfected into the kidney cells of human embryo cells. Six hours later the culture were transferred into completed DMEM culture medium containing 10% bovine serum and incubated for 7-10 days. The culture solution was refreshed every 3 days, and the expression of the targeted genes was observed regularly.

2.3.2. Identification of adenovirus vector

On the seventh day after the transfection, the HEK293A cells were collected using conventional method under aseptic condition. after being resuspended by PBS, the cells were repeatedly freezed and freeze-thawed for 4 times at -20 ℃ and 37 ℃, and then centrifuged (1 2000 r/min) for 10 min at 4 ℃ to obtain the suspension. Five micro liters of suspension was digested by K Protease at 55 ℃ for an hour, and boiled for 5 min, and the identification was performed by PCR. Two primers were used in the identification of hGDNF gene, thus, upstream: 5'-CCACCATGAAGTTATGGGATGTCGT-3' and downstream: 5'-TCAGATACATCCACACCTT-3'. The hGDNF gene was pre-denatured for 3 min at 94 ℃ and then 45 seconds at 94 ℃, 1 min at 60 ℃, 1 min at 72 ℃, the total cycles were 30, and finally extended at 72 ℃ for 10 min. For the identification of sTNFR Ⅰ gene, the primers are upstream 5'-CAGGATCCGATAGTGTGT2GTCCCCAAG-3', and downstream 5'-CCCAAGCTTCTCAGTGCC2CTTAACATTC-3'. The reactions were carried out as follows: 3 min initial

denaturation of sTNFR Ⅰ gene at 94 ℃, followed by 30 cycle of 30 second denaturation at 94 ℃, 1 min annealing at 60 ℃ for 1 min, and 1 min extension at 68 ℃. Finally extended at 68 ℃ for 7 min.

2.4. Transfect ion of adenovirus into neural stem cells and the identification of the expressed product of the targeted gene

After neural stem cell propagation for 48 h, 1×10^8 PFU (colony forming unit) titers of the virus supernatant were added into two bottles of the cell culture, of which one was added transfected virus with targeted gene hGDNF and sTNFR Ⅰ to serve as experimental group and the other was added empty vector virus supernatant as control. After two days of transfection. The supernatant of the cell culture in both experimental group and control group were collected to detect the expression of gGDNF and sTNFR Ⅰ gene (ELISA kit purchased from R&D Company). And the culture temperature were altered from 37 ℃ to 30 ℃ to decreased the output of the targeted gene. The total cell proteins were collected two days later to detect the expression status of the targeted gene using WB methods (antibodies purchased from Santa Cruz Company). Fluorescence microscope was employed to observe the expression status of the targeted gene in the transfected neural stem cells before protein collection.

2.5. Identification of the neural stem cells and its differentiation

Immunofluorescence staining was performed to identify neural stem cells and its differentiation. Neurospheres were stained by Nestin antibody and immunofluorescence positive staining shows the presence of neural stem cells. Glial fibrillary acidic protein (GFAP) antibody and Tubulin antibody were used to stain four-week trangenic cells. Positive staining of both the proteins showed the presence of neuron and axons. The staining methods were followed as the corresponding antibody instruction.

3. Results

3.1. Construction and identification of hGDNF and sTNFR I gene recombinant adenovirus vector

By digesting pLNCX2/GDNF plasmid using *Bgl* Ⅱ and *Hind* Ⅲ enzymes, hGDNF gene of 558 bp was obtained (Figure 1). The length of the amplified sTNFR Ⅰ cDNA was around 504 bp (Figure 1B). As shown in figure 1C, after digested by *Bam*H Ⅰ and *Hind* Ⅲ enzymes the vector fragment and targeted gene which were consistent with theoretical value were obtained from recombinant pET-28a/sTNFR Ⅰ plasmid (Lane 3 and 2 are targeted gene fragment). After recombinating with adenovirus and being digested by Pac Ⅰ enzyme, two fragments of 3.0 kb and 2.9 kb were obtained from pAdeasy-hGDNF plasmid and pAdeasy-sTNFR Ⅰ plasmid, respectively (Figure 1D lane 3 and lane 2).

