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Gene transfer into primary cultures of fetal neural stem cells by a recombinant adenovirus carrying the gene for green fluorescent protein*

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Abstract: Objective: To evaluate the transduction efficiency of a recombinant adenovirus carrying the gene for green fluorescent protein (Ad-GFP) into the primary cultures of fetal neural stem cells (NSCs) by the expression of GFP. Methods: The Ad-GFP was constructed by homologous recombination in bacteria with the AdEasy system; NSCs were isolated from rat fetal hippocampus and cultured as neurosphere suspensions. After infection with the recombinant Ad-GFP, NSCs were examined with a fluorescent microscopy and a flow cytometry for their expression of GFP. Results: After the viral infection, flow cytometry analysis revealed that the percentage of GFP-positive cells was as high as 97.05%. The infected NSCs sustained the GFP expression for above 4 weeks. After differentiated into astrocytes or neurons, they continued to express GFP efficiently. Conclusion: We have successfully constructed a viral vector Ad-GFP that can efficiently infect the primary NSCs. The reporter gene was showed fully and sustained expression in the infected cells as well as their differentiated progenies.

Key words: Recombinant adenovirus vector, Viral infection, Fetal neural stem cells, Green fluorescent protein
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INTRODUCTION

Neural stem cells (NSCs) have many merits that enable them to be used not only as a source of transplantation for cell-based therapy, but also as targeting cells in gene therapy of the nervous system (Gage, 2000; Rubio *et al*., 1999). They migrate well and are able to reach the distant diseased tissues; they are compatible with the host tissue and can sustain the expression of an exogenous gene; they are stem cells that can be differentiated into certain mature cell types for regeneration of diseased tissues; they do not cause immune reactions after being transplanted via auto-grafting.

Adenovirus is a type of non-enveloped, double-strained DNA virus. It is easy to prepare the virus in higher concentration with good infection efficiency. Currently, adenovirus has been widely used as gene delivery tool for gene therapy (Seki *et al*., 2002). NSCs have been infected by the virus carrying an exogenous gene and transplanted into central nervous system or/and diseased tissues. They stably expressed the exogenous genes from the virus in vivo. Therefore, NSCs have been shown to be suitable for infection by recombinant adenoviruses (Arnhold *et al*., 2003; Iwai *et al*., 2001; Yang *et al*., 2005).

In the present study, we constructed the recom-

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binant adenovirus carrying the gene for green fluorescent protein (Ad-GFP) and used it to infect cultures of NSCs primarily isolated from rat embryos.

MATERIALS AND METHODS

The plasmid pAdTrack carrying the gene for *GFP*, and bacterial strain *Escherichia coli* BJ5183 transformed with the plasmid pAdEasy-1 were gifted from Dr. Bert Vogelstein at the Tumor Research Center of Johns Hopkins Hospital, USA. 293 cells were maintained by our laboratory. The restriction endonucleases were purchased from New England Biolabs Co. (USA). B27, fibroblast growth factor-basic (bFGF), epidermal growth factor (EGF) and lipofectamine 2000 were all from Invitrogen (USA). Plasmid Kits were purchased from Huashun Biology Engineering (Shanghai, China). DMEM/F12 (Dulbecco's Modified Eagle Medium/F12 nutrient medium) was from Hyclone (USA), and the rabbit anti-nestin antibody and Cy3-conjugated goat anti-rabbit IgG were from Sigma (USA). The experimental animals, postnatal day 14 (P14) Sprague Dauley rats, were provided by Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

The Ad-GFP was constructed by the homologous recombination in bacteria with the AdEasy system that comprises two plasmids, the shuttle plasmid pAdTrack carrying the GFP gene and the backbone plasmid pAdEasy-1 containing most of the viral genome, the bacterial strain *E. coli* BJ5183, and the packaging cell line 293 cells. Briefly, the shuttle plasmid with GFP was linearized by Pme I digestion, and subsequently electroporated into *E. coli* BJ5183 that has been previously transformed with pAdEasy-1. After homologous recombination, recombinants were selected with kanamycin and screened by restriction enzyme analysis. Recombinant adenoviral DNA was then extracted from the correctly recombined clones, digested with Pac I to expose its ITR (inverted terminal repeats) and transfected into packaging 293 cells with Lipofectmine 2000 reagent to produce viral particles.

The resulted recombinant adenovirus was tested for the expression of GFP in 293 cells under fluorescent microscopy. After propagation in 293 cells, the virus particles were collected from the culture supernatants, and purified by chlorinated cesium (CsCl) gradient ultracentrifugation. The viral titers were determined by plaque assays, and viruses were stored at –80 °C.

