Alizadeh et al., Clon Transgen 2015, 4:3
http://dx.doi.org/10.4172/2168-9849.1000142

Cloning & Transgenesis
ISSN: 2168-9849

Research Article Open Access

Lentiviral Mediated Overexpression of NGF in Adipose-derived Stem Cells

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Abstract

Introduction: Human adipose-derived stem cells (ADSCs) are multipotent stem cells that can self-renew and differentiate into various types of cells such as adipocytes, osteocytes, and neural cells. These stem cells can be isolated by minimally invasive technique in large amounts. ADSCs are a useful resource for cell therapy and regenerative medicine. Nerve growth factor (NGF) is the first neurotrophin factor discovered and characterized for its anti-apoptotic role in neural development. NGF can promote neuronal survival and neurite outgrowth and it also promotes neuron differentiation and migration. Moreover, research showed that NGF could protect axons from inflammatory damage, improve cognitive function in damaged brain models, and function in the prevention and treatment of neurological diseases like Alzheimer's disease. In this study we use Lentiviral vector-mediated gene transfer technique to deliver NGF gene to ADSCs and overexpress this factor in ADSCs.

Method and Materials: ADSCs extracted from human adipose tissue after lipoaspiration by digestion method. ADSCs characterized with Flowcytometry and differentiation assay in adipogenic and osteogenic differential media. The NGF gene was cloned in pCDH-513B-1 (System Bioscience, Mountain View, CA, United States) under a cytomegalovirus (CMV) promoter. Recombinant lentiviruses were produced according to the Prof. Trono lab protocol with some modifications in HEK 293T cells. The spinfection method was used to transduce ADSCs. NGF expression was assayed using fluorescent microscope to trace green fluorescent protein (GFP) marker, RT-PCR and western blotting.

Results: Extracted ADSCs had mesenchymal morphology and differentiated into adipocytes and osteocytes in differentiating media. HEK293T easily transfected with pCDH-513B-1 and over 99% of them expressed GFP so we gathered pseudoviruses from the supernatant. ADSCs transduced with these pseudoviruses transferred NGF and after transduction expressed GFP, as seen under fluorescent microscope. RT-PCR and western blotting verified NGF overexpression in them.

Conclusion: ADSCs can be transduced with pseudo lentiviruses transferring NGF leading to overexpression of NGF.

Keywords: ADSCs; NGF; Pseudo lentiviruses

Introduction

Cell therapy is an important field in regenerative medicine. In this field several kinds of cells attract researchers' attention. The growing interest focuses on the mesenchymal stem cells (MSCs). These stem cells have multipotent differentiation and immunosuppressive properties that make them an ideal candidate in cell based therapies. Human adipose derived stem cells (ADSCs) are a source of multipotent stem cells that can self-renew and differentiate into various types of cells such as adipocytes, osteocytes, and neural cells [1,2]. These stem cells can be isolated by minimally invasive technique in large amounts. Approximately 0.5×10^4 to 2×10^5 ADSCs can be isolated per gram of adipose tissue from different patients [3]. So, ADSCs are a useful resource for cell therapy and regenerative medicine. In clinic ADSCs are used in soft tissue defects, post mastectomy repair, lipodystrophy and have some cosmetic applications [4]. Several clinical trials are currently ongoing around the world using these stem cells [5,6].

Nerve growth factor (NGF) is the first neurotrophin factor that was discovered and characterized for its anti-apoptotic role in neural development [7]. NGF can promote neuronal survival and neurite outgrowth and it also promotes neuron differentiation and migration [8,9]. Moreover, researches showed that NGF could protect axons from inflammatory damage, improve cognitive function in damaged brain models, and function in the prevention and treatment of neurological diseases like Alzheimer's disease [10,11]. So, NGF is a therapeutic agent for preserving and restoring neuronal function during neurodegenerative disorders, traumatic brain injury (TBI) and spinal cord injury (SCI) [12-15]. However this agent has a short half-life so its direct application is limited [11].

