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Effective Gene Transfer into Regenerating Sciatic Nerves by Adenoviral Vectors: Potentials for Gene Therapy of Peripheral Nerve Injury

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Replication defective adenoviral vectors have been demonstrated as an effective method for delivering genes into a variety of cell types and tissues both *in vivo* and *in vitro*. Transfecting genes into neuronal cells has proven to be difficult because of their lack of cell division. Since the major problem in neurological disease is the degeneration of the terminally differentiated neuronal cells, the adenoviral vector's ability to transfer genes into differentiated post-mitotic cells makes them advantageous for a gene delivery system for the nervous system. Here we showed that a replication defective recombinant adenovirus carrying the *lacZ* gene could infect the neuronal stem cells and even the differentiated neuronal cells derived from the central nervous system. The *lacZ* gene delivered into the neuronal cells was expressed efficiently. In addition, the recombinant virus also infected Schwann cells in intact and injured nerves *in vivo*. The expression of the *lacZ* gene lasted for 5 weeks, within which nerve regeneration is accomplished in the rat. Adenoviral vectors might thus be used to modulate Schwann cell gene expression for treating peripheral nerve injury or peripheral neuropathy.

Keywords: Neuronal Stem Cell; Regeneration; Replication Defective Adenovirus; Sciatic Nerve.

Introduction

Gene therapy has attracted considerable interest as a molecular tool for neurological research and also as a potential treatment for human neurological diseases that range from simple monogenic disorders to complex diseases. The effectiveness of gene therapy depends among other factors on the efficient delivery of the desired genes to the cell population of interest. A Reliable approach that has so far been developed to transfer significant numbers of neuronal cells is the use of non-replicating virus as a vector. Several viral vector systems based on DNA viruses, such as adenovirus, adeno-associated virus and herpes simplex virus, and on retroviral systems such as human immunodeficiency virus, have demonstrated a potential for direct gene transfer to the nervous system *in vivo* (Slack and Miller, 1996; Federico, 1999). Among them replication defective adenoviruses are attractive candidate vectors because they can be obtained at a high titer and have a low pathogenicity in humans as well as a high efficacy for the *in vivo* delivery of exogenous genes (Benihoud *et al*., 1999).

Adenoviruses (Ads) are non-enveloped double stranded DNA viruses whose natural host is the airway epithelium. It has, however, a broad range of potential hosts including a wide variety of proliferating and terminally differentiated cells (Federoff *et al*., 1997; Giger *et al*., 1997; Haddada *et al*., 1995). This makes the adenoviral vector advantageous to target genes to the site of disease and injury of the nervous system, because the major problem in neurological disease is the degeneration of the terminally differentiated neuronal cells that do not divide.

Recently, however, it has been reported that neuronal stem cells proliferate in several regions of the rat and human adult brain such as the hippocampal dentate

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Abbreviations: Ad, adenovirus; β-gal, β-galactosidase; PDGF, platelet-derived growth factor; p.f.u., plaque-forming unit; PNS, peripheral nervous system.

gyrus and the subventricular zone of the cerebral cortex (Johansson *et al.*, 1999). Promotion of the proliferation and differentiation of the stem cells in the injury sites of the adult brain may replace the degenerating cells and ultimately lead to regeneration. Thus, the delivery of genes required for proliferation and differentiation into neuronal stem cells is promising for therapy. A similar strategy can be applied to treat peripheral nerve injury or peripheral neuropathy. In the injury sites of the peripheral nervous system (PNS), axons re-grow toward the target by the guidance of Schwann cells, the supporting glia in the PNS (Salzer and Bunge, 1980). Thus, delivery of genes such as neurotrophic factors, that can influence axonal regeneration into Schwann cells, would facilitate nerve regeneration.

In an attempt to apply an adenoviral vector to the gene therapy of neurological disease we investigated whether such a vector efficiently delivers reporter genes into neuronal stem cells and differentiated neurons *in vitro*. The adenovirus carrying the *lacZ* gene infected both cell types and mediated a strong expression of β-galactosidase activity. In addition we showed that the replication-defective adenovirus vector transferred the *lacZ* gene efficiently into differentiated and proliferating Schwann cells in intact and injured sciatic nerves of the rat. The stable expression of β-gal was observed for up to 5 weeks *in vivo*. These results demonstrated the feasibility of using adenovirus to transfer therapeutic genes into the PNS for the treatment of nerve injury.