NSCs were prepared as described by Fu *et al*. (2007). Briefly, female rats at gestational day 14 were painlessly euthanized, and embryos were removed. The hippocampal tissues were transferred to neurosphere culture medium (DMEM/F12 supplemented with 2% B27, 20 ng/ml bFGF, 20 ng/ml EGF), washed with 0.01 mol/L phosphate buffered saline (PBS) at pH 7.4 once, and completely dissociated by pipetting. Cell suspension containing 8×10^6 cells was added into 50 ml culture bottle and incubated in a tissue culture incubator at 37 °C under 5% CO_2. At Day 3, primary spheres were collected, spun down at 800 r/min for 5 min, then resuspended in neurosphere culture medium, and completely dissociated by pipetting. The resulting cell suspension of 8×10^6 cells was cultured for three more days, exactly as for the primary spheres. On Day 6, secondary spheres were collected for identification and infection.

To identify the cell types, the secondary spheres were resuspended in neurosphere culture medium and plated into the 24-well culture plates coated with poly-L-lysine. After one day in culture, the expression of nestine protein was detected by immuno-fluorescence.

For the infection, the multiplicity of infection (MOI) at 0, 10, 100 and 200 was used to infect the secondary NSCs. Starting from one day after the viral infection, the expression of GFP was examined three times a week for a total of 4 weeks under fluorescence microscopy. Cells from 4 corners and the central foci in each culture well were viewed, and numbers of cells showing GFP signals were recorded. The efficiency of infection was expressed as the percentage of cell numbers expressing bright green fluorescence in the total cell numbers.

In one infection group with MOI of 100, cells were collected at the 3rd day by centrifugation at 800 r/min for 5 min, washed by PBS, and completely dissociated by pipetting. The resulting cell suspension (at a density of 1×10^6 cells/ml) was detected for the expression of GFP by flow cytometer analysis and the percentage of GFP-positive cells was graphed by the flow cytometer program.

The MTT assay was used to measure the growth

rate of the secondary NSCs infected at MOI of 100 or the control cells non-infected. Measurements were taken everyday for 7 d, then the growth curve was plotted.

After infection at MOI of 100, the secondary NSCs were plated into the poly-L-lysine-coated 24-well plate, and cultured in the differentiating medium (DMEM/F12 and 10% fetal bovine serum (FBS) without any added growth factors). After the cells attached to the well, the expression of GFP was observed under fluorescent microscopy 3 and 7 d after differentiation.

RESULTS

The adenoviral plasmid has a size of 34 kb, and after homologous recombination between the pAd-Track plasmid and the adenoviral plasmid in bacteria, the GFP was inserted into the recombinant adenovirus. The recombinant adenoviral DNA was extracted and digested by Pac I, and one band of bigger size about 30 kb and another band about 4.5 kb were obtained, indicating that the recombination was successful (Fig.1). The band of higher molecular weight (MW) appeared close to the 21 kb marker band, because the standard agarose gel was not sufficient to separate and accurately estimate DNAs of high MW. Later, this recombinant viral DNA was confirmed for its correct functionality since it performed well in the virus production, and subsequently reporter gene expression from the viruses was obtained.

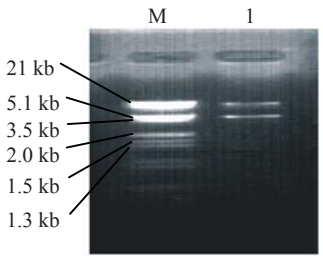


Fig.1 Image of agarose gel stained by ethdium bromide showing Pac I-digested DNA fragments from the recombinant viral vector. There are two bands, the one with higher molecular weight migrated near the 21 kb band of the standard marker (M), and the other one was about 4.5 kb (lane 1)

One day after transfection with the linearized DNA, the packaging 293 cells began to express green fluorescence. The intensity of fluorescence gradually increased over time in each GFP-positive cell, and the percentage of cells expressing the fluorescence also increased gradually. On the 7th day, typical grape cluster aggregation of infected cells was observed due to the known cytopathic effect (CPE) (Fig.2).

The cells from the hippocampal tissues of rat embryos were cultured in neurosphere culture medium free of serum. After 7 d, nest-like clusters of neural stem cells were obtained in suspension and the cells grew vigorously (Fig.3a). There were tens to hundreds of cells in one cell cluster. The cell cluster was identified to be of neuronal origin by the positive staining with nestin immunofluorescence (Fig.3b). After 12 passages in culture, the cell clusters retained the main characteristics of NSCs and their growth rates unchanged.