Moreover, when this factor was delivered peripherally, its efficacy dropped due to the blood–brain barrier. Therefore, a new approach to long term delivery of NGF into central nervous system (CNS) has to be developed, maybe one based on transplantation of ADSCs which overexpress it.

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Received July 24, 2015; **Accepted** August 25, 2015; **Published** August 27, 2015

Citation: Alizadeh A, Soleimani M, Ai J, Fallah A, Hashemian SJ, et al. (2015) Lentiviral Mediated Overexpression of NGF in Adipose-derived Stem Cells. Clon Transgen 4: 142. doi:10.4172/2168-9849.1000142

In this regard use of viral vector-mediated gene transfer techniques, particularly based on lentiviruses, have some useful features. Lentiviral vectors can deliver sequence up to 8 kb and mediate gene transfer into any cell type with sustained expression and normal cellular functions in vitro and in vivo. No significant immune responses or unwanted side effects of the vectors have been reported yet [16,17]. Hohesfield et al compared five methods of gene transfer: cationic lipid-mediated transfection, classical electroporation, nucleofection, protein delivery (Bioporter) and lentiviral vectors for overexpression of NGF. They illustrate that lentiviral infection can successfully produce effective NGF secretion [18]. In this study we transduced ADSCs with pseudo lentiviruses to overexpress NGF.

Method and Materials

Isolation and expansion of human ADSCs

Human adipose tissue was obtained from patients after lipoaspiration plastic surgery under a bioethics agreement. The tissue was transferred to a laboratory. After three washings with PBS containing Penicillin/Streptomycin and Amphotericin, Dulbecco's Modified Eagle's Medium (DMEM)(GIBCO-BRL, Tokyo, Japan) with dispase (50 U/ml) and collagenase I (250 U/ml) (Sigma-Aldrich, St. Louis, MO), was added to the tissue and then it was shaken for 30 minute at 37°C. The solution was then centrifuged for 3 minutes at 1500 rpm. Mononuclear cells, which were separated, were washed with erythrocyte lysis buffer and PBS. Finally isolated cells were suspended in DMEM supplemented with 10% fetal bovine serum (FBS) (GIBCO-BRL, Tokyo, Japan) and 1% Penicillin/Streptomycin and cultured in flasks. The medium was changed every 48 h and at 80-90% confluence, the cells were passaged.

Human ADSCs characterization

Flowcytometry analysis: To characterize hADSCs, Flowcytometry was used. After 4 passages the isolated cells were fixed in paraformaldehyde 2% for 30 min. After washing two times with PBS, the fixed cells were incubated 30 min with CD90 (Specific mesenchymal stem cell surface marker) and CD45 (Hematopoietic stem cell surface marker) antibodies. CD90 antibody was directly conjugated with the allophycocyanin (APC). The Rat IgG2b was used as a control isotope of CD90. For CD45 antibody, Goat anti-Rabbit IgG-FITC was used as a secondary antibody and Rabbit polyclonal IgG was used as a substitute control antibody. Flowcytometry was performed with a BD FACScalibur Flowcytometer device (BD Biosciences, USA). Details of used antibodies are summarized in Table 1.

Adipogenic differentiation: At the fourth passage the ADSCs were incubated with adipogenic maintenance medium containing 0.5 mM isobutylmethylxanthine (IBMX), dexamethasone (10-7 M), insulin (66 nM), and indomethacin (0.2 mM) for 21 days. The medium was changed every three days throughout the study. The adipogenic differentiation was assayed using of Oil Red O (Sigma, Belgium). When the adipogenic medium was removed the cells were washed three times with PBS. Then the cells were fixed by 10% formalin for 30-60 minutes at room temperature, washed with distilled water and treated with 2 mL isopropanol (60%) for 5 minutes. Fixed cells were stained with Oil Red O (2 mL to each well) at room temperature for 5 minutes [19,20]. Finally, the cells were washed with tap water and photographed with inverted microscope (Olympus, Japan).

