Recombinant adenovirus-mediated expression of GHS-R1a in HEK 293 cells

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Abstract: Objective To construct the recombinant adenovirus vector carrying human growth hormone secretagogue receptor type 1a (GHS-R1a), for genetic transfection. **Methods** The full-length human GHS-R1a gene was obtained by PCR amplification and then cloned into the shuttle plasmid pAdTrack-CMV. The linearized plasmid pAdTrack-CMV-GHS-R1a was co-transformed into *Escherichia coli* (*E. coli*) BJ5183 cells along with an adenoviral backbone plasmid pAdEasy1. The HEK293 cells were then infected with adenoviruses. The expression of GHS-R1a was indicated by green fluorescent protein (GFP), and confirmed by Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Western blot. **Results** Enzymatic digestion of pAdGHS-R1a yielded a large fragment (approximately 30 kb) and a small fragment (4.5 kb), indicating the successful construction of recombinant adenovirus expression vector. Expression of GFP was observed by confocal laser scanning microscopy at 24 h after infection. RT-PCR and Western blot further confirmed that GHS-R1a was efficiently expressed in 293 cells. **Conclusion** Recombinant adenovirus (AdGHS-R1a) is successfully constructed, and the target gene can be expressed efficiently in 293 cells, which provide a valuable tool for further studying the function of GHS-R1a.

Keywords: adenovirus vector; homologous recombination; GHS-R1a

1 Introduction

Gene therapy is potentially a powerful approach to treat neurological diseases^[1]. Constructing the gene transfer vectors can provide efficient delivery and expression of target genes. Viruses can efficiently transfer genes that allow them to survive and replicate in mammalian hosts^[2]. Currently, the most widely used viral vectors include recombinant adenovirus (rAd), recombinant adenoassociated viruses (rAAV), herpes simplex viruses (HSV) and lentiviruses (Lv)^[3-5]. Among these vectors, adenovirus vectors are prominent since they

can infect both dividing and non-dividing cells, have extremely high viral titers, and can contain large DNA inserts. Also, the transferred DNA does not integrate into the host genome, but remains as a non-integrated episome in the nucleus, thus being adequate for transient expression of transgenes in non-dividing cells^[6]. Therefore, in the present study, adenovirus vectors were employed to transfer the target gene. Previous studies have demonstrated that ghrelin, a newly identified 28-amino acid brain-gut peptide, can inhibit apoptosis in Parkinson's disease (PD) models both *in vitro* and *in vivo*, through its receptor: growth hormone secretagogue receptor type 1a (GHS-R1a)^[7,8]. Besides, it has been reported that ghrelin receptor agonists elicit pro-cognitive effects and can enhance spatial learning and memory. These findings suggest that the ghrelin receptor may be a new tar-

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get for drug application. The present study was aimed to construct the recombinant adenovirus containing both human GHS-R1a gene and a *gfp* reporter gene encoding the green fluorescent protein (GFP), and to inject the virus into mouse substantia nigra to examine the expression of GHS-R1a.

2 Materials and methods

2.1 Materials Shuttle plasmid pAdTrack-CMV containing a *gfp* reporter gene, E1/E3-deleted replication-defective adenoviral backbone plasmid pAdEasy1, *Escherichia coli* (*E.coli*) BJ5183, JM109 and DH10B cells were all kept in our laboratory. HEK293 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology for Biological Sciences. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Hyclone. Plasmid Mini Kit was purchased from Omega Biotek Inc. Gel Extraction Kit was from QIAGEN GmbH. Restriction enzyme and T4 DNA Ligase were both from New England Biolabs. Inc.