Materials and Methods

Adenoviral vectors E1-deleted replication defective adenovirus from a single plaque was amplified in 293 cells that supplement E1 in trans, and was purified twice by cesium chloride density gradient centrifugation. The stock was then dialyzed against Phosphate buffered saline (PBS) and kept as aliquots at −80°C until use. Viral titers were determined by plaque assay in 293 cells and expressed as a plaque-forming unit (p.f.u.).

Cell culture and adenoviral infection Mammalian precursor stem cell line HiB5 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Hyclone) at 33°C. Cells were plated in a 24 well plate (around $3–4 \times 10^4$ cells/well) 48 h before infection at 33°C, which was performed using 5 to 50 p.f.u. per cell. Stock solutions (8×10^{10} p.f.u./ml) of adenoviral vectors were diluted in serum free medium to various dilutions and directly added to the medium. In order to induce differentiation to neurons, HiB5 cells were moved to 39°C, and incubated in N2 serum-free medium, a mixture of F12 and DMEM medium at the same ratio, containing 100 μg/ml transferrin, 20 nM progesteron, and 20 ng/ml of PDGF. Cells were infected with LacZ-Ad in N2-serum medium 24 and 48 h later as described above.

Direct injection of adenovirus into the sciatic nerve of the rat Male Sprague Dawley rats weighing 210–230 g (Daehan experimental animal center, Seoul, Korea) were anesthetized with an intraperitoneal injection of entoval® (pentobarbital sodium; Hallym Pharmaceuticals, Seoul, Korea). Using an aseptic technique, the right sciatic nerve was exposed at the sciatic notch, and 5 μl of PBS or a purified LacZ-Ad suspension at 8×10^{10} p.f.u./ml were directly injected into the sciatic nerve at each of two sites using a 10 μl Hamilton microsyringe (Polylabo, Strasbourg, France). To examine the efficacy of adenoviral vector to transfer foreign genes into the injured peripheral nerve system, the right sciatic nerve of rat was transected at 5 d prior to the administration of virus. Adenoviral stock was injected into sciatic nerves at the regenerated site of the injured nerve as described above. Control nerves were injected with the same volume of PBS.

Immunocytochemistry Undifferentiated or differentiated HiB5 cells were fixed in 4% paraformaldehyde in PBS and washed twice with PBS. After permeabilization in cold methanol for 5 min, cells were incubated with monoclonal antibody against acetylated tubulin (Sigma, 1:1,000) overnight, and subsequently incubated with FITC-conjugated secondary antibody for 30 min. Stained cells were washed with PBS three times and mounted in PBS containing 25 mM NaI to reduce fading before examination under a Zeiss Axiovert 35 fluorescence microscope.

X-gal staining

Cells Cells were fixed 48 h after infection in 0.25% glutaraldehyde in PBS for 10 min at 4°C and washed with PBS twice, and then incubated in X-gal staining solution composed of 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-D-Galactoside overnight at 37°C.

Sciatic nerves The animals were sacrificed at various times between 2 d to 5 weeks postinjection by intracardiac perfusion with a cold fixing solution containing 0.5% glutaraldehyde, 2 mM $MgCl_2$, and 5 mM EGTA. Sciatic nerves were then excised and placed in 30% sucrose in PBS containing 2 mM $MgCl_2$, and incubated overnight at 4°C. The sciatic nerves were embedded in blocks prior to freezing. Cryosections were cut out longitudinally with a thickness of 7–8 μm and permeabilized in a solution containing 0.01% sodium deoxycholate, 0.02% NP-40 for 10 min, and reacted with X-gal staining solution as described above. Sections were then mounted and examined under a microscope for the presence of blue color.

Determination of cell numbers β-gal positive cells in 6 high-power fields (×200) were counted at each section, and the numbers were averaged for each time point.

