Construction of a recombinant AAV vector encoding human alpha-synuclein gene with myelin basic protein Promoter

Tian Huijun[1], Wang Xiangqing[2], Lang Senyang[2]

[1]The 309th hospital of Chinese People's Liberation Army
[2]The General Hospital of People's Liberation Army

Abstract: Objective: To construct recombinant adeno-associated virus carrying human α-synuclein gene under the control of cytomegalovirus (CMV) or murine myelin basic protein (MBP) promoter and to measure the virus titer and verify the recombination. **Methods:** Human α-Synuclein and MBP cDNA were amplified by polymerase chain reaction (PCR), digested and subcloned into shuttle plasmid pSNAV2.0 to obtain recombinant plasmid pSNAV2-CMV-α-Synuclein and pSNAV2-MBP-α-Synuclein. The resulting recombinant plasmids were co-transfected into the 293T cells by calcium-phosphate precipitation method to complete rAAV2-α-Synuclein packaging. The titer of the recombinant rAAV2-α-Synuclein was determined by dot-blot assay. The recombinant rAAV-α-Synuclein was verified by PCR of the exogenous interest genes. **Results:** The recombinant AAV vector containing α-Synuclein genes under the control of CMV or MBP promoter were successfully constructed. The physical particle titer of rAAV2-CMV-α-Synuclein and rAAV2-MBP-α-Synuclein were respectively 5.6×10^{11} vg/ml and 3.1×10^{11} vg/ml. The recombinant virus was confirmed by PCR of exogenous α-Synuclein and MBP promoter gene. **Conclusion:** rAAV2-α-Synuclein was successfully constructed with a high virus titer, which may offer foundation for in vitro and in vivo experiments and provide a new method for the study of pathogenetic mechanisms and exploration of new therapeutic targets of particular relevance to human neurodegenerative disorders.
[Tian Huijun, Wang Xiangqing, Lang Senyang. **Construction of a recombinant AAV vector encoding human alpha-synuclein gene with myelin basic protein Promoter.** *Life Sci J* 2014;11(3):214-219]. (ISSN:1097-8135). http://www.lifesciencesite.com. 30

Key words: α-synuclein;myelin basic protein;recombinant adeno-associated virus; plasmid.

1. Introduction

α-Synuclein is a 140 amino acid neuronal protein, which is highly enriched within presynaptic terminals that may function in modulating synaptic transmission. α-Synuclein was initially isolated from Torpedo cholinergic synaptic vesicles and phylogenetically conserved and abundant with widespread expression throughout the central nervous system(ockenstein et al.,2001). Recent studies have shown that aggregation of α-synuclein in neurons or glial cells is a hallmark lesion in a subset of neurodegenerative disorders such as Parkinson's disease (PD), and multiple systemic atrophy (MSA) (Kahle 2008). Because alterations in α-synuclein turnover may be causative for neurodegenerative diseases, animal models were generated to identify factors infuencing α-synuclein metabolism, aggregation, and cytotoxicity. These findings have prompted the development of animal models based on overexpression of human α-synuclein.

Adeno-associated viruses (AAV) are single-stranded DNA parvoviruses that offer several advantages over other gene transfer vectors. Recombinant AAV vectors lack pathogenicity in humans, show a broad range of infectivity and can infect both dividing and quiescent cells. rAAV in vivo gene transfer studies have also demonstrated efficient longterm transduction and expression (Xiao et al., 1996). Recently, viral-mediated gene delivery has proved to be the most efficient means of gene transfer. Lentivirus vectors and AAV vectors are useful for this purpose because they transduce non-dividing neurons with high efficiency in the CNS, and in particular do not seem to induce inflammatory or immune responses (Samulski et al., 1999; Naldini and Verma 1999).

Control of gene expression using tissue- and cell-specific promoters has been tested extensively as a means of targeting transgene expression. Several different promoters were used to express α-synuclein in transgenic mouse model such as Tyrosine hydroxylase (TH) promoter, Platelet-derived growth factor-β (PDGFβ) promoter, and MBP promote (Rich Weld et al.,2002; Masliah et al.,2000; Shults et al.,2005). Recent study demonstrated that transgenic mice overexpressing hα-Synuclein under the control of the MBP promoter developed abundant hα-Synuclein-immunoreactive inclusions in oligodendrocytes, supporting a more general role of α-Synuclein accumulation in the pathogenesis of MSA (Shults et al.,2005).