2.2 PCR amplification PCR was used to amplify the full-length human GHS-R1a gene from pcDNA3.1-GHS-R1a. The primers were designed according to the full sequence of human GHS-R1a from GenBank, and synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd: forward 5'-AAGCTTCCACCATGTGGAACGCGACGC-3'; reverse 5'-GATATCTGTATTAATACTAAT-3'. PCR was performed at 94 °C for 5 min and then repeated 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. A final extension step was taken at 72 °C for 10 min. The size of PCR products was 1.1 kb. Bands of PCR products were stained with ethidium bromide and scanned using Tanon Image Software. Gel Extraction Kit provided a simple, rapid and effective method for purifying GHS-R1a fragments.

2.3 Plasmid PMD18-T-GHS-R1a construction and sequence analysis The full-length purified human GHS-R1a gene was cloned into a PMD18-T simple vector and transformed into JM109 cells. The transformed cells were plated on LB/Amp plates and cultured at 37 °C overnight (16-20 h). The plasmid was extracted from ampicillin-resistant clones, and examined by digestion with EcoR V and Hind III enzymes. Digestion products were run on 1.0% agarose gel. Sequence analysis

of GHS-R1a was also made.

2.4 pAdTrack-CMV-GHS-R1a construction Human GHS-R1a gene digested with EcoR V and Hind III from PMD18-T-GHS-R1a was cloned into the shuttle plasmid pAdTrack-CMV containing a *gfp* reporter gene, CMV promoters, and Pme I and Pac I sites. The ligation of purified GHS-R1a fragment to pAdTrack-CMV at the molar ratios of 1:1, 2:1 and 3:1, was achieved via incubation with T4 DNA Ligase at 16 °C overnight. The plasmid was extracted from positive clones and screened by restriction endonuclease reaction.

2.5 Construction of recombinant adenoviral plasmids by homologous recombination in *E.coli* BJ5183 cells Shuttle plasmid digested at Pme I was co-transformed into *E.coli* BJ5183 cells, together with adenoviral backbone vector. The transformed cells were plated on LB/Kan plates, and grown at 37 °C overnight (16-20 h). Plasmids extracted from small colonies were run on 0.8% agarose gel and the positive plasmids were identified by restriction endonuclease Pac I. The recombinant adenoviral plasmid was named as pAdGHS-R1a and transformed into *E.coli* DH10B for amplification.

2.6 Transfection of recombinant adenovirus into HEK293 cells and identification of GHS-R1a expression by Reverse Transcription Polymerase Chain Reaction (RT-PCR) HEK293 cells were cultured in DMEM supplemented with 10% calf serum at 37 °C in a humidified atmosphere with 5% CO₂. Plasmids pAdGHS-R1a were digested with Pac I, and transfected into HEK293 cells using Lipofectamine according to the manufacturer's instructions. At 10-14 d after transfection, the cells were collected and underwent 3 cycles of freeze/thaw/vortex, including freezing at -80 °C, thaw in 37 °C water bath, and vigorous vortex. Then, the recombinant adenovirus stably expressing GHS-R1a was collected and again used for cell transfection. After 24-h culture, GFP expression was observed. After 48-h culture, total RNA was isolated from 293 cells by using TRIzol reagent according to the manufacturer's instructions. GHS-R1a cDNA was generated using the AMV reverse transcription system (Promega Corporation, Madison, WI, USA), and amplified. Primers were as follows: GHS-R1a forward 5'-AGGGACCAGAACCA-CAAGCAAA-3', reverse 5'-TGGCAGCACTGATTTCGG-TAGAAGAGG-3'; GAPDH forward 5'-TTCACCACCAT-

GGAGAAGGC-3', reverse 5'-GGCATGGACTGTGGTCATGA-3'. PCR was performed at 94 °C for 5 min and repeated 30 cycles at 94 °C for 30 s, 68 °C for 30 s and 72 °C for 30 s. The final extension step was taken at 72 °C for 10 min. PCR products were run on gel and stained with ethidium bromide. The sizes of GHS-R1a and GAPDH RT-PCR products were 170 bp and 236 bp, respectively. The intensity of each band was quantified using Tanon Image Software and normalized to that of GAPDH.