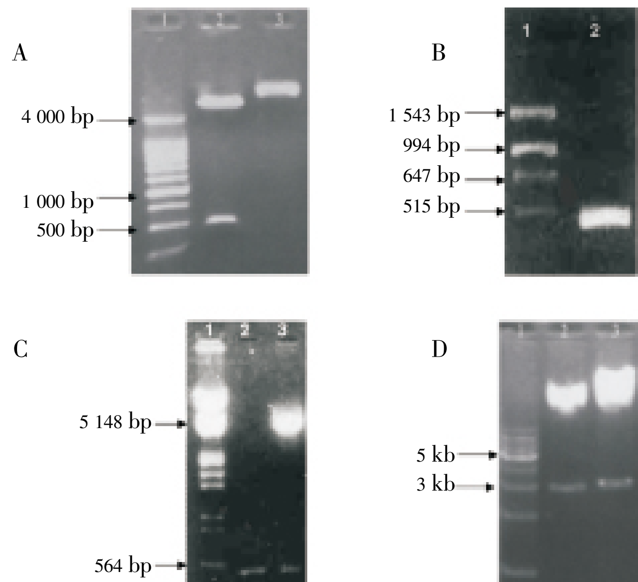

Figure 1. Construction and identification results of hGDNF and sTNFR Ⅰ gene recombinant adenovirus vector.
A: pLNCX2/GDNF plasmid was digested by *Bg1* Ⅱ and *Hind* Ⅲ enzymes to obtain gene vector and 558 bp hGDNF gene fragment (Lane 2), lane 3 represent the undigested vector. B: 504 bp sTNFR Ⅰ cDNA obtained using RT-PCR amplification. C: Identification result of pET-28a/sTNFR Ⅰ enzyme digestion. Lane 2: 504 bp targeted gene, lane 3: Targeted gene after enzyme digestion and vector fragment. D: The obtained fragment after the digestion by Pac Ⅰ enzyme in pAdeasy-hGDNF (Lane 3) and pAdeasy- sTNFR Ⅰ (Lane 2).

3.2. Packaging and identification of adenovirus containing targeted gene

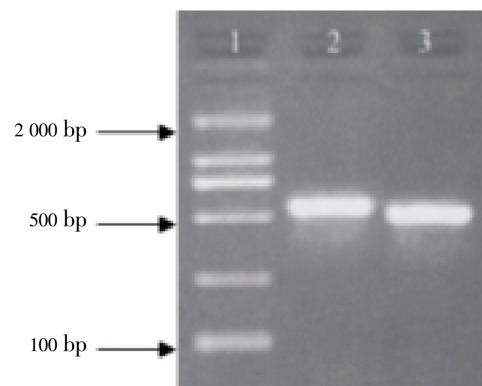

Figure 2. Identification of hGDNF and sTNFR Ⅰ genes in supernatant of virus using PCR. Lane 2: 558 bp hGDNF gene, lane 3: 504 bp sTNFR Ⅰ gene.

Five days after the transfection into 293A cells, obvious hGDNF gene infected cells were observed with presence of green fluorescence protein using fluorescence microscope. And sTNFR Ⅰ gene infected cells were observed with presence of red fluorescence protein. A great quantity of EGFP and RFP were observed in some neural stem cells, which indicated the expression of both GDNF and sTNFR Ⅰ. The supernatant of the virus were collected on the eighth day and detected by PCR. The obtained specific 558 bp targeted band was hGDNF gene (Figure 2, lane 2), and the 504 bp band was sTNFR Ⅰ gene (Figure 2, lane 3).