Results and Discussion

Efficacy of the adenoviral vector to transfer the LacZ gene into neuronal stem cells and differentiated neuronal cells *in vitro* To investigate whether the adenoviral vector efficiently transfers and expresses foreign genes in neuronal stem cells and differentiated neurons, a

hippocampal stem cell line, HiB5, was infected with the adenoviral vector expressing β-galactosidase (β-gal). HiB5 cells were established by immortalizing the primary cultured cells of rat embryonic hippocampus using a temperature-sensitive oncogene, the tsA57 allele of the SV40 large T antigen (Renfranz *et al*., 1991). These cells proliferate at the permissive temperature, 33°C, and show the shape of neuroepithelial cells and express a stem cell marker, the nesting intermediate filament (Fig. 1A). However, they stop proliferation and differentiate to various shapes of cells at the non-permissive temperature, 39°C. When HiB5 cells were injected into adult rat brain, spinal cord, or developing brain, they migrated into the proper sites. There they differentiated into various neuronal and glial cell types including pyramidal cells, granule cells, astrocytes and oligodendrocytes, probably depending on the external cues that they receive from the endogenous microenvironment (Renfranz *et al*., 1991). Thus these HiB5 cells are termed multipotent stem cells. We have previously reported that a platelet-derived growth factor (PDGF) stimulates HiB5 cells to express the brain-derived neurotrophic factor at the non-permissive temperature (Kwon *et al*., 1997). Addition of PDGF also promoted HiB5 cells to differentiate into neuronal cells showing the morphological characteristics of neurons such as neurite outgrowth and the expression of neuronal markers such as acetylated tubulin (Fig. 1E), neurofilaments, and GAP43 (data not shown).

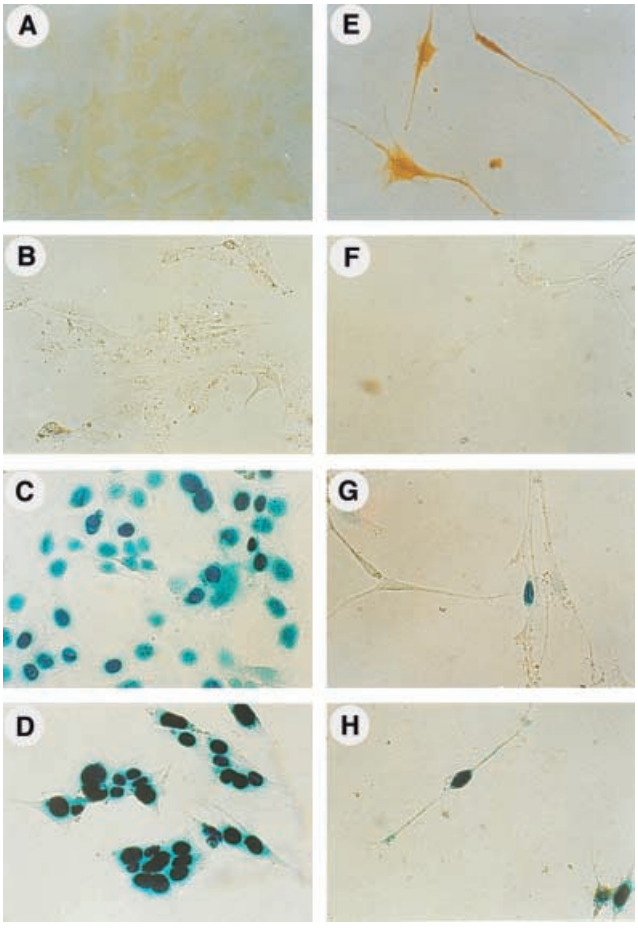

Fig. 1. Infection of the proliferating neuronal stem cells, HiB5 or differentiated HiB5 cells by LacZ-Ad. The proliferating or differentiated HiB5 cells were immunostained by anti-acetylated β-tubulin (**A** and **E**). Expression of β-galactosidase in LacZ-Ad infected HiB5 cells (**B**–**D**, **F**–**H**) were localized in the nucleus of virus infected cells. Cells were either uninfected (**B**) or infected with LacZ-Ad at a m.o.i. of 10 (**C**) and 50 (**D**) at 33°C. Neuronal differentiation of HiB5 cells was induced by shifting the incubation temperature to 39°C and adding PDGF for 24 h and then infected with LacZ-Ad at a m.o.i. of 10 (**G**) and 50 (**H**). (**F**) shows differentiated HiB5 cells not infected with LacZ-Ad.