Antibody	Cat. number	Company	Dilution
CD 90	559869	BD Biosciences	1/100
CD 45	Ab10558	Abcam	1/100
Rat IgG2b	17-4031	eBioscience	1/200
Rabbit polyclonal IgG	Ab27478	Abcam	1/100
Goat anti-Rabbit IgG-FITC	AP156F	Millipore	1/100

Table 1: Details of used antibodies.

Osteogenic differentiation: For differentiation of ADSC (at the fourth passage) toward osteogenic phenotype, the ADSCs culture medium was changed to osteogenic maintenance medium containing 10 mM β-glycerophosphate, 0.2 mM ascorbic acid and 10-7 M dexamethasone for 21 days [19]. The cultured cells were fed every three days throughout the study. For confirming the osteogenic differentiation, we used Alizarin Red staining. Osteogenic medium was removed and the cells washed three times with PBS. The cells were fixed by 70% ethanol at 4°C for 1 h. Then the cells were washed with deionized water and allowed to air dry. The fixed cells were stained with 2% Alizarin Red S (pH 7.2, Sigma, Belgium) at 37°C for 1 h, then washed in deionized water and photographed with inverted microscope (Olympus, Japan).

Plasmid construction: In this experimental study, we purchased human NGF (EBI3) cDNA from Open Biosystems (Huntsville, AL, United States). The gene was cloned in pCDH-513B-1 lentivector (System Bioscience, Mountain View, CA, United States) under a cytomegalovirus (CMV) promoter. pCDH-513B-1 had copa GFP (copepod green fluorescent protein) under EF1 promoter with T2A self-cleavage peptide and resistance to puromycin for selecting stably transduced cells. All cloning procedures into XbaI and EcorI sites were performed according to the common digestion-ligation protocol. We verified the pCDH-CMV-hNGF-EF1-CopaGFP- T2A -Pur construct by digestion and subsequent sequencing [20].

Production of recombinant pseudo lentiviruses: Recombinant lentiviruses were produced according to the Prof. Trono lab protocol with some modifications [19,21,22]. Briefly, 1×10^6 HEK 293T cells (Invitrogen, Carlsbad, CA, United States) were cultured in a 10 cm plate in DMEM medium with 10% FBS one day prior to transfection. We replaced the medium 2 hours before transfection with fresh medium. Then 21 µg of pCDH-CMV-hNGF-EF1-copGFP-T2A -Pur, 21 µg of pCMV-dR8.2, 10.5 µg of pMD2, 33 µl of TE 1X, 105 µl of 2.5 M CaCl2, and 1050 µl of 2x HEPES-buffered saline (HeBS) were added to 10 cm^2 plate (the HeBS added during the solution was vortexed). The HEK 293T cells with this solution were kept in incubator for 14 hours. The transfection medium was replaced with fresh medium 14 hours after transfection. 24 h later the cells were assayed for GFP expression with fluorescent microscope (Leica, German). GFP expression indicated that transfection was efficient. Under fluorescent microscope five fields were selected randomly and the transfection rate was presented as a percentage of GFP-positive cell number to total cell number. After first infection, viral titer was sufficient for use in the following experiments and the supernatant of cells containing viruses was collected at three times (24, 48 and 72 hours after transfection). These collected supernatants were centrifuged at 1500 rpm and filtered through a 0.25 µm filter before transduction. The titer of viruses was determined using Prof. Trono lab protocol.