In the present study, We have developed the recombinant AAV vector system capable of sustained expression of α-Synuclein which may provide fundamental understanding and a platform

for future applications of rAAV2-α-Synuclein to generate animal models by targeting α-synuclein expression to the substantia nigra (SN). This viral vector will serve to interpret the role of α-Synuclein in the neurodegenerative process by specially increasing the expression of α-Synuclein gene.

2. Materials and methods

2.1 Materials

The plasmid pSNAV2.0 and pSNAV-GFP was purchased from Beijing Vector Gene Technology Company (Beijing, China). The plasmid pSNAV-GFP is an rAAV-GFP proviral construct that was constructed by inserting GFP gene derived from pGREEN LANTERN-1 (GIBCO BRL) into the cloning site of an rAAV vector pSNAV[9]. 293T cell line were purchased from American Type Culture Collection. T4 DNA ligase reagent were purchased from Invitrogen (Carlsbad, CA, USA). Restriction endonucleases XhoI, KpnI and SalI were purchased from New England Biolabs (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO BRL (Gaithersburg, MD, USA). Human α-synuclein (GenBank accession number NM_000345) and MBP promoter gene (GenBank accession number M24410) were amplified by PCR. PCR primers synthesis and DNA sequencing was performed by Beijing AuGCT biotechnology Company (Beijing, China).

2.2 Construction of recombinant plasmid

The recombinant AAV2 packaging plasmid pSNAV(Wu et al.,2000) was constructed by deleting all viral open reading frames and introducing the sequences for human cytomegalovirus promoter/enhancer, a multiple-cloning site, a neomycin-resistant gene, a simian virus 40 promoter and a polyadenylation signal, retaining the 2 inverted terminal repeats (ITR), which contain the palindromic sequences necessary in cis for replication of the intact viral genome. The resulting α-synuclein cDNA were cloned into the KpnI and SalI restriction sites of pSNAV vector. All constructs were verified by DNA sequencing and subcloned into expression plasmid pSNAV2. Expression and purification of the recombinant proteins were done as described (Jakes et al.,1994). pSNAV-α-Synuclein was transformed into E. coli XL10 Gold (Stratagene, La Jolla, Calif.) in accordance with the manufacturer's protocol, with selection for Ampicillin (Amp)-resistant transformants, and the insert sequence was verified. Plasmid pSNAV-MBP-hα-Synuclein encoding MBP promoter for comparison were constructed by replacing the sequences of the CMV promoter of pSNAV-α-Synuclein in XhoI and KpnI site. The plasmid map of the constructed pSNAV-CMV-α-Synuclein and pSNAV-MBP-α-Synuclein were illustrated in Figure 1 and 2. Plasmid pSNAV-EGFP was constructed as the same methods.

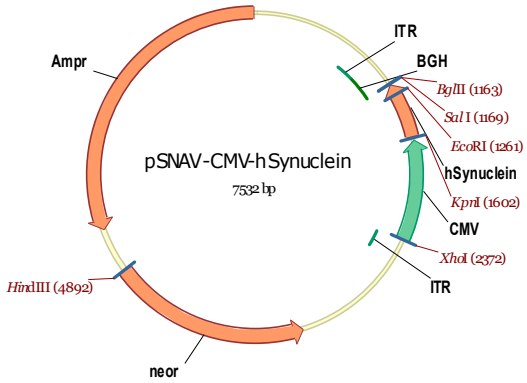

Figure 1.The plasmid map of the constructed pSNAV-CMV-α-Synuclein.

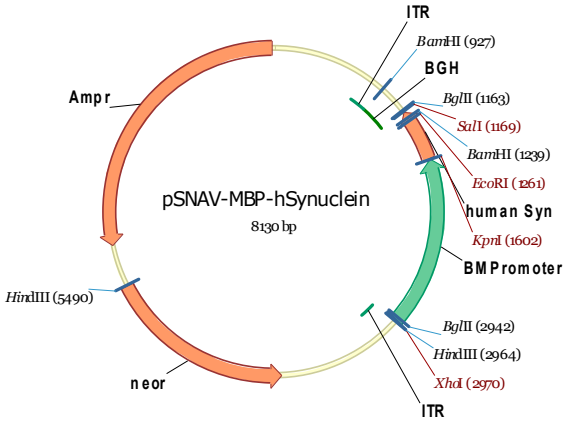

Figure 2. The plasmid map of the constructed pSNAV-MBP-α-Synuclein.

