



Research article

Establishment of a HEK293T cell line able to site-specifically integrate and stably express GDNF by rAAV-2 vector



Jinju Zhang¹, Yun Zhang¹, Xiaomei Liu, Jingjing Xiang, Chun Zhang*

CAS Key Lab of Bio-Medical Diagnostics, Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences, Suzhou 215163, PR China
University of Chinese Academy of Sciences, Beijing 100049, PR China

ARTICLE INFO

Article history:

Received 30 January 2016

Accepted 6 April 2016

Available online 27 May 2016

Keywords:

Glial cell line-derived neurotrophic factor

Multiplicity of infection

Recombinant adeno-associated virus 2

Site-specific integration

Transduction

ABSTRACT

Background: Using recombinant adeno-associated virus 2 (rAAV-2), we attempted to establish a HEK293T cell line that is able to site-specifically integrate and stably express glial cell line-derived neurotrophic factor (GDNF). **Results:** Recombinant vector with enhanced green fluorescent protein (EGFP) and GDNF (pTR-P5-EGFP-IRES-GDNF), as well as that carrying Rep genes and SV40 promoters (pSVAV2) were constructed and packed. HEK293T cells were co-infected with rAAV-2/EGFP-GDNF and rAAV-2/SVAV2 virus separately at 1×10^4 , 1×10^5 , and 1×10^6 of multiplicity of infection (MOI). The efficiency of transduction was detected using flow cytometry. Additionally, the infected HEK293T cells were separately validated by touchdown polymerase chain reaction (PCR) and Western-blot. After 72 h of transduction, the rate of EGFP positive cell was 22%, 45% and 49% at the MOIs of 1×10^4 , 1×10^5 and 1×10^6 , respectively. On the 3rd, 6th and 9th day of cell passage, there was no significant difference in the cell viability and proliferation rate between transduction and control groups. Importantly, touchdown PCR showed that there was a specific PCR amplified product band in the lane of infected cells. Furthermore, GDNF expression was detected in the infected cells after 15 and 180 d of cultivation.

Conclusions: A HEK293T cell line able to site-specifically integrate and stably express GDNF was established.

© 2016 Pontificia Universidad Católica de Valparaíso. Production and hosting by Elsevier B.V. All rights reserved.

1. Introduction

Gene therapy is being used in treatment for multiple kinds of inherited diseases [1,2]. By transferring viruses carrying genes of interest into the target cells, gene therapy can result in an improvement of the clinical status of a patient or even cure a disease [3]. Neurotrophic factors are crucial for the development, function, and survival of neurons. Glial cell line-derived neurotrophic factor (GDNF), a kind of multiple efficient neurotrophic factor separated from rat glioma cell line B49 by Lin et al. [4], can activate the MAP kinase signaling pathway in cultured sympathetic neurons and support the survival of sympathetic neurons as well as sensory neurons of the nodose and dorsal root ganglia [5]. Furthermore, GDNF is considered as the most potent survival factor for motor neurons [6]. A recent study has shown that GDNF is important for the differentiation of Schwann cells into their native phenotype in order to improve functional nerve regeneration [7]. Therefore, GDNF is an ideal candidate gene for nerve injury and neurodegenerative diseases.

Adeno-associated virus (AAV), a small single-stranded DNA virus, is one of the four *Parvoviridae* family genera and has been the vector of choice in recent clinical trials of neurological diseases [8,9]. AAV is able to integrate into a specific site on human chromosome 19 (19q13.4), which is known as AAVS1 [10]. Due to multiple advantages, including high efficiency of transduction of postmitotic tissues *in vivo*, long-term stable transgene expression and high security without inflammation or immune response, AAV vectors and recombinant AAV (rAAV) vectors have been widely applied to the establishment of transgenic cell lines and gene therapy [11,12,13].

In this study, using rAAV-2 vector, we attempted to establish a stable HEK293T cell line, which is able to site-specifically integrate and express exogenous GDNF. The established stable cell line may contribute to the further study of GDNF gene therapy.

2. Materials and methods

2.1. Recombinant vector construction

Both pTR-P5-EGFP-IRES-GDNF and pSVAV2 recombinant plasmid vectors [14] were constructed using standard molecular cloning method [15]. The GDNF target gene of 738 bp was amplified by

* Corresponding author.

E-mail address: chunzhd@163.com (C. Zhang).

¹ These two authors contributed equally.