The replication defective adenoviral vector, LacZ-Ad, used in this study contains the gene for β-gal of *E. coli* fused to the amino acid sequence of SV 40 for nuclear localization under the control of the RSV LTR promoter (Stratford-Perricaudet *et al*., 1992). When viruses were added at m.o.i. of 50 at 33°C, most of the HiB5 cells were infected, showing the strong expression of β-gal activity detected by histochemical staining in the nucleus (Fig. 1D). There is no observed cytopathic effect. Most of the undifferentiated HiB5 cells were also infected with a lower amount of viruses (m.o.i. of 10), although the expression level was little reduced (Fig. 1C). Next, we investigated whether LacZ-Ad can infect the differentiated HiB5 cells. Neuronal cell differentiation of HiB5 cells was induced by shifting the incubation temperature to 39°C in the presence of PDGF for 24 h. When infected with LacZ-Ad with an m.o.i of 10 or 50, 10% of the differentiated cells expressed LacZ in the nucleus (Figs. 1G and 1H). When HiB5 cells were infected with the same m.o.i of the virus at 33°C and then induced to differentiate into neuronal cells at 39°C, the infection efficiency increased up to 40% (data not shown). These results indicate that the recombinant adenovirus is a feasible tool for delivering foreign genes into neuronal stem cells and differentiated neuronal cells as well.

Expression of β-gal in the intact sciatic nerves injected with adenoviral vector In order to apply adenoviral vector to transfer genes to PNS nerves, we examined whether these viruses can infect Schwann cells in sciatic nerves. LacZ-Ad was injected into sciatic nerves and β-gal activity was examined in histological sections from the sciatic nerves at various magnifications. The rats injected showed a strong expression of β-gal in the vicinity of the injection site 2 d after viral administration.

Histologically, the expression was prominent mainly in Schwann cells and to some extent in perineural tissue because there was no neuronal nuclei in the sciatic nerves (Fig. 2B). β-gal activity remained as strong even

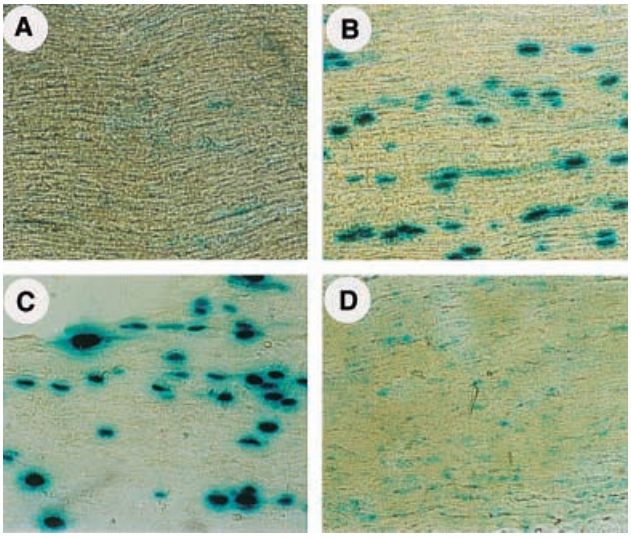

Fig. 2. Infection of intact sciatic nerves by LacZ-Ad. Intact sciatic nerves were injected with PBS (**A**) or LacZ-Ad (**B**, **C**, and **D**) and the expression of β-gal activity was monitored by X-gal staining at 2 d (**B**), 6 d (**C**), and 5 weeks (**D**) after the injection. The blue color visualizes the cells expressing β-gal.

at 6 d post-injection (Fig. 2C). Then, the intensity of staining and the number of stained cells were reduced at later time points (5 weeks, Fig. 2D). In rats receiving PBS as sham controls, there was no detectable or localized β-gal activity (Fig. 2A).