2.3 Cell culture

293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in humidified 5% CO2 atmosphere and sub-cultured when confluence was reached. The medium was changed every 5 d.

2.4 AAV vector construction and production

Large quality of AAV2 vectors were prepared by using the three-plasmid cotransfection system as previously described (Su et al., 2000), using plasmids pSNAV2 expressing hα-Synuclein under the control of the CMV or MBP promoter, a plasmid supplying adenovirus helper functions, and a third plasmid containing the AAV-2 *rep* and *cap* genes. We also prepared rAAV-EGFP (enhanced green fluorescent protein) vector for control experiments, as described previously (Yasuda et al.,2007). Briefly, the plasmid pSNAV-α-Synuclein was transfected into 293T cells

by the calcium phosphate method and cultured in RPMI 1640 medium supplemented with 10% FBS. 72 hours later, cells and media were harvested,cell lysate was produced by using three freeze and thaw cycles 3 days after the transfection, chloroform was added at 10% (v/v), and the resulting mixture was shaken vigorously to release rAAV particles. The rAAV containing supernatant was harvested following centrifugation at 12,000 r/m for 15 min. An appropriate amount of solid PEG8000 was added to the supernatant to obtain a final concentration of 10% (w/v). Following overnight incubation at 4°C, the rAAV particles were precipitated by centrifugation at 11,000g for 15 min at 4°C. The precipitated rAAV particles were resuspended in PBS solution, and this suspension was referred to as crudely purified rAAV-EGFP stock. An equal volume of chloroform was added to the suspension which was then shaken vigorously for 2 min. The organic and aqueous phases were separated by centrifugation at 12000 r/m for 5 min at 4°C. The upper layer was harvested and referred to as the purified rAAV-EGFP stock.

2.5 Determination of rAAV titres

A dot-blot assay was used to determine the total particle titer as follows (Synder et al.,1996): The rAAV stock was treated with DNasel to degrade any unencapsidated DNA, treated with proteinase K in the presence of 0.5% SDS and 10 mm EDTA to liberate the rAAV genomes, phenol extracted, ethanol precipitated and applied to a nylon membrane. Dilutions of the corresponding vector plasmid pSNAV2-GFP were used as standards to determine the rAAV virion copy number. Titers are given as vector genomes (vg)/ml. The supernatant containing rAAV was stored in aliquots at -80°C and thawed for use immediately before each experiment.

2.6 Verify of the recombinant virus rAAV2-α-Synuclein

To confirm that α-Synuclein gene was successfully inserted into the recombinant AAV, we verified the recombinant virus by PCR of the exogenous interest genes. Primers 1 were: human α-Synuclein (expected product size 326 bp): upstream, 5′-TTAGGCTTCAGGTTCGTA-3′; downstream, 5′-CAAAAGAGGGTGTTCTCT-3′; Primers 2 were: MBP promoter (expected product size 614 bp): upstream, 5′-GGAAGCTGCTGTGGGGTCTT-3′; downstream, 5′-TCTGGGGCTTGTGCACTGGT-3′. PCR reaction conditions were as follows: 94°C for 5 minutes and 30 cycles of 94°C for 30 seconds, 56°C for 40 seconds, and 72°C for 40 seconds, with extension for 5 minutes at 72°C. PCR products were separated by 1.0% agarose gel electrophoresis and the bands photographed.

3. Results

3.1 Identification of plasmid pSNAV-α-Synuclein

The hα-Synuclein and MBP promoter gene was successfully amplified and verified from plasmid pSNAV-CMV-α-Synuclein and pSNAV-MBP-α-Synuclein. Sequencing of the α-Synuclein gene yielded 435 bp and was in accordance with what had been previously published in Genbank (Figure 3). Sequencing of the MBP promoter yielded 1366 bp and was in accordance with what had been previously published in Genbank (Figure 4). The pSNAV-α-Synuclein and pSNAV-MBP-α-Synuclein were successfully constructed.