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

polymerase chain reaction (PCR), and a gel fragment of 726 bp was extracted using a QIAquick Gel Extraction kit (OMEGA Bio-Tek, Doraville, USA) after Cla I digestion. Similarly, internal ribosome entry site (IRES) sequence with a length of 695 bp was synthesized. Then, Not I and Cla I Enzyme cutting sites were separately inserted into 5' and 3' ends of the IRES sequence. Subsequently, a gel fragment of 684 bp was extracted using a QIAquick Gel Extraction kit (OMEGA Bio-Tek, Doraville, USA) after double digestion. After being connected using DNA connection kit (TaKaRa, Tokyo, Japan), the connection product of plasmid vector pTR-P5-EGFP (6297 bp), GDNF target gene fragment and IRES sequence fragment were transferred to the competent cells Sure-2 prepared and saved by our laboratory. Positive clone was selected and cultivated. Afterwards, plasmids were extracted using a Plasmid DNA Extract kit (Qiagen, Valencia, CA, USA) and purified using a Plasmid DNA Purification kit (Qiagen, Valencia, CA). Then, recombinant intermediate plasmid pTR-P5-EGFP-IRES-GDNF was validated by PCR and sequencing (sequenced by Sangon Biotech Co., Ltd., Shanghai, China) after digestion with Not I and Cla I (Takara, Tokyo, Japan). Similarly, pSVRep carrying rep gene and SV40 promoter were digested with EcoR V and Hind III (Takara, Tokyo, Japan), and then it was inserted into pAV2 digested by BmgB I and Hind III (Takara, Tokyo, Japan) to obtain recombinant intermediate plasmid pSVAV2. After digestion, the recombinant plasmid was also validated by PCR and sequencing (sequenced by Sangon Biotech Co., Ltd., Shanghai, China).

2.2. Packing, purification and identification of recombinant virus

According to the molar ratio of 1:1 (N/P = 20), pTR-P5-EGFP-IRES-GDNF and pSVAV2 were separately co-infected with pDG into HEK293T cells using the calcium phosphate coprecipitation method [16]. Thus, rAAV-2/EGFP-GDNF and SVAV2 viruses were synthesized, respectively. Then, viruses were purified and concentrated by the method of cesium chloride gradient centrifugation [17]. In addition, titers of rAAV-2/EGFP-GDNF and rAAV-2/SVAV2 recombinant virus solutions were measured by fluorescent quantitative PCR in an ABI 7500 Real-Time PCR system (Applied Biosystems, USA), respectively, using a fluorescence quantitative PCR kit (Tiangen Biotech Co. Ltd., Beijing, China). The PCR program was followed as follows: 94°C for 20 s, 40 cycles of 95°C for 3 s and 60°C for 40 s.

2.3. Recombinant virus transduction

HEK293T cells purchased from cell bank of Typical culture preservation commission, Chinese academy of sciences, were cultured in 24-well plates filled with 90% Dulbecco's modified eagle medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, Grand Island, NY, USA). When the cells spread to 70–80% of the plate, the culture solution was removed, and 200 μ L DMEM containing 2% FBS was added to the plates. Then, rAAV-2/EGFP-GDNF and rAAV-2/SVAV2 (rAAV-2/EGFP-GDNF:rAAV-2/SVAV2 = 50:1) recombinant virus was added to the new media with the multiplicity of infection (MOI) of 0, 1×10^4 , 1×10^5 and 1×10^6 , respectively. MOI = 0 was taken as non-transduction control group. After 24 h of transduction in a humidified 5% CO₂ incubator (Thermo Scientific BB150, USA) at 37°C, EFGP expression was observed and recorded under an inverted fluorescence microscope (ZEISS, Shanghai, China). When EGFP expression was stable after 72 h of transduction, the transduction efficiency of rAAV-2/EGFP-GDNF to HEK293T cell was detected by a LSRFortessa flow cytometer, equipped with 405 nm, 488 nm, 561 nm and 640 nm lasers (Becton Dickinson, CA, USA). The cells received a passage every three days. On the 3rd, 6th and 9th day of cell passage, cell viability was tested using trypan blue stain assay [18] and calculated as follows: amount of viable cells / (amount of viable cells + amount of dead cells) \times 100%; meanwhile, cell

proliferation rate was counted for each group as follows: amount of viable cells on the 3rd, 6th or 9th day/amount of viable cells of original culture.