Stable and widespread expression of β-gal in regenerating sciatic nerves after injection of LacZ-Ad To explore the ability of adenoviral vector to transfer genes into Schwann cells during nerve regeneration, we monitored the expression of β-gal by LacZ-Ad within nerve segments over the period of regeneration. To induce the regeneration process we surgically transected a rat sciatic nerve leaving the main artery intact at the sciatic notch. Five days later LacZ-Ad was injected into the nerves. Axons degenerated in the distal stump of the injury site after surgery. The proliferation of the dedifferentiated Schwann cells also occurred and peaked 5 days later as detected by BrdU staining (unpublished observation, Kwon). We also observed that axons in the proximal stump of the injury site re-grew later and Schwann cells differentiated and re-myelinated the growing axons when they contacted them. This phenomenon became most prominent 5 weeks after the injury. Therefore, we monitored the expression of β-gal for 5 weeks after the injection (about 6 weeks after the injury) which is enough time for recovery in this system.

Of the 31 rats injected with adenovirus into the injury site, all showed strong β-gal activities. Peak expression of *lacZ*, measured by both X-gal staining and counting the number of β-gal positive Schwann cells, occurred between 10 d to 3 weeks. The expression lasted up to 5 weeks post-injection, although it gradually decreased over the time period. At 2 and 7 d, nerves showed strong but localized β-gal activity mainly at Schwann cells in the area of the injection (Figs. 3A and 3B). At later time points (10 d to 5 weeks), however, Schwann cells expressing β-gal were found to be scattered in the vicinity of the injection site (Figs. 3C–3F) and in areas distant from the injection site. The number of Schwann cells expressing β-gal also increased continuously until 10 d post-injection at the distal end of nerves as well as the injection site (Fig. 4). These results indicate that more than one virus infected single Schwann cells at the time of injection and that the viral DNAs remained stable over the period of Schwann cell proliferation during regeneration, in which they divided into daughter cells and continued to express the *lacZ* gene. From 3 to 5 weeks after injection, the expression gradually decreased in terms of the intensity of staining and the number of stained cells (Figs. 3D–3F), but the reduction was not as extensive as that observed in intact nerves at

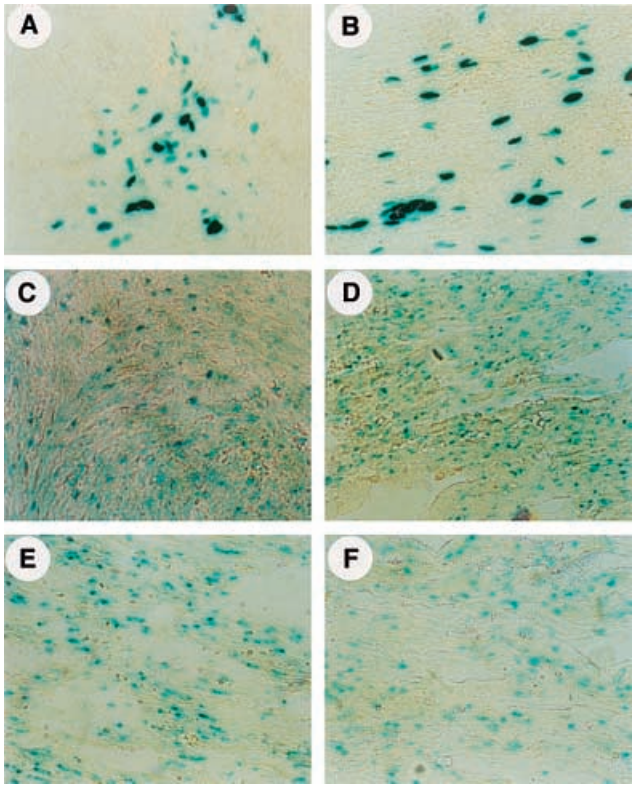

Fig. 3. Expression of β-gal following intraneural injection of LacZ-Ad into injured sciatic nerves. The expression of β-gal activity was monitored by X-gal staining at the injection sites. The expression of β-gal was localized at the injection site at 2 d (**A**) and 7 d (**B**) post-injection and then extended to the vicinity of the injection site in nerve sections from 10 d (**C**), 3 weeks (**D**), and 4 weeks (**E**). The expression of β-gal was markedly decreased at 5 weeks after viral administration, but no significant tissue degeneration was observed (**F**).

