Construction of lentiviral vector carrying Rab9 gene and its expression in mouse brain

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Abstract Rab proteins and their effectors facilitate vesicular transport by tethering donor vesicles to their respective target membranes. Rab9 mediates late endosome to trans-Golgi-network trafficking. To explore the possibility of Rab9-related gene therapy for neurodegenerative diseases, we packed lentivirus encoding Rab9 cDNA. The expressing plasmid pCDH1-MCF1-Rab9-EF1-copGFP was constructed by using molecular biological techniques. The lentivirus encoding Rab9 cDNA was packed by LifeTectamine-2000 mediated co-transfection of the plasmid pPACKH1-GAG, pPACKH1-REV and pVSV-G into 293T cells. DNA sequencing proved the successful construction of pCDH1-MCF1-Rab9-EF1-copGFP. After 72 h, the expression of GFP could be detected in BV-2 cells. Western blotting revealed that the Rab9 gene expression in BALB/c mice brain was up-regulated significantly 4 weeks after injection with lentivirus encoding Rab9 cDNA, which evidenced a satisfactory increasing effect of this virus. Administration of lenti-Rab9 to postnatal day 3 Niemann-Pick disease type C (NPC) mice reduced motor defects and prevented the weight loss associated with female NPC mice, as well as modulated the death rate of Purkinje neurons. It is concluded that the packaging of lentivirus encoding Rab9 cDNA was successful. lentivirus encoding Rab9 can increase the expression of Rab9 cDNA gene effectively, which might offer a novel means for the treatment of neurodegenerative diseases.

Keywords Rab9; lentivirus; gene therapy; gene transfer

1 Introduction

Niemann-Pick disease type C (NPC) is an autosomal recessive disorder of intracellular cholesterol trafficking with tragic consequences, which is of interest in its own right and is a “juvenile” model for Alzheimer disease [1,2]. The majority of NPC cases arise from mutations in the NPC1 gene [3]. A characteristic of NPC is the massive accumulation of cholesterol and glycosphingolipids within late endosomes and lysosomes [4–6]. In normal cells, endocytosed low density lipoproteins are delivered to endosomes, where they are hydrolyzed and free cholesterol is released. This cholesterol is transported rapidly out of endosomes to the plasma membrane and endoplasmic reticulum [7,8].

In NPC cells, the cholesterol does not exit the endocytic pathway and it accumulates within lysosomes [9]. So far, no effective therapy has been available. Studies have demonstrated that NPC1 human skin fibroblasts over-expressing endosomal Rab9 protein shows a correction in the storage disease phenotype [10,11]. To learn more about the role of Rab9 protein in modulating lipid traffic and accumulation in NPC cells, and to develop the therapeutic potential of this system, an alternative method for conventional DNA transfection is required to introduce the Rab9 gene into living cells. The lentiviral vector mediated transgenic technique that developed rapidly in recent years has the advantages of easy-operation, high-rate integration, no selection in cell cycle of host cells compared with traditional microinjection [12–14]. In this study, lentivirus encoding Rab9 gene was generated and introduced into BALB/c mice brain to evaluate the efficacy of gene transfer and its function.

2 Materials and methods

2.1 Materials

The plasmid p-Rab9-EGFP was purchased from Addgene Inc (USA). The plasmid pCDH1-MCF1-EF1-copGFP and the 293T cell line were provided by KangChen Bio-tech Inc (China). T4 DNA ligase and the restriction enzymes of
BamHI and NheI were products of New England Biolab (USA). DNA markers were bought from Beijing Tiangen Biochemical Technology Co. (China). The plasmid extract kit was from Qiagen (Germany). The primers were synthesized by Shanghai Bioengineering Co. (China). Fetal bovine serum (FBS) and high glucose Dulbecco’s modified Eagle’s medium (DMEM) powder were procured from Hyclone Co., USA and Gibco Co., USA, respectively. Lifetactamine-2000 was the product of Invitrogen Inc (USA). The pPACKH1 Packaging Plasmid Mix consisting of an optimized mixture of three plasmids — pPACKH1-GAG, pPACKH1-REV and pVSV-G was from System Biosciences (SBI), USA. BALB/c mouse strain was obtained from the Jackson Laboratory (Bar Harbor, ME). Protease inhibitor cocktail was purchased from Sigma Co, USA. Rabbit-anti-mouse Rab9 and β-actin antibody were obtained from Santa Cruz Biotechnologies, USA, and Cell Signaling Co, USA, respectively.