2.4. Screening and identification of the stable cell line

The infected HEK293T cells expressing EGFP were separated by the LSRFortessa flow cytometer and separately cultivated in 96-well plates filled with 90% DMEM with 10% FBS. The cell culture media were changed every two days. When the cells spread to 80% of the plate, they were transferred to 24-well plates to expand the culture dose and establish cell lines for identification. Then, nuclear DNA of the infected HEK293T cells was extracted using a DNeasy blood and tissue Kit (Cat. No. 69504; QIAGEN, Valencia, CA, USA). Meanwhile, the DNA of non-infected HEK293T cells was extracted as control. Touchdown PCR was conducted using a Biometra PCR machine (Biometra, Germany). The specific primer pairs used are AAV D-sequence sense primer HAIJP1 (5'-AGGAACCCCTAGTGATGGAG-3') and human chromosome 19 AAVS1 site-specific primer HAIJP2 (5'-TCAGAGGACA TCACGTGGTG-3') [14] which were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), and the program was detailed as follows: 94°C for 2 min, 5 cycles of 98°C for 10 s and 74°C for 30 s, 5 cycles of 98°C for 10 s and 72°C for 30 s, 5 cycles of 98°C for 10 s and 70°C for 30 s, 30 cycles of 98°C for 10 s and 68°C for 30 s, as well as 68°C for 7 min. The enzyme used in PCR was KOD-Plus-Neo enzyme (Toyobo Life Science Co., Ltd., Tokyo, Japan). After PCR amplification, electrophoresis on 1% agarose gel was conducted to detect whether pTR-P5-EGFP-IRES-GDNF had been integrated into AAVS1 locus in human chromosome 19. After 15 and 180 d of cultivation, the screened infected cells were collected and then tested using standard Western-blot as described previously by Ausubel et al. [19], to identify whether they had GDNF or not. The blots were probed with rabbit anti-GDNF (1:500; Santa Cruz, USA) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:10,000; Abgent, USA) antibodies.

2.5. Statistical analysis

The experimental data were expressed by mean \pm standard deviation (SD). The one-way analysis of variance (ANOVA) was used to conduct comparison among groups and the SPSS 21.0 software was applied to perform statistical tests on the experimental data. The $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Vector construction and titer determination

After GDNF was digested by Cla I, as well as IRES was digested by Not I and Cla I, they were linked to pTR-P5-EGFP vector, which produced pTR-P5-EGFP-IRES-GDNF recombinant vector (7703 bp) (Fig. 1a). Meanwhile, pSVRep was digested by EcoR V and Hind III, subsequently, it was linked to pAV2 digested by BmgB I and Hind III to obtain pSVAV2 recombinant vector (8669 bp) (Fig. 1b). Positive clones were examined by colony PCR, and DNA sequencing after digestion declared that GDNF and pSVRep were separately integrated into the pTR-P5-EGFP vector and the pAV2 vector (Fig. 1d). The pTR-P5-EGFP-IRES-GDNF and pSVAV2 were respectively co-infected with pDG into HEK293T cells, after 48 h, most of the cells showed EGFP expression (Fig. 1c). Titters of rAAV-2/EGFP-GDNF and SVAV2 recombinant virus solutions were 1×10^{12} vg/mL and 8×10^{11} vg/mL, respectively.

3.2. Efficiency of transduction

To investigate the transduction efficiency of the recombinant virus, HEK293T cells were infected at different MOIs (1×10^4 , 1×10^5