2.7 Western blot Protein expression of GHS-R1a was analyzed by Western blot. Cells were lysed in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 150 mmol/L NaCl, 1% Nonidet P-40, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 µg/mL aprotinin. The protein concentration was determined using Bradford assay kit (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved in SDS-polyacrylamide gel and then transferred to polyvinylidene fluoride (PVDF) membrane. After being blocked in 5% non-fat milk at room temperature for 2 h, the membranes were incubated with rabbit anti-rat GHS-R1a antibody (Phoenix Pharmaceuticals, Inc., USA; 1:10 000) at 4 °C overnight, or with rabbit anti-rat β-actin (BIOS, China; 1:4 000) antibody at room temperature for 2 h. The membranes were washed and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:10 000; Santa Cruz Biotechnology, Inc., USA). The proteins were visualized with ECL chemiluminescence kit (Millipore, USA). The protein levels were quantified through scanning densitometry by Tanon Image System.

3 Results

3.1 Amplification of full-length human GHS-R1a gene by PCR The full-length sequence of GHS-R1a was amplified by PCR. PCR products were run on agarose gel. As shown in Fig. 1, the size of PCR products was 1.1 kb, consistent with the expected size of GHS-R1a.

3.2 Human GHS-R1a gene was cloned into PMD18-T simple vector The recombinant plasmid PMD18-T-GHS-R1a was digested by enzymes EcoR V and Hind III, and then electrophoresed on agarose gel. As shown in Fig. 2, the products included 2 fragments with sizes of 2.6 kb and 1.1 kb, respectively. The fragment of 2.6 kb corresponded to PMD18-T,

Fig. 1 Amplification of human GHS-R1a gene. M: DNA marker. Lane 1: GHS-R1a gene (1.1 kb).

Fig. 2 Identification of recombinant PMD18-T-GHS-R1a from ampicillin-resistant clones, by digestion with enzymes EcoR V and Hind III. M: DNA marker. Lanes 1 and 2: digestion products of PMD18-T-GHS-R1a. The fragment of 2.6 kb corresponded to PMD18-T, and the other fragment of 1.1 kb corresponded to GHS-R1a fragment.

and the other 1.1 kb fragment corresponded to GHS-R1a fragment. Sequence analysis also confirmed that the fragment inserted into PMD18-T was just the human GHS-R1a cDNA fragment obtained by PCR.

3.3 Human GHS-R1a gene was cloned into the shuttle plasmid pAdTrack-CMV The GHS-R1a fragment from PMD18-T-GHS-R1a digestion was inserted into adenovirus shuttle vector pAdTrack-CMV. The recombinant plasmid was extracted from kanamycin-resistant clones and digested by enzymes Hind III and EcoR V. As shown in Fig. 3, the recombinant plasmid pAdTrack-CMV-GHS-R1a was digested into 2 fragments: GHS-R1a (1.1 kb) and adenovirus shuttle vector (approximately 9.2 kb), confirming the successful construc-

Fig. 3 Identification of recombinant plasmid pAdTrack-CMV-GHS-R1a by digestion with enzymes *EcoR V* and *Hind III*. M: DNA marker. Lanes 1-3: digestion products of pAdTrack-CMV-GHS-R1a. Candidate clones produced a small fragment of 1.1 kb, indicating that human GHS-R1a gene was cloned into the shuttle plasmid.

tion of pAdTrack-CMV-GHS-R1a.

3.4 Identification of recombinant adenovirus plasmid pAdGHS-R1a Recombinant adenovirus vector containing GHS-R1a fragment was confirmed by *Pac I* endonuclease analysis. As shown in Fig. 4, pAdGHS-R1a could be digested by *Pac I* into 2 fragments: a large fragment and a small fragment (approximately 4.5 kb), indicating the successful construction of recombinant adenovirus expression vector pAdGHS-R1a.