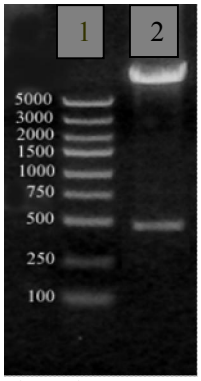

Figure 3. Identification of pSNAV-α-Synuclein. Lane 1, DNA ladder; Lane 2, target gene α-Synuclein DNA (435bp).

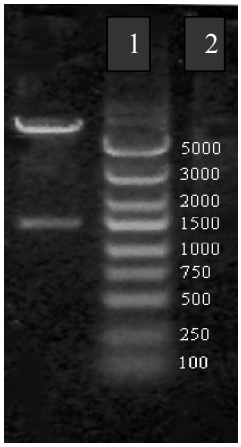

Figure 4. Identification of pSNAV-MBP-α-Synuclein. Lane 1, target gene MBP DNA (1366bp); Lane 2, DNA ladder.

3.2 Packaging efficiency of recombinant AAV

We tested the functionality of these AAV constructs by infecting 293T cells *in vitro*. Infected cells were identified by GFP fluorescence (Figure 5). 24 h after the transfection, the color of the medium obviously changed from red to orange or yellow.

Some of the 293T cells rounded up and detached from the plate, and were seen floating in the medium.

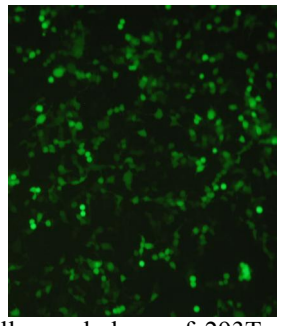

Figure 5. Cell morphology of 293T cells with co-transfection of AAV plasmids and two other help plasmids

3.3 Viral titer measurement of recombinant AAV

The viral titer of rAAV2-CMV-α-Synuclein and rAAV2-MBP-α-Synuclein were respectively calculated as 5.6×10^{11} vg/ml and 3.1×10^{11} vg/ml (Figure 6 and 7).

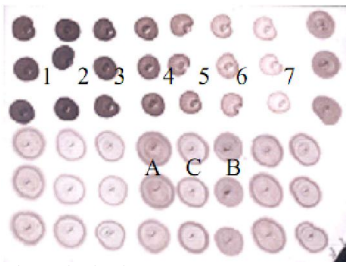

Figure 6. The viral titre rAAV2- CMV- α-Synuclein (5.6×10^{11} vg/ml).
1: 3×10e12vg/ml; 2: 2×10e12vg/ml; 3: 1×10e12vg/ml;4: 5×10e11vg/ml;
5: 2.5×10e11vg/ml; 6: 1.25×10e11vg/ml; 7: 6.25×10e10vg/ml;
A, Undiluted;B, dilution 1 : 2; C, dilution 1: 4.

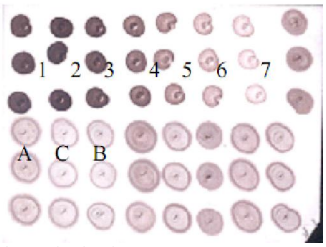

Figure 7. The viral titre rAAV2- MBP- α-Synuclein (3.1×10^{11} vg/ml).
1: 3×10e12vg/ml; 2: 2×10e12vg/ml; 3: 1×10e12vg/ml;4: 5×10e11vg/ml;
5: 2.5×10e11vg/ml; 6: 1.25×10e11vg/ml; 7: 6.25×10e10vg/ml;
A, Undiluted;B, dilution 1 : 2; C, dilution 1: 4.

3.4 Identification of rAAV2-α-Synuclein by PCR

As shown in Figure 8, The 326 bp and 614 bp target band were separated by 1% agarose gel electrophoresis in experimental group, respectively in accordance with the length of exogenous hα-Synuclein gene and MBP promoter gene fragment. There was no target band in control group. rAAV-CMV-α-Synuclein and rAAV-MBP-α-Synuclein were confirmed.