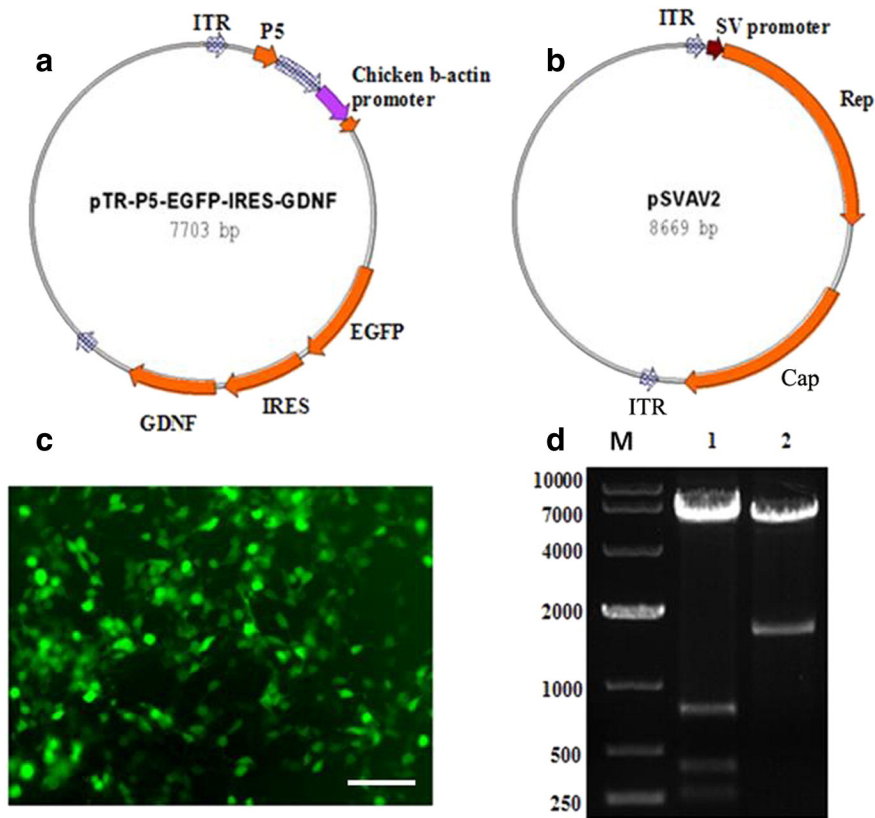


Fig. 1. Vector construction (bars: 100 μm). (a) Schematic representation of pTR-P5-EGFP-IRES-GDNF recombinant vector. (b) Schematic representation of pSVAV2 recombinant vector. (c) Expression of EGFP in HEK293T cells observed under a fluorescence microscope. (d) Electrophoresis result of colony PCR products for pTR-P5-EGFP-IRES-GDNF and pSVAV2 recombinant vector. Lane M: DNA marker; Lane 1: pTR-P5-EGFP-IRES-GDNF; Lane 2: pSVAV2.

and 1×10^6). After 72 h, the green fluorescence of EGFP-expressing HEK293T cells was measured using flow cytometer to assess transduction efficiency. The rate of EGFP positive cells at the MOIs of 1×10^5 and 1×10^6 was 45% (Fig. 2b) and 49% (Fig. 2c), respectively,

whereas just 22% (Fig. 2a) of the cells were EGFP positive at the MOI of 1×10^4 . In addition, there was no significant difference of the cell viability and proliferation rate between the transduction groups and the control group ($P < 0.05$) (Table 1).

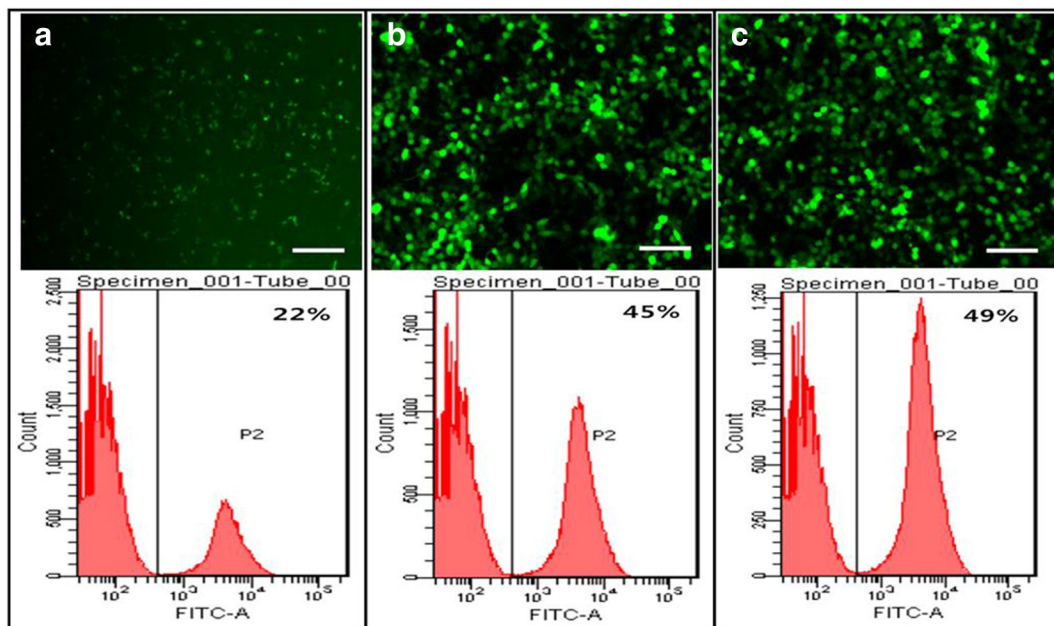


Fig. 2. Fluorescence microscopic images of EGFP expression after 24 h of transduction (bars: 100 μm) and flow cytometry results of EGFP expression after 72 h of transduction in HEK293T cells with the ratio of rAAV-2/EGFP-GDNF to rAAV-2/SVAV2 at 50:1 when MOI was 1×10^4 (a), 1×10^5 (b) and 1×10^6 (c), respectively.