Genetic engineering of ADSCs

After four passages ADSCs were cultured in six well cell culture plates. Before adding fresh recombinant viruses, ADSCs were washed with PBS. Recombinant viruses that were collected from transfected HEK 293T cells supernatant, with MOI of 50 were added to each well. The spinfection method (2000 rpm for 60 minutes at a temperature of 25°C) was used for efficient transduction. After spinfection, the

Name	Forward Primer	Reverse Primer	Tm	Product Size
NGF	5-CAGTTTTACCAAGGGAGCAG-3	5-TAGAAGGGGCAGGGGGAG-3	61.8	814bp
GAPDH	5-CAAGGTCATCCATGACAACTTTG-3	5-GTCCACCACCCTGTTGCTGTAG-3	66	495bp

Table 2: Primer sequences used.

plate was placed in a 37°C incubator and the medium was changed 14-20 hours after spinfection. Transduced cells were assayed for GFP expression with fluorescent microscope (Leica, German) at 24, 48 and 72 hours after spinfection and weekly for 8 weeks. GFP expression indirectly indicated expression of NGF and transduction efficiency. Under fluorescent microscope five fields were selected randomly and the transduction rate was presented as a percentage of GFP-positive cell number to total cell number.

Overexpression of NGF

RT-PCR: Total RNA was extracted from 2×10^6 transduced ADSCs after purification with puromycin and cDNA was synthesized by Qiagen kits (Alameda, CA, United States) using oligo-dT primer. The cDNA was used for PCR following to the manufacturer's protocols. Specific primer pairs were used for NGF and GAPDH. The expression of NGF mRNA was evaluated in the lentiviral engineered ADSCs. GAPDH expression was used as an endogenous reference gene. The PCR product (after 30 cycles) was separated by electrophoresis on 2% agarose gels, subsequently visualized with a UV transilluminator (UV-tec, Germany) and digitally photographed. The amplicon was quantified densitometrically. Primer sequences used in this study are provided in Table 2.

Western blotting: To confirm NGF overexpression, total cellular protein from transduced ADSCs with NGF-GFP and GFP pseudo lentiviruses, was extracted using lysis buffer and a cocktail protein inhibitor (Gibco, USA). The concentration of protein was analyzed with Bio-rad protein assay buffer. Proteins were run in 8% glycine gels. After electrophoresis, separated proteins were transferred to nitrocellulose membrane and blocked with 5% non-fat milk in TBST buffer for 1 hour. Then immunoblotting was performed with NGF (Abcam) and actin (Abcam) antibodies at 1:1000 dilutions in 5% non-fat milk at 4°C overnight. Anti-rabbit IgG monoclonal antibody, conjugated with horseradish peroxidase at 1:2000 dilution, was used for 1 h at room temperature. Finally protein bands were visualized by ECL, scanned and analyzed.

Statistical analysis: Differences between groups were analyzed using the Student's t test or appropriate level of ANOVA. Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS, version 18). A $p < 0.05$ was considered statistically significant.

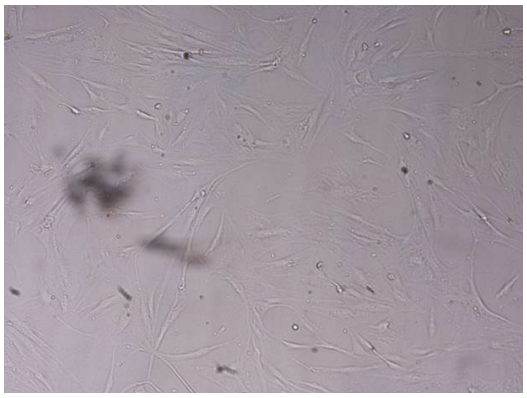

Figure 1: ADSCs after first passage, ×10 magnification, inverted phase-contrast microscope (original picture).

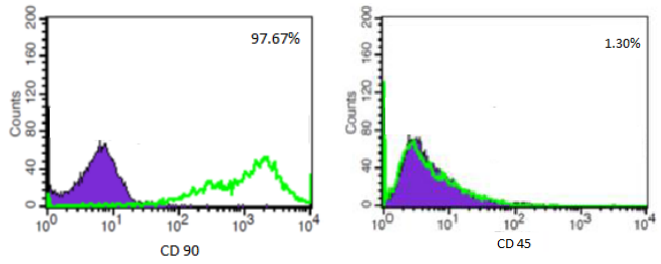

Figure 2: Flowcytometry analysis of isolated hADSCs: about 98% of the cells expressed CD 90 as specific mesenchymal stem cell surface marker.