2.2 Methods

2.2.1 Preparation of plasmid and Rab9 gene fragment

The Rab9 cDNA was obtained from plasmid p-Rab9-EGFP by polymerase chain reaction (PCR) amplification. The primers of Rab9 for complete CDS (coding sequence) were: 5'-TAGCTAGCGCCACCATGGCAGGAAAATCATCACTTTTA-3' (sense) (cleavage site of NheI was added to 5') and 5'-TACGATCTCACAAGCAAGAT-GAGCTAGG-3' (anti-sense) (cleavage site of BamHI was added to 5'). The PCR product (678 bp) was analyzed by electrophoresis on a 1% agarose gel, and then extracted and purified according to the protocols of the gel extracted mini-kit.

2.2.2 Ligation of Rab9 fragment with shuttle vector

The Rab9 fragment was ligated with pMD 19-T vector at the NheI and BamHI site. The product was transformed into the competent cells DH5α. The plasmid prepared from the positive clone was digested by restriction enzymes NheI and BamHI and analyzed by electrophoresis on 1% agarose gel containing ethidium bromide (0.5 mmol/L). The extracted and purified Rab9 fragment and the linearized shuttle vector pCDH1-MCF1-EF1-copGFP (digested with NheI and BamHI) were ligated in a mole ratio of 1.5:1 by T4 DNA ligase at 16℃ for 16 h. The construction plasmid was amplified, analyzed and sequenced.

2.2.3 Generation of lentivirus

The packaging procedure was performed according to SBI’s User Manual. 293T cells were cultured in 10% high glucose DMEM at 37℃ in an incubator with 5% CO2. The growth status and morphological characteristics were observed under an inverted phase-contrast microscope. Four plasmids pCDH1-MCF1-Rab9-EF1-copGFP, pPACKH1-GAG, pPACKH1-REV and pVSV-G were co-transfected into 293T cells with approximately 60% confluence by using the Lipofectamine 2000™ method. The green fluorescent protein (GFP) expression in the cells was observed under an inverted fluorescent microscope so as to confirm the success of co-transfection. After a 72 hour transfection, the medium was harvested and concentrated by centrifugation at 3000 r/min for 5 min at 15℃, and then filtered with a 0.45 μm filter membrane. The filtrate was concentrated by centrifugation at 25 000 r/min for 2 hours. The supernatant was discarded. The precipitate was diluted with phosphate buffered saline (PBS) and stored at –80℃. The lentivirus was named Lenti-Rab9. The final virus titer was determined by dot blot assay.

2.2.4 Expression of recombinant lentivirus in BV-2 cells

Microglioma cells/BV-2 (BV-2 cells) were cultured in 24-well plastic plates at an initial density of 1.5×10⁶ cells/well. Sixteen hours after plating, the old medium was removed, and the cells were washed with high glucose DMEM twice. The BV-2 cells were transfected by Lentivirus-Rab9 at different multiplicities of infection (MOI) (MOI = 1×10², 1×10³, 1×10⁴, 1×10⁵) in a pre-mixed incomplete culture medium of 200 μL per well respectively, and incubated for 2 hours at 37℃. Then the medium was removed and replaced with complete culture medium. In the control group, the Lentivirus-Rab9 was replaced by the same volume of culture medium. The expression of GFP in the two groups was observed under an inverted fluorescent microscope 3 days later and the transfection efficiency was calculated.

2.2.5 Cerebella injection of recombinant lentivirus in BALB/c mice

Twelve 4-week-old BALB/c mice were randomly divided into an experimental (virus) group and a control (saline) group (n = 6 per group, male:female = 1:1). Four microliters (2.0×10⁵ TU/mL) of lentivirus suspended in PBS was injected into the cerebella bilaterally in the experimental group. In the control group, 4 μL saline was administered. Mice were sacrificed and the brains were dissected for biochemical analysis 4 weeks after injection. The GFP expression in the brain was observed under a fluorescent microscope.

2.2.6 Western blot analysis

Western blot was performed as reported previously [15]. Briefly, frozen sagittal cerebella halves were homogenized in lysis buffer. Protein concentration was determined by Bradford assay. Aliquots of total protein (100 μg) were