Table 1

The cell viability and proliferation rate between the transduction groups and the control group (when cultivated for 3, 6 and 9 d).

Groups	Cell viability (%)			Proliferation rate		
	3 d	6 d	9 d	3 d	6 d	9 d
MOI = 0	96.07 ± 1.61	96.01 ± 1.21	96.22 ± 1.35	8.35 ± 0.14	24.46 ± 0.28	42.40 ± 0.21
MOI = 1 × 10 ⁴	96.24 ± 1.65	96.26 ± 1.22	96.10 ± 1.22	8.37 ± 0.20	24.38 ± 0.19	41.18 ± 1.09
MOI = 1 × 10 ⁵	96.38 ± 1.64	96.33 ± 1.31	96.18 ± 1.34	8.42 ± 0.23	24.35 ± 0.26	41.30 ± 1.10
MOI = 1 × 10 ⁶	96.05 ± 1.68	96.09 ± 1.42	96.01 ± 1.58	8.39 ± 0.12	24.29 ± 0.25	42.40 ± 0.85

Note: All the transduction groups were compared with the control group at the same time. Cell viability was calculated as follows: amount of viable cells / (amount of viable cells + amount of dead cells) × 100%; cell proliferation rate was counted for each group as follows: amount of viable cells on the 3rd, 6th or 9th d / amount of viable cells of original culture.

3.3. Screening and identification of the stable cell line

The infected HEK293T cells expressing EGFP were separated by flow cytometry fluorescence instrument, and cell strains for identification were established. The fluorescent expression of monoclonal cell strain was seen under fluorescence microscope (Fig. 3a). Meanwhile, the rate of fluorescent cell was 99.7% when tested with FCM (Fig. 3b), which proved that cell lines integrated with exogenous genes were successfully established. Then, cells were collected and nuclear DNA was extracted. Afterwards, touchdown PCR was conducted, and only in the lane of the infected cells, a distinct PCR amplified product band (1000 bp) was detected, but there was not in the lane of the non-infected cells (Fig. 4). According to the Western-blot test, there was a distinct electrophoretic band in the lanes of the infected cells after 15 and 180 d of cultivation, respectively (Fig. 5).

4. Discussion

GDNF is an ideal candidate gene for the therapy of nerve injury and neurodegenerative diseases [20]. In this study, to establish a stable cell line able to express GDNF, rAAV-2 vector was used for the delivery of GDNF into HEK293T cells. A recombinant vector carrying EGFP and GDNF (pTR-P5-EGFP-IRES-GDNF), as well as pSVAV2 were constructed. Afterwards, they co-infected HEK293T cells at different MOIs, and cell viability and proliferation rate were calculated. Besides, the stable infected cells were screened and validated by touchdown PCR and Western-blot.

Results showed that the rate of EGFP-positive cells at the MOIs of 1 × 10⁵ and 1 × 10⁶ was obviously higher than that at the MOI of 1 × 10⁴. Transduction of AAV-2 into target cells was mediated by heparan sulfate proteoglycan, which is a type of cell-surface receptor [21]. Thus, efficiency of transduction enhanced along with the enlargement of MOI before binding between rAAV-2 and its receptor reached equilibrium. When binding between rAAV-2 and its receptor achieved a balance, efficiency of transduction was no longer

of obvious enhancement along with the amplification of MOI. These declared that 1 × 10⁵ was the optimal MOI.

Furthermore, on the 3rd, 6th and 9th d of cell passage, there was no significant difference of the cell viability and proliferation rate between the transduction groups and control group, indicating that after infection of rAAV-2 vector, the cell viability and proliferation rate of HEK293T cells were still stable. A similar result was previously reported by Yoshioka et al. [22]. The stable cell viability and proliferation rate are likely due to the high biosafety of rAAV-2 vector [23,24]. Besides, in each group, the cell viability was stable (keeping about 96%) over time after transduction, indicating that the difference of MOI was not an influential factor of cell viability and proliferation after transduction. Taken together, it proved that the survivability of HEK293T cells was not affected by the transduction of rAAV-2 vector carrying GDNF.