Ethical considerations: Human adipose tissue was obtained in accordance with the Declarations of the Shahid Beheshti University of Medical Science and Stem Cell Research Center Committee.

Results

Isolation and expansion of human ADSCs

After isolation ADSCs were cultured in DMEM supplemented with 10% FBS and 1% Penicillin/Stereptomycin. Like other mesenchymal stem cells immediately after isolation these cells had a round shape and floated. 24 h later, the floating cells attached to flask and formed fibroblast-like colonies during the first week. During the first three days lipid granules of cells were released into the culture medium and deleted. After six days the cells were confluent so first passage was performed (Figure 1). The cells proliferation rate was rapid as with bone marrow mesenchymal stem cells. After 2 weeks (at 3-4 passages) the cells were used for differentiation assay and other manipulations.

Flowcytometry analysis

About 98% of the isolated cells were CD 90 positive cells and only a tiny population of them (1.3%) was CD 45 positive. Results are showed in Figure 2.

Adipogenic and osteogenic differentiation

In vitro differentiation of ADSCs into adipocytes and osteocytes using inductive cocktail medium has been shown in Figure 3. The differentiated into adipocytes cells gained small lipid vacuoles or droplets during the differentiation process and were of various sizes due to the different amounts of lipid and were stained with Oil Red O (Figure 3A). After 21 days in osteogenic inductive medium ADSCs differentiated into osteocytes which were capable of mineralizing extracellular matrix and were stained with Alizarin Red dye (Figure 3B).

Production of recombinant pseudo lentiviruses

We cloned human NGF cDNA in a pCDH-513B-1 lentiviral vector. The construct was co-transfected in HEK 293T cells with the helper packaging vectors using CaCl2 protocol. The transfection efficiency shown by the GFP marker under fluorescent microscope was more than 90% (Figure 4). The viral particle titer was approximately $1.5\text{-}2\times10^6$.

Genetic engineering of ADSCs

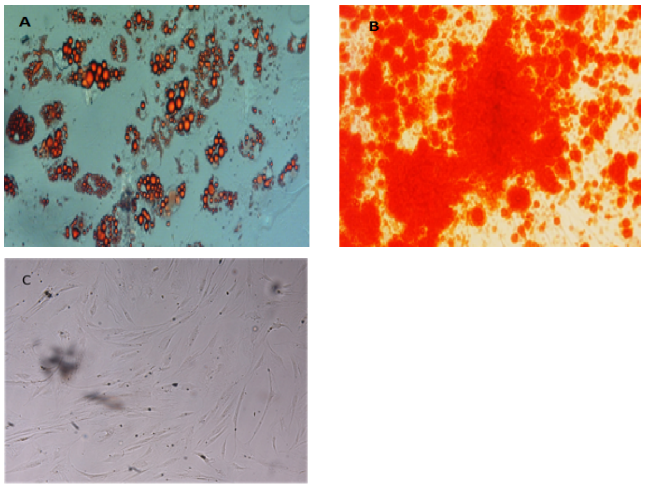

Figure 3: (A) Differentiated ADSCs into adipocytes, (**B)** Differentiated ADSCs into osteocytes and **C:** Control ADSCs, ×10 magnification, inverted phase-contrast microscope (original picture).