With MOI of 100, after 1 d, about 50% of infected cells expressed green fluorescence under fluorescent microscopy, but the fluorescent intensities were weak. After 3 d, over 90% of NSCs expressed bright green fluorescence (Fig.4), indicating a remarkable increase in the number of infected cells. The fluorescent intensities decreased gradually after 20 d, but the faint green fluorescence sustained after 4 weeks. In the control cells without viral infection (MOI=0), there were no green fluorescent signals. With the MOI of 200, the infected cells displayed bright green fluorescence, but their morphologies changed dramatically. They appeared as a chunk of floc, which differed greatly from the natural neurospheres, indicating the death of a large number of NSCs. Flow cytometer detected that as high as 97.05% of the cells infected at MOI of 100 were GFP-positive (Fig.5). Under this condition, although the cells were less vigorously growing compared with the controls without infection (Fig.6 in p.303), they are still healthy and viable.

The secondary NSCs infected by recombinant adenovirus were induced to differentiate in 10% FBS without growth factors. After 2~4 h, the cell clusters began to attach to the wells of the culture plate and differentiated toward the morphology of radial cells. At the 3rd day, the cell clusters and the radial cells both expressed green fluorescence (Fig.7a). After 7 d, the majority of cells differentiated into neurons with one or two tubers, or astrocytes with a few tubers. All the differentiated cells had bright green fluorescence (Fig.7b).

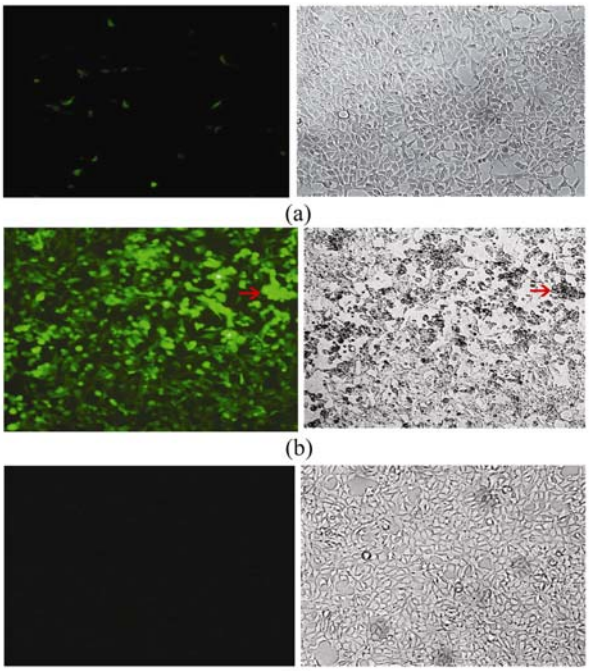
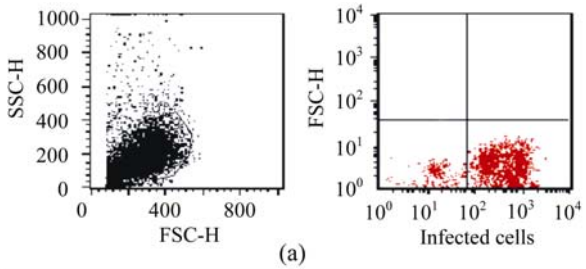


Fig.2 Expression of GFP in 293 cells after transfected by the recombinant viral DNA pAd-GFP under fluorescent microscope at 20× magnification. (a) Images of cells at Day 1 post-infection under the fluorescent channel (left) or bright field (right). The number of cells expressing the GFP was small and the fluorescent intensity was weak; (b) Images of cells at Day 7 post-infection under the fluorescence channel (left) or the bright field (right). The intensity of fluorescence and the percentage of cells expressing the fluorescence gradually increased, and typical CPE (arrow) could been observed; (c) Images of control cells without viral infection under the fluorescence channel (left) or the bright field (right)



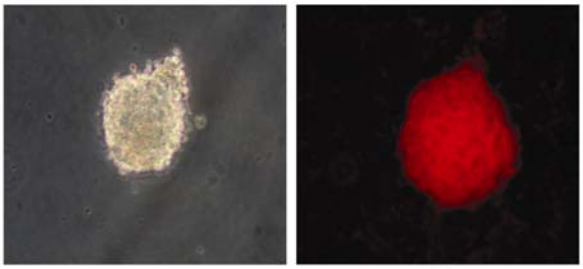
(a)