In this study, the touchdown PCR assay showed that pTR-P5-EGFP-IRES-GDNF had been integrated into AAVS1 locus on human chromosome 19. AAVS1 locus is a specific site that can be integrated by AAV [10]. A previous study reported by us has demonstrated that rAAVSVAV2 carrying green fluorescent protein (GFP) gene is able to integrate at AAVS1 locus on chromosome 19 in the HeLa cell genome, and GFP is expressed stably for 35 passages [14]. Additionally, there was GDNF expression in the infected cells after 15 and 180 d of cultivation, suggesting that GDNF was expressed for a long time in HEK293T cells. A similar study has used a rAAV-GDNF vector to express GDNF long-term (6 months) nigral dopamine neurons and striatal target cells [25]. These results demonstrated that rAAV is a highly efficient vector system for long-term expression of GDNF.

Previous studies on gene therapy of nervous system diseases have used lentiviral vectors [26], AAV2 [27] or AAV5 [28] for GDNF delivery into eukaryotic cells. Compared with AAV vectors, lentiviral vectors have a large cloning capacity, whereas, they can integrate into the cell genomes, which are highly favorable for long-term expression of transgenes in the nervous system, but meanwhile it raises a set of important safety issues that may limit their clinical use [29]. Both

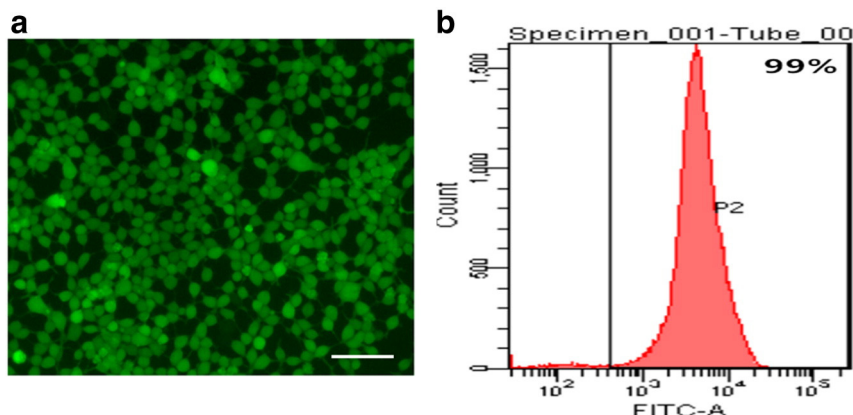


Fig. 3. Fluorescence microscopic image of EGFP expression in HEK293T cells (bars: 100 μm) (a) and the rate of EGFP positive cells (b) after cultivation for 180 d. MOI used is 1 × 10⁵.

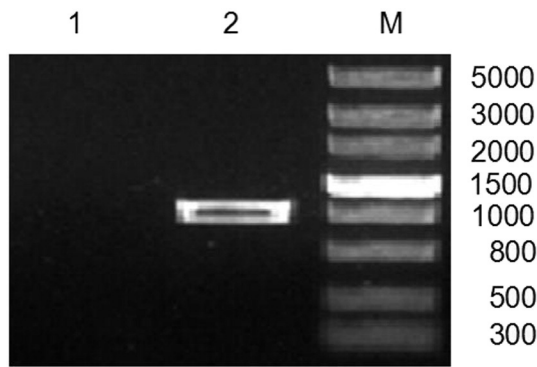


Fig. 4. Electrophoresis result of touchdown PCR products of DNA from infected HEK293T cells. Lane M: DNA marker; Lane 1: touchdown PCR products of DNA from non-infected HEK293T cells; Lane 2: touchdown PCR products of DNA from infected HEK293T cells. MOI used is 1×10^5 .

AAV2 and AAV5 have a highly effectivity in the delivery of its transgene into nerve cells and have been widely used for the gene therapy study. However, by contrast, rAAV vectors have a higher biosecurity due to the removal of 96% of viral genome [29], and they are considered as the most promising and commonly-used vectors in gene therapy research. Therefore, in this study, rAAV2-mediated GDNF transfer in HEK293T cells is promising in the application of further clinical study. We can use this HEK293T cell line to extract GDNF, and purify it for the application of further GDNF-related studies, such as gene therapy for nerve injury and neurodegenerative diseases.

Despite the aforementioned results, this study has several limitations. For example, the GDNF expression in the infected cells needs to be detected quantitatively. The Rep and Cap expression in the infected cells should have been determined. In our further study, we will solve these issues.

In conclusion, a cell line able to stably express GDNF was established using rAAV-2, and the HEK293T cell line was expected to be applied to further GDNF-related studies, such as gene therapy for nervous system diseases.