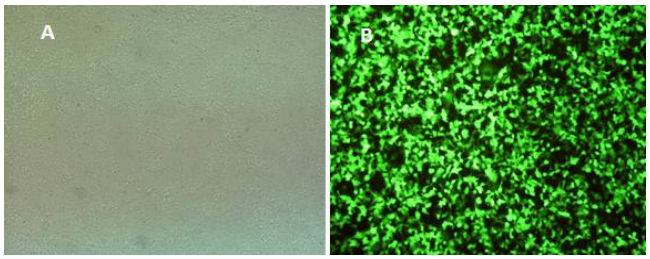

Figure 4: HEK 293T cells 24 hours after transfection, ×10 magnification (original picture). (**A)** Transfected HEK 293T cells in inverted phase-contrast microscope, (**B)** The same field after florescent illumination.

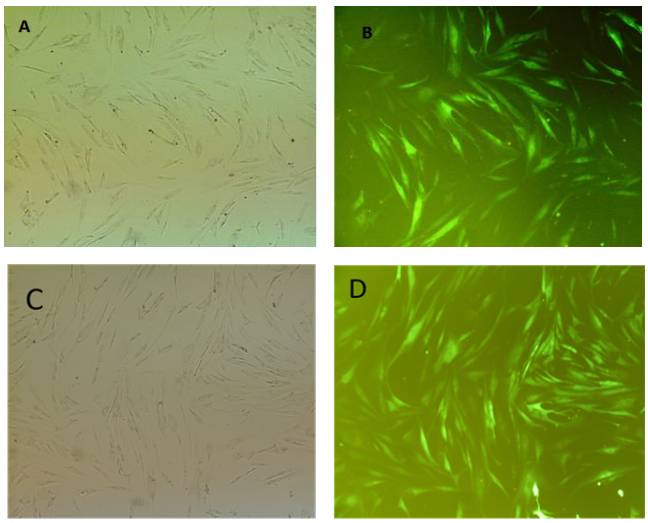

Figure 5: Transducted hADSCs by lentiviral particles, ×10 magnification (original picture). (**A,B)** Transducted hADSCs before and after fluorescent illumination; 72 h after transduction, (**C,D)** Transducted hADSCs before and after fluorescent illumination; 8 weeks after transduction.

24 h after transduction of ADSCs some adherent cells were expressing weak green fluorescence under fluorescent microscope. After 48 h stronger fluorescence was observed in whole-cell cytoplasm and nucleus of more cells. 72 h after transduction about 80% of the cells expressed strong fluorescence (GFP) when assayed under fluorescent microscope (Figure 5A and 5B). Therefore, human ADSCs were successfully transduced with pCDH-513B-1 lentiviral vector with high efficiency. The GFP marker provided a good index for the transfection, transduction and purification processes and indirectly indicated NGF expression in the cells. Transduced cells were selected via puromycin. The selection curve determined that 2 μg of puromycin was sufficient to generate approximately 90% true transduced cells after 3 days. Tracing transduced ADSCs with fluorescent microscope for 8 weeks, showed that the percentage of GFP positive ADSCs did not change and GFP stably expressed in them (Figure 5C and 5D).

Overexpression of NGF

RT-PCR: RT-PCR analysis confirmed overexpression of NGF mRNA in ADSCs after transduction with NGF-GFP pseudo lentiviruses in contrast with the control cells (Figure 6).

Western blotting: Western blotting was performed to confirm the overexpression of NGF protein. Figure 7 shows that control ADSCs expressed NGF at low level. Transduction of ADSCs with lentiviral vector without NGF increased the NGF expression slightly. After transduction with lentiviral vector transferring NGF, expression of NGF protein in ADSCs significantly increased.

Discussion

Regeneration of the central nervous system (CNS) is the most important target of regenerative medicine. A large population in the world suffers from neurological diseases caused by loss of cells in different parts of CNS. Transplantation of mesenchymal stem cells (MSCs) improves the recovery in some neurological diseases [21]. ADSCs as an easily accessible source of MSCs are the best candidate in CNS regeneration. It demonstrated that ADSCs have neurotrophic activity and secrets NGF at low level [22]. NGF is a therapeutic agent for preserving and restoring neuronal function in CNS disorders.