3.5 Generation of adenoviral recombinants in HEK293 cells The adenoviral recombinant Ad-GHS-R1a was transfected in

Fig. 4 The plasmid pAdTrack-CMV-GHS-R1a was linearized by restriction endonuclease *Pme I*, and subsequently co-transformed into *E. coli* BJ5183 cells with an adenoviral backbone plasmid pAdEasy-1. DNA was extracted from clones and restrictively digested by *Pac I*. M: DNA marker. Lanes 1 and 2: digestion products of pAd-GHS-R1a, including a large fragment (approximately 30.0 kb) and a small fragment (approximately 4.5 kb).

293 cells and expression of GHS-R1a was indicated by GFP. As observed under confocal microscopy, the number of cells expressing GFP increased time-dependently, and most cells exhibited specific morphological alteration. The dramatic morphological alteration was the cytopathic effect (CPE), including accumulation of membranous vesicles, rounding up of the infected cells, *etc* (Fig. 5).

Fig. 5 GFP expression was observed in HEK293 cells at 24 h after infection with Ad-GHS-R1a, as observed under confocal microscopy. A: Morphological observation of 293 cells. B: GFP expression in 293 cells. Scale bar: 100 μ m.

3.6 RT-PCR analysis of GHS-R1a mRNA expression To further investigate GHS-R1a expression in Ad-GHS-R1a-infected HEK293 cells, the mRNA level of GHS-R1a was examined. Results demonstrated that the mRNA level of GHS-R1a in infected HEK293 cells was significantly higher than that in control (Fig. 6).

3.7 Western blot analysis of GHS-R1a protein level The protein level of GHS-R1a in HEK293 cells was investigated by Western blot. The result showed that the protein level of GHS-R1a in infected HEK293 cells was higher than that of control (Fig. 7), indicating that GHS-R1a could be expressed efficiently in HEK293 cells infected with Ad-GHS-R1a.

Fig. 6 GHS-R1a mRNA expression in HEK293 cells. At 48 h after infection with Ad-GHS-R1a, total RNA was isolated from infected HEK293 cells by using TRIzol reagent according to the manufacturer's instructions. GHS-R1a mRNA level was significantly higher in Ad-GHS-R1a group than in control group. A: RT-PCR analysis of GHS-R1a expression in infected HEK293 cells. GAPDH served as an internal control. B: Quantification of GHS-R1a mRNA level. Data were presented as mean $\pm$ SEM ($n = 3$). * $P < 0.01$ vs Control.

Fig. 7 GHS-R1a protein expression in HEK293 cells. At 48 h after infection with Ad-GHS-R1a, Western blot was conducted. The protein level of GHS-R1a was significantly higher in Ad-GHS-R1a group than in control group. A: Western blot analysis of GHS-R1a expression in infected HEK293 cells. β -Actin served as an internal control. B: Quantification of GHS-R1a protein level relative to β -actin. Data were presented as mean $\pm$ SEM ($n = 3$). * $P < 0.01$ vs Control.