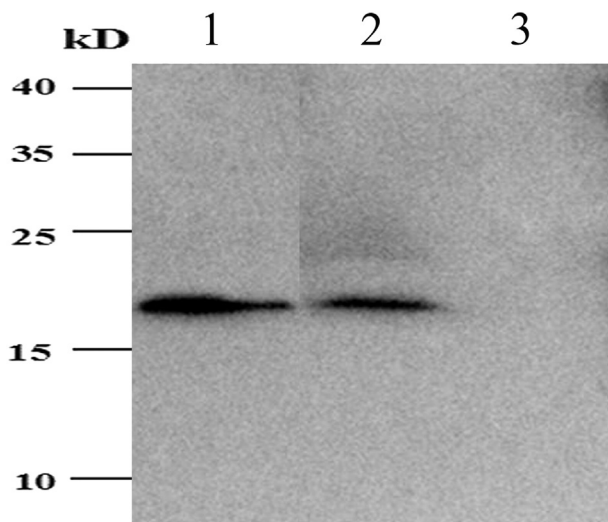


Fig. 5. GDNF expression in the infected HEK293T cells after 15 and 180 d of cultivation, respectively. Lane 1: GDNF expression in the infected HEK293T cells after 15 d of cultivation; Lane 2: GDNF expression in the infected HEK293T cells after 180 d of cultivation; Lane 3: GDNF expression in the non-infected normal HEK293T cells. MOI used is 1×10^5 .

Financial support

This work was partially supported by the National Natural Science Foundation of China [Grant No. 81371670] and Jiangsu Planned Projects for Postdoctoral Research Funds [1301069C]; and was partially supported by the Natural Science Foundation of Jiangsu Province [Grant No. BK2012629 and Grant No. BK20140381].