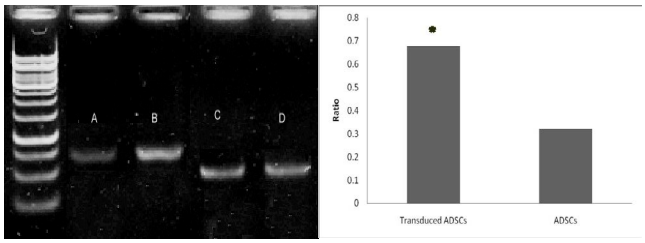

Figure 6: Overexpression of NGF mRNA (72 h after transduction) in ADSCs. (**A)** NGF mRNA in ADSCs (control), (**B)** NGF mRNA in ADSCs after transduction, (**C,D)** GAPDH in ADSCs (control) and ADSCs after transduction (1kb DNA Ladder).˙P<0.01.

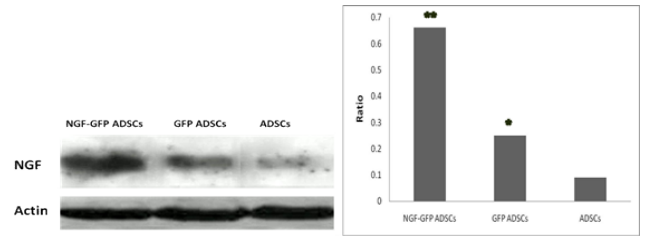

Figure 7: Overexpression of NGF (72 h after transduction) in ADSCs. ˙P<0.01, ¨p<0.001.`

But direct use of NGF has limitations. So transplantation of ADSCs overexpressing NGF could be a good method for long term delivery. In this study we isolated ADSCs and tried to increase NGF expression in them. The results showed that ADSCs can be easily isolated from human adipose tissue by digestion method. Isolated cells with this method had specific mesenchymal surface marker; CD 90 and a very small number of them were hematopoietic stem cells and had CD45 (Figure 2A and 2B). ADSCs expanded in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. These cells were able to differentiate into adipocytes and osteocytes under in vitro differentiating conditions such as other MSCs.

To overexpress NGF we used lentiviral vector: pCDH-CMV-hNGF-EF1-CopaGFP- T2A –Pur. Lentiviral vector transduction is the most efficient method for generating NGF-secreting cells. Hohesfield, et al. illustrated that lentiviral infection can successfully transduce and produce effective NGF secretion [18].

This lentiviral vector had a bright form of green fluorescent protein (GFP): copa GFP. Numerous studies have shown that copa GFP as a new version of GFP with boosted fluorescence that is more useful for enhanced visualization *in vivo* and *in vitro* [23]. In our study the results of fluorescent microscope assay confirmed it. GFP was very useful in analysis of transfection efficiency in HEK 293T cells after packaging to produce pseudoviruses and assessment of transduction efficiency in transduced ADSCs. In addition a gene for resistance to puromycin was beneficial for an ideal therapeutic application that was previously reported with this method of transduction [19,24]. It could purify transduced cells over 90%, however, transduction efficacy was about 80%. Our results of fluorescent microscopy showed that ADSCs after transduction with pseudo lentiviruses overexpress GFP stably for long time (8 weeks in this study). GFP expression indirectly indicated NGF overexpression. To confirm this overexpression on mRNA and protein level for NGF, RT-PCR and western blotting were performed. Figure 6 shows NGF mRNA naturally express in ADSCs at low levels but after transduction, NGF-GFP cells expressed NGF mRNA at a significantly higher level. As showed in Figure 7, western blotting confirmed that transduction of ADSCs with lenti vector transferring NGF-GFP increased NGF expression significantly.

Conclusion

In conclusion, the findings of this study have shown that by using lentiviral vector, ADSCs can be successfully transduced and overexpress NGF. This method of transferring gene into ADSCs is more efficient and may be an effective strategy for delivering NGF into CNS long term in neurological disorders.

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