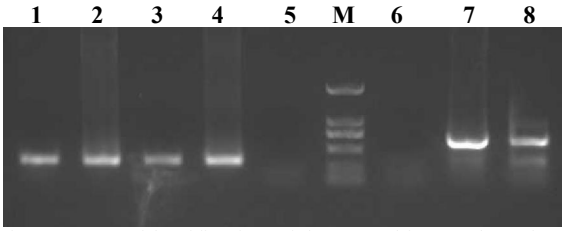

Figure 8. PCR identification of the recombinant adenovirus
M：DL2000Marker(2000、1000、750、500、250、100bp)
Lane 1：PCR product of rAAV2- CMV- α-Synuclein (primer 1)
Lane 2：PCR product of pSNAV2-CMV-α-Synuclein (primer 1)
Lane 3：PCR product of rAAV2-MBP-α-Synuclein (primer 1)
Lane 4：PCR product of pSNAV2.0-MBP-α-Synuclein (primer 1)
Lane 5：negative control (primer 1)
Lane 6：negative control (primer 2)
Lane 7：PCR product of rAAV2-MBP-α-Synuclein (primer 2)
Lane 8：PCR product of pSNAV2.0-MBP-α-Synuclein (primer 2)

4. Discussion

In our study, we developed a new plasmid vector containing human α-Synuclein cDNA, ie, pSNAV2-α-Synuclein. Because the genome of plasmid pSNAV contained inverted terminal repeats (ITR) of rAAV and cytomegalovirus (CMV) immediate early promoter followed by a multiple clone site (MCS), and the MCS included several sites of restriction enzyme, such as NotI and EcoRV et al (2005), it is also easy to develop a recombinant AAV vector for α-Synuclein gene transfer as a perfect gene transfer vector.

α-Synuclein might be involved in the pathogenesis of neurodegenerative disorders. Recent studies have begun to address this issue by generating transgenic mice that express α-Synuclein under the regulatory control of oligodendroglial promoters such as MBP promoter. The MBP promoter was originally demonstrated to restrict transgene expression specifically to myelin, forming oligodendrocytes in a transgenic mouse model(Gow et al.,1992). An oligodendrocyte-specific promoter has also been used to drive rAAV expression. An early study showed that injected a rAAV–MBP

cassette containing the GFP reporter gene into the mouse corpus callosum, resulting in transduction of oligodendrocytes with expression persisting for greater than 3 months (Chen et al.,1999). A recent study found that the MBP promoter can induce high levels of α-Synuclein expression in oligodendrocytes (Kirik et al.,2003). In the present study, on the basis of pSNAV2-CMV-α-Synuclein construction, the CMV promoter of pSNAV2 has been replaced by the MBP promoter, and constructed a new cloning vehicle, pSNAV2-MBP-α-Synuclein. These results provide impetus for further studies into the construction of AAV vector.

Recently, direct gene transfer may be carried out using either viral vectors or recombinant plasmid DNA carrying cloned genes. Recombinant adeno-associated virus (rAAV) is most commonly generated in cell culture by co-transfecting 293 or 293T cells with a rAAV vector plasmid and an AAV rep-cap plasmid followed by helper adenovirus infection (Clark 2002). rAAV vector is non-pathogenic, and be easily obtained in high yield. To date, the most commonly used vector has been based on AAV serotype 2. rAAV2 vectors has been widely used as a gene transfer agents in preclinical studies and as well has been reported in early-phase clinical trials (Büning et al.,2004;Büning et al.,2003). Our in vitro experiments demonstrated that 293T cells infected with three-plasmid cotransfection system. rAAV2-α-Synuclein were able to be generated and measured the titre of rAAV2-MBP-α-Synuclein and rAAV2-CMV-α-Synuclein. We have now generated a high titer (10^{11}vg/mL) rAAV2-α-Synuclein preparation from this vector that can be used for further degeneration studies.

The development of an rAAV vector that expresses α-Synuclein makes it feasible to investigate the effects of rAAV2-α-Synuclein gene transfer alone and incombination with other drug agents in a variety of murine models of neurodegenerative disorders. rAAV vector can transduce nondividing neurons with high efficiency in the CNS. An recent study showed that rAAV2-CMV-α-Synuclein vector system can be used to overexpress α-Synuclein in the nigrostriatal neurons in adult rats, and provides a transgenic primate model of nigrostriatal α-synucleinopathy, which closely resembles pathogenic changes in PD (Kirik et al.,2002). Overexpression of human α-synuclein using recombinant adeno-associated viral (rAAV) vectors provides a novel tool to study neurodegenerative processes seen in Parkinson's disease and other synucleinopathies. In this regard, we will examined the effectiveness of rAAV2-MBP-α-Synuclein to develop a more successful degeneration model in vivo. This model offers new opportunities for the study of pathogenetic mechanisms and exploration of new therapeutic targets of particular relevance to human PD.