Quadrant	Number of cells (%)	Quadrant	Number of cells (%)
UL	0.00	LL	2.95
UR	0.00	LR	97.05

(b)

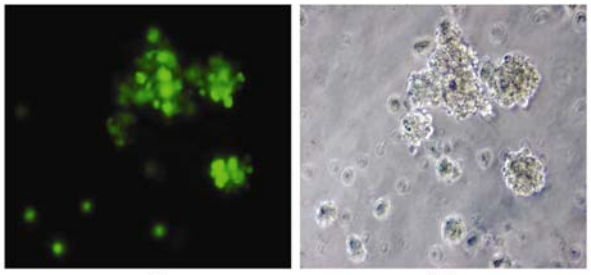
Fig.5 Flow cytometer analysis of NSCs infected by Ad-GFP at MOI of 100. (a) The scatter plots of the infected cells; (b) The analysis results of (a). The quadrant of LR shows 97.05% of GFP-positive cells, and LL shows 2.95% of GFP-negative cells

FSC-H: Forward scatter-height; SSC-H: Side scatter-height; UL: Upper of the left; UR: Upper of the right; LL: Lower of the left; LR: Lower of the right



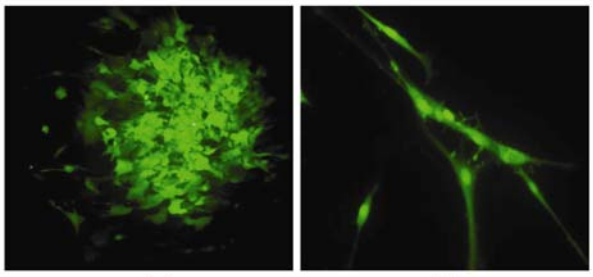
(a) (b)

Fig.3 (a) Nest-like clusters of neural stem cells were obtained in suspension culture with the neurosphere media. Images were taken under a light microscope at 200× magnification; (b) The immuno-fluorescence for nestin. After cultured in neurosphere media for one day, NSC clusters were stained for the expression of the neuronal lineage marker, nestin, with the Cy3-conjugated antibody. Image was taken under the red fluorescent channel in a fluorescent microscope at 200× magnification, showing red signals in nestin positive cells



(a) (b)

Fig.4 Expression of GFP in viral infected NSCs in suspension. When infected at the MOI of 100, more than 90% of infected cells expressed bright green fluorescence at the 3rd day. (a) Under the green fluorescent channel; (b) Under the bright field (200× magnification)



(a) (b)

Fig.7 The expression of GFP. (a) GFP in the infected NSCs attached to the culture dish and staring to differentiate. At the 3rd day of induction by the differentiating media, the cell clusters and the radial cells both expressed green fluorescence (200× magnification); (b) GFP in the differentiated cells. The secondary NSCs were induced to differentiate into neurons and astrocytes. At Day 7, their bright green fluorescence signals were observed (400× magnification)

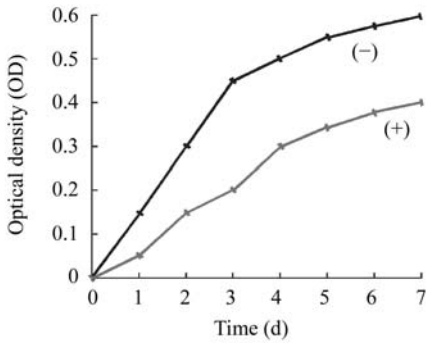


Fig.6 The growth curves of secondary NSCs without (−) or with (+) the infection by Ad-GFP recombinant virus

DISCUSSION

There are three major approaches to obtain long term cultures of NSCs (Gage *et al*., 1995; Steghaus-Kovac, 1999): (1) The primary NSCs may be immortalized by transduction with oncogene such as *v-myc* and SV40 via recombinant retroviral vector. (2) NSCs primarily isolated from adult or embryonic tissues may be cultured in media without serum but supplemented with bFGF and EGF, or other factors. (3) They may be derived from the differentiated embryonic stem cells under specific conditions. In our experiments, the primarily isolated cells from the rat embryonic hippocampus (P14) showed a strong capacity of division, multiplication and self-renewal when cultured in the serum-free neurosphere culture media containing 20 ng/ml bFGF and 20 ng/ml EGF. The cells retained the main characteristics of NSCs after 12 passages in culture.