4 Discussion

Ghrelin is a newly discovered gut-brain peptide composed of 28 amino acids, and has been identified as the endogenous ligand for GHS-R^[9]. GHS-R1 is a G-protein-coupled receptor with 7 transmembrane domains^[10]. There are 2 variants of GHS-R1: GHS-R1a and GHS-R1b, resulting from the alternative processing of pre-mRNA of the same gene^[11]. GHS-R1a acts as the receptor for ghrelin, and mediates a wide spectrum of ghrelin actions^[12,13]. Therefore, exploring the mechanisms underlying the regulation of GHS-R1a would contribute to a better understanding of the physiological role of ghrelin^[14]. Besides being present in the peripheral tissue, GHS-R mRNA has also been detected in the arcuate, ventromedial nuclei, hippocampus, the substantia nigra, the ventral tegmental area, *etc*^[15]. Recent studies have reported that ghrelin could inhibit apoptosis in several cell lines via GHS-R1a^[16-18]. Our previous study has also revealed the neuroprotective effects of ghrelin on dopaminergic neurons against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity *in vivo* and on MES23.5 cells against 1-methyl-4-phenylpyridinium ion (MPP⁺)-induced apoptosis *in vitro* via its fully functional receptor, GHS-R1a^[7,8]. Zeenat Atcha *et al.* have reported that ghrelin receptor agonists can elicit pro-cognitive effects in recognition and spatial learning and memory tests^[19]. Recently, it has been demonstrated that GHS-R1a is constitutively active to 50% of its maximal capacity *in vitro*. Also, it has been mentioned that GHS-R1a is constitutively active and could induce signaling even in the absence of its ligand^[20], although the underlying mechanisms remain largely unclear. This indicates the important role of GHS-R1a in ghrelin-mediated neuroprotective effects and GHS-R1a might be a new drug target for therapeutic approaches. Based on the above findings, we speculate that if GHS-R1a is highly expressed in the surviving dopaminergic neurons of the substantia nigra after MPTP treatment, it may act as a survival factor for dopaminergic neurons. In order to examine the protective effects of GHS-R1a, it is necessary to construct a vector carrying GHS-R1a gene.

There are 2 kinds of vectors for gene therapy: nonviral vector and viral vector. The viral vector has become an in-

creasingly popular approach for gene therapy. Adenoviral vectors can contain large DNA inserts and mediate transient but high levels of protein expression. Besides, adenoviral vectors can transfect both dividing and non-dividing cells, and high titers of adenovirus can be easily produced, making it relatively easier for vector construction. Moreover, the virus can not be integrated into the chromosome of host cells and will not induce cancerogenesis^[5,21].

According to the advantages of adenovirus vector mentioned above, in the present study, we constructed the recombinant adenovirus vector carrying GHS-R1a, using the E1/E3-deleted Ad vector. E3 region is not necessary for viral replication *in vitro*, and E3-deleted virus may minimize the risks of viral latency or establishment of persistence in the vaccinated host^[22,23]. E1 sequences encode proteins involved in DNA replication, and E1-defective viruses can be complemented in 293 cells, a cell line containing E1 sequences. Thus, the E1-deleted viruses can replicate and be amplified in this cell line^[24], which enables us to get enough adenoviruses for further study.

In the present study, we confirm that GHS-R1a can be expressed efficiently in 293 cells. The successful construction of recombinant adenovirus containing GHS-R1a provides a valuable tool for further exploring the functions of GHS-R1a.

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重组腺病毒携带的人 GHS-R1a 基因在人胚肾 293 细胞中的表达

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摘要: **目的** 构建携带 GHS-R1a 基因的重组腺病毒, 为转基因做准备。**方法** 采用 PCR 法扩增 GHS-R1a 基因片段, 随后亚克隆至腺病毒穿梭质粒 pAdTrack-CMV, 形成重组质粒 pAdTrack-CMV-GHS-R1a。线性化的 pAdTrack-CMV-GHS-R1a 和腺病毒骨架质粒 pAdEasy1 在感受态大肠杆菌 BJ5183 内同源重组。将线性化重组质粒 pAdGHS-R1a 转染进 HEK293 细胞, 并用激光共聚焦显微镜、RT-PCR 和 Western blot 技术检测绿色荧光蛋白及 GHS-R1a 的表达。**结果** 重组质粒 pAdGHS-R1a 经 Pac I 酶切后得到一个大于 30 kb 的大片段和一个 4.5 kb 的小片段, 证明重组腺病毒质粒 pAdGHS-R1a 构建成功。Ad-GHS-R1a 转染 293 细胞后 24 h 能观察到绿色荧光蛋白的表达。RT-PCR 和 Western blot 均证实 GHS-R1a 基因能在 293 细胞中有效表达。**结论** 携带 GHS-R1a 基因的重组腺病毒构建成功, 为进一步研究 GHS-R1a 的功能提供了基础。

关键词: 腺病毒载体; 同源重组; 生长激素促分泌素受体 1a 亚型