References

- [1] Fischer A, Cavazzana-Calvo M. Gene therapy of inherited diseases. *Lancet* 2008;371:2044–7. [http://dx.doi.org/10.1016/S0140-6736\(08\)60874-0](http://dx.doi.org/10.1016/S0140-6736(08)60874-0).
- [2] Tachibana M, Amato P, Sparman M, Woodward J, Sanchis DM, Ma H, et al. Towards germline gene therapy of inherited mitochondrial diseases. *Nature* 2013;493:627–31. <http://dx.doi.org/10.1038/nature11647>.
- [3] Ginn SL, Alexander IE, Edelstein ML, Abedi MR, Wixon J. Gene therapy clinical trials worldwide to 2012 – An update. *J Gene Med* 2013;15:65–77. <http://dx.doi.org/10.1002/jgm.2698>.
- [4] Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F. GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 1993;260:1130–2. <http://dx.doi.org/10.1126/science.8493557>.
- [5] Kottbauer PT, Lampe PA, Heuckeroth RO, Golden JP, Creedon DJ, Johnson Jr EM, et al. Neurturin, a relative of glial-cell-line-derived neurotrophic factor. *Nature* 1996;384:467–70. <http://dx.doi.org/10.1038/384467a0>.
- [6] Tarn CY. Glial cell line-derived neurotrophic factor (GDNF) protein expression and distribution in skeletal muscle of mice and rats. Kalamazoo College; 2014.
- [7] Jesuraj NJ, Marquardt LM, Kwasa JA, Sakiyama-Elbert SE. Glial cell line-derived neurotrophic factor promotes increased phenotypic marker expression in femoral sensory and motor-derived Schwann cell cultures. *Exp Neurol* 2014;257:10–8. <http://dx.doi.org/10.1016/j.expneurol.2014.04.005>.
- [8] Muzyczka N, Berns K. Parvoviridae: The viruses and their replication. *Fields Virol* 2001;2:2327–59.
- [9] Weinberg MS, Samulski RJ, McCown TJ. Adeno-associated virus (AAV) gene therapy for neurological disease. *Neuropharmacology* 2013;69:82–8. <http://dx.doi.org/10.1016/j.neuropharm.2012.03.004>.
- [10] Surosky RT, Urabe M, Godwin SG, McQuiston SA, Kurtzman GJ, Ozawa K, et al. Adeno-associated virus Rep proteins target DNA sequences to a unique locus in the human genome. *J Virol* 1997;71:7951–9.
- [11] Zentilin L, Giacca M. Adeno-associated virus vectors: Versatile tools for *in vivo* gene transfer. *Contrib Nephrol* 2008;159:63–77. <http://dx.doi.org/10.1159/000125584>.
- [12] Zsebo K, Yaroshinsky A, Rudy JJ, Wagner K, Greenberg B, Jessup M, et al. Long-term effects of AAV1/SERCA2a gene transfer in patients with severe heart failure. Analysis of recurrent cardiovascular events and mortality. *Circ Res* 2014;114:101–8. <http://dx.doi.org/10.1161/CIRCRESAHA.113.302421>.
- [13] Carlon MS, Vidović D, Dooley J, Mori da Cunha M, Maris M, Lampi Y, et al. Immunological ignorance allows long-term gene expression after perinatal recombinant adeno-associated virus-mediated gene transfer to murine airways. *Hum Gene Ther* 2014;25:517–28. <http://dx.doi.org/10.1089/hum.2013.196>.
- [14] Zhang C, Cortez N, Berns K. Characterization of a bipartite recombinant adeno-associated viral vector for site-specific integration. *Hum Gene Ther* 2007;18:787–97. <http://dx.doi.org/10.1089/hum.2007.056>.
- [15] Sambrook J. *Molecular cloning: A laboratory manual*. Joseph Sambrook, David W. Russell. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 2001.
- [16] Dowdy SC, Mariani A, Janknecht R. HER2/Neu- and TAK1-mediated up-regulation of the transforming growth factor β inhibitor Smad7 via the ETS protein ER81. *J Biol Chem* 2003;278:44377–84. <http://dx.doi.org/10.1074/jbc.M307202200>.
- [17] Lawrence JE, Steward GF. Purification of viruses by centrifugation. *Manual of Aquatic Viral. Ecology* 2010:166–81.
- [18] Strober W. Trypan blue exclusion test of cell viability. *Curr Protoc Immunol* 2001. <http://dx.doi.org/10.1002/0471142735.ima03bs21>.
- [19] Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al, editors. *Current protocols in molecular biology*. New York: Wiley; 2000.
- [20] Allen SJ, Watson JJ, Shoemark DK, Barua NU, Patel NK. GDNF, NGF and BDNF as therapeutic options for neurodegeneration. *Pharmacol Ther* 2013;138:155–75. <http://dx.doi.org/10.1016/j.pharmthera.2013.01.004>.
- [21] Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol* 1998;72:1438–45.
- [22] Yoshioka Y, Abe T, Wakusawa R, Moriya T, Mochizuki S, Saigo Y, et al. Recombinant AAV-transduced iris pigment epithelial cell transplantation may transfer vector to native RPE but suppress systemic dissemination. *Invest Ophthalmol Vis Sci* 2006;47:745–52. <http://dx.doi.org/10.1167/iovs.05-0398>.
- [23] Engelhardt JF, Duan D. Adeno-associated virus vectors. *Google Patents*; 2002.
- [24] Gafni Y, Pelled G, Zilberman Y, Turgeman G, Apparailly F, Yotvat H, et al. Gene therapy platform for bone regeneration using an exogenously regulated, AAV-2-based gene expression system. *Mol Ther* 2004;9:587–95. <http://dx.doi.org/10.1016/j.ymthe.2003.12.009>.
- [25] Kirik D, Rosenblad C, Björklund A, Mandel RJ. Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: Intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. *J Neurosci* 2000;20:4686–700.
- [26] Chen SS, Yang C, Hao F, Li C, Lu T, Zhao LR, et al. Intrastriatal GDNF gene transfer by inducible lentivirus vectors protects dopaminergic neurons in a rat model of

- parkinsonism. *Exp Neurol* 2014;261:87–96. <http://dx.doi.org/10.1016/j.expneurol.2014.06.022>.
- [27] Eberling JL, Kells AP, Pivrotto P, Beyer J, Bringas J, Federoff HJ, et al. Functional effects of AAV2-GDNF on the dopaminergic nigrostriatal pathway in parkinsonian rhesus monkeys. *Hum Gene Ther* 2009;20:511–8. <http://dx.doi.org/10.1089/hum.2008.201>.
- [28] Blits B, Kirik D, Petry H, Hermening S. Gene therapy for Parkinson's disease: AAV5-mediated delivery of glial cell line-derived neurotrophic factor (GDNF). *Gene Deliv Ther Neurol Disord* 2015;98:67–83. http://dx.doi.org/10.1007/978-1-4939-2306-9_3.
- [29] Björklund A, Kirik D, Rosenblad C, Georgievska B, Lundberg C, Mandel RJ. Towards a neuroprotective gene therapy for Parkinson's disease: Use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model. *Brain Res* 2000;886:82–98. [http://dx.doi.org/10.1016/S0006-8993\(00\)02915-2](http://dx.doi.org/10.1016/S0006-8993(00)02915-2).