Currently, many researchers believe that the neurons and astrocytes in the central nervous system are originated from common neural stem cells. The cell differentiation is a process resulted from many procedures involving cell division and transfer, and also largely dependent on many factors such as bFGF and EGF or other growth factors (Gage, 2000). EGF is mainly responsible for the long-term survival of NSCs, and bFGF for their differentiation towards neurons and astrocytes. The long-term cultures of primary NSCs often require more than one factor. It is important for neurophysiologists to understand how these factors regulate the behaviors of NSCs and how their regulations change the cell functions spatially and temporally.

Recombinant adenoviruses are replication-defective adenoviral vectors that have proven to be useful for gene therapy, vaccine therapy and many basic biological applications. Several features make recombinant adenoviruses particularly appealing as vectors for gene transfer. For example, high-titer preparations of adenoviruses can be readily prepared and used to achieve a high level of transgene expression in a broad spectrum of host cells and tissues, including primary and non-dividing cells (Luo *et al*., 2007). In our study, the reporter gene *GFP* was used to track the successful viral transduction, and the percentage of GFP-expressing cells after infection was indicator of the infection efficiency. The recombinant adenovirus vector (Ad-GFP) was constructed via homologous recombination in bacteria. With the AdEasy system, the backbone plasmid and the shuttle plasmid share large homologous DNA fragments, and can be efficiently recombined to generate a new viral DNA comprising sequence from both the backbone and shuttle vectors. The recombinant viral DNA can be transfected into mammalian packaging lines for the virus production. These approaches have made it possible to generate large quantities of recombinant adenoviruses in a timely and predictable fashion. As having higher successful rate, simpler operation and shorter experimental period, this method has been widely used in the research on gene therapy (Luo *et al*., 2007).

When cultured in serum-free media, NSCs accumulate, and then form suspended cell clusters and some conglomerations. Therefore, it is difficult to obtain high transfection efficiency by the standard transfection methods. Li *et al*.(2005) reported that the highest transfection efficiency in these cells was 39.9% through lipofectamine 2000. Studies reported that the transfection efficiencies through lipofectamine 2000 or routine electroporation were lower than 20%, or sometimes even lower than 5% (Falk *et al*., 2002; Guo *et al*., 2003; Hung *et al*., 2004; Lemkine *et al*., 2002). On the other hand, the transfection efficiencies via the recombinant adenoviral or recombinant retroviral vectors were much higher. For example, the efficiency of infection by the recombinant adenoviral vector was greater than 90%, sometimes was even close to 100%. However, when the recombinant adenovirus vector was transferred into NSCs, the viability of NSCs has been shown to be

reduced and the cells were prone to differentiate into astrocytes (Hughes *et al*., 2002; Wu *et al*., 2002).

In our experiments, the recombinant adenoviral vector was used to transfer a reporter gene *GFP* into the secondary NSCs. When MOI was 100, the infection efficiency was the highest, up to 97.05%, as determined by flow cytometry, and the infected NSCs were still vigorously growing. The expression of GFP lasted for about 4 weeks, and the reporter gene expression was at its peak from the 3rd day to the 12th day. The MTT analysis revealed that although the infection restrained the growth of NSCs, the cells still maintained their ability to divide, multiply and self-renew. Upon withdrawal of the growth factors, NSCs cultured in neurosphere culture media have been shown to differentiate into a variety of mature neurons and astrocytes (Fu *et al*., 2007; Gage *et al*., 1995). In this study, the FBS in addition of all known needed factors was used, NSCs differentiated into neurons and astrocytes simultaneously. When the growth factors (EGF and bFGF) were withdrawn from the culture media, the infected secondary NSCs became attached to the culture dish, and started to differentiate towards the neurons and astrocytes, two most abundant cell types in the nervous system. These differentiated cells all expressed bright green fluorescence, suggesting that the infected NSCs can be efficiently differentiated, and the differentiated cells can retain the expression of GFP.

In order to promote NSC proliferation and differentiation to neurons, some researchers tempted to infect NSCs by recombinant adenoviruses with exogenous genes or developmental factors (Zeng *et al*., 2007; Wang *et al*., 2007). It will also be our future studies to decrease the adverse effects of adenoviral infection on NSCs, and increase the proportionment of neurons differentiated from the infected NSCs.

CONCLUSION

In summary, we successfully constructed the recombinant adenovirus vector Ad-GFP, and obtained the high infection efficiency into primary cultures of NSCs. The infected NSCs retained their abilities of division, multiplication and self-renewal, and expressed the reporter GFP for a relatively long time. Our study suggested that NSCs are suitable target cells for recombinant adenoviral infection. The infected NSCs carrying a target gene may be potentially used in cell-based therapy, particularly in the neuronal systems.

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