Acknowledgement

This work was supported by Beijing Natural Science Foundation Proposed Program(7093135).

Correspond to:
Lang Senyang
28# Fuxing Road, Haiding district, Beijing 100853, China
Tel.:+86-10-66876328;
Fax:+86-10-66876328;
Email: langsenyang@163.com

References

1. R, Hansen LA, Mallory M. Altered expression of the synuclein family mRNA in Lewy body and Alzheimer's disease. Brain Res. 2001;914:48-56.
2. K.alpha-Synucleinopathy models and human neuropathology:similarities and differences. Acta Neuropathol. 2008;115:87-95.
3. Xiao X, Li J, Samulski RJ. Efficient long term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. J Virol. 1996;70: 8098-8108.
4. Samulski RJ, Sally M, Muzyczka N. in The development of human gene therapy, ed. Friedmann, T. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).1999; pp. 131-170.
5. Naldini L, Verma IM. in The development of human gene therapy, ed. Friedmann, T. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).1999; pp. 47-60.
6. RichWeld EK, Thiruchelvam MJ, Cory-Slechta DA. Behavioral and neurochemical eVects of wild-type and mutated human α-synuclein in transgenic mice. Exp Neurol. 2002;175:35–48.
7. Masliah E, Rockenstein E, Veinbergs I.Dopaminergic lossand inclusion body formation in α-synuclein mice: implications for neurodegenerative disorders. Science. 2000;287:1265–1269.
8. Shults CW, Rockenstein E, Crews L. Neurological and neurodegenerative alterations in a transgenic mouse model expressing human α-synuclein under oligodendrocyte promoter: implications for multiple system atrophy.J Neurosci. 2005;25:10689–10699.
9. Wu ZJ, Wu XB, Hou YD. Construction of a series of adeno-associated virus vectors and

their expression of β-galactosidase gene. Chin J Virol 2000; 16: 1–6.
10. Jakes R, Spillantini MG, Goedert M. Identification of two distinct synucleins from human brain. FEBS Lett. 1994;345:27–32.
11. Su H, Lu R, Kan YW. Adeno-associated viral vector-mediated vascular endothelial growth factor gene transfer induces neovascular formation in ischemic heart. Proc Natl Acad Sci USA. 2000;97:13801–13806.
12. Yasuda TS, Miyachi R, Kitagawa K. Neuronal specificity of alpha-synuclein Toxicity and effect of Parkin co-expression in primates. Neuroscience. 2007;144:743–753.
13. Synder RO, Xiao X, Samulski RJ. Production of recombinant adeno-associated viral vectors. In: Dracopoli N(ed). Current Protocols in Human Genetics. John Wiley: New York,1996; pp.12.1.1-20.
14. Y, Ma QJ, Dang GT.Adeno-associated virus-mediated bone morphogenetic protein-7 gene transfer induces C2C12 cell differentiation into osteoblast lineage cells.Acta Pharmacol Sin. 2005;26:963-968.
15. G, Friedrich VL Jr, Lazzarini RA.Myelin basic protein gene contains separate enhancers for oligodendrocyte and Schwann cell expression.J Cell Biol. 1992;119:605-616.
16. C, McCarty DM, Bruce AT. Oligodendrocyte-specific gene expression in mouse brain: use of a myelin-forming cell type-specific promoter in an adeno-associated virus. J Neurosci Res. 1999;55:504-513.
17. K, Annett LE, Burger C.Nigrostriatal alpha-synucleinopathy induced by viral vector-mediated overexpression of human alpha-synuclein: a new primate model of Parkinson's disease.Proc Natl Acad Sci USA. 2003;100:2884-2889.
18. Clark KR.Recent advances in recombinant adeno-associated virus vector production. Kidney Int. 2002;61:S9-15.
19. B, Braun-Falco M, Hallek M. Progress in the use of adeno-associated viral vectors for gene therapy. Cells Tissues Organs. 2004;177:139-150.
20. Büning H, Nicklin SA, Perabo L, et al. AAV-based gene transfer. Curr Opin Mol Ther. 2003;5:367-375.
21. K, Rosenblad C, Burger C.Parkinson-like neurodegeneration induced by targeted overexpression of alpha-synuclein in the nigrostriatal system.J Neurosci. 2002;22:2780-2791.

2/8/2014