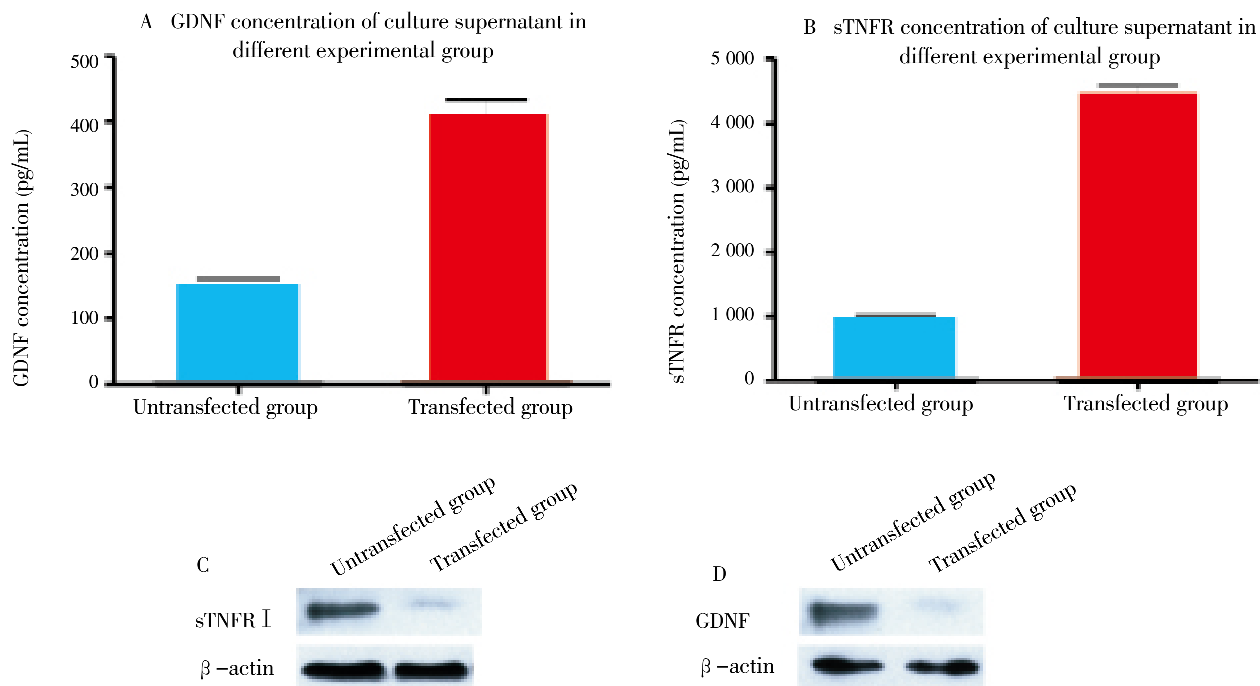

Figure 3. Expression status of hGDNF and sTNFR Ⅰ gene in culture supernatant and adenovirus infected NSCs.

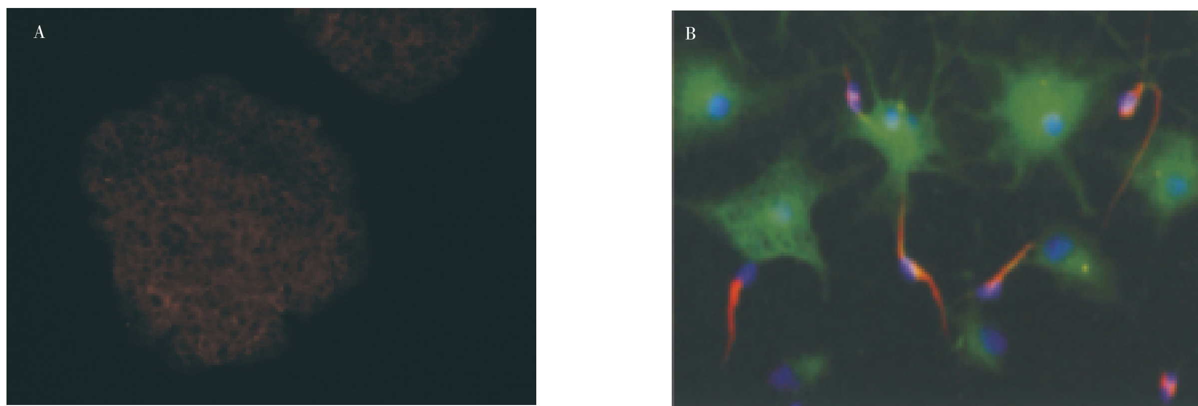

Figure 4. Immunofluorescence staining of NSCs and its differentiation.
A: Nestin antibody staining in neurosphere, B: Four weeks after the infection of adenovirus, GFAP (green) and Tubulin antibody staining.

3.3. Expression status of the targeted gene in adenovirus infected neural stem cells

After the infection of adenovirus, the expression of GDNF and sTNFR Ⅰ gene was observed to be increased obviously in the supernatant of the culture by ELISA detection (Figure 3A and B). Increased expression could be detected by Western blot methods in cells cultured under low temperature to decrease the output of targeted protein (Figure 3C and D), which validated the effectiveness of this method.

3.4. Detection of the neural stem cells and its differentiation

A large quantity of cells showed Nestin positive staining after the immunofluorescence staining in neurosphere (Figure 4A), which showed that the neurosphere retained the characteristics of neural stem cell in vitro.Four weeks after the infection of adenovirus, the cells were stained with immunofluorescence. The GFAP positive cells (green) also showed Tubulin positive areas (red), which meant that most neural stem cells differentiated into neuron at that condition (Figure 4B).

4. Discussion

The studies of NSC are mainly focused on NSC transplant to treat brain lesion and spinal cord injury, which is considered to be a promising therapy[8-10]. However, to achieve an ideal effect, the problems of long term survival and directional differentiation of the NSCs must be solved. While, to induce the committed differentiation of NSCs into neurons and to optimize the niche for the regeneration of the nerves will greatly enhance the effect of stem cell transplant.

A relatively high concentration of neurotrophic factor around the transplanted cells is important for the survival and committed differentiation of the cells[11].It has been shown that many neurotrophic factors including nerve growth factor, eurotrophy (NT-3), brain-derived neurotrophic factor, *etc*[12] can facilitate the differentiation of the NSCs into neurons. As a peptides growth factor in TGF-α family, GDNF is a kind of secretory protein which can promote the growth of neuron, increase the nerve fiber and enhance the secretion level of dopamine[4,5]. It can also protect dopamine neurons and motor neurons from injury and improve the function of central nervous system [13]. In

our earlier studies, GDNF gene recombinant adenoviruses were successfully imported into NSCs and expressed, increasing the neurotrophy concentration around the NSCs and inducing the differentiation of NSCs towards neurons.

However, except the obvious influence of the local neurotrophy factor on the committed differentiation of NSCs, the negative effect of the inflammatory mediators in the growth niche should also be eliminated [14,15]. After the spinal cord injury, the massive release of inflammatory mediators will lead to the destroy of nervous structure and the formation of glial scar, worsening the niche for nervous regenaration.It has been proven that TGF-α is the main medium in the inflammatory reaction.After the injury of the surrounding nerves, the TGF-α in the vervous system is mainly synthesized and released by macrophage and schwann cell or by astroglia and microglia in central nervous system, which plays an important role in the pathological physiology process in the injury of central nervous system. TGF-α realizes its biological function by combining with the TGF-α receptors on the cell membrane and activating signaling pathway in the cells. Soluble TNFR can combine with TGF-α specifically and decrease the biological activity of TGF-α, which make it a therapy for the treatment of TGF-α mediated diseases[2,3,16]. Hence, in the present study, except for providing high concentration of growth factor for NSCs through transinfecting GDNF gene, soluble TGF-α receptor was also transplanted to block the injury of excessive TGF-α on NSCs, optimizing the growth and differentiation niche in two aspects.

The study of optimizing NSCs regeneration niche includes direct injection of growth factor in pivot, transplant of brain tissue processed by growth factors in embryo, local injection of transplant and virus mediated growth factor gene therapy, *etc*[17]. And gene therapy receives widely emphasis because of it obvious application prospects[18]. In gene therapy, recombinant adenovirus with cloning defects has been used widely in clinical treatment as effective gene vector because of its high transfer efficiency and non chromosomal integration incidence. Although the most widely used vector in GDNF mediated gene therapy is retrovirus vector because its exogenous gene could be integrated into cell genomes to realize its expression, retrovirus only infect cells in split period and has very low infection rate in NSCs[19]. While recombinant adenovirus has a relatively high infection rate in NSCs and cells in quiescent and split period. In addition, brain and spinal cord are partial immune privilege organ, adenovirus can express for several weeks without eliciting inflammatory reactions[20]. Hence, the present study employed adenovirus to transfect GDNF gene and obtained expected effect. And good effect were also achieved in the transplant of sTNFR Ⅰ using adenovirus. The differentiation of NSCs into neurons and formation of axons proves that the present gene therapy could achieve a well effect in vitro, which facilitates the further in vivo study.

Conflict of interest statement

We declare that we have no conflict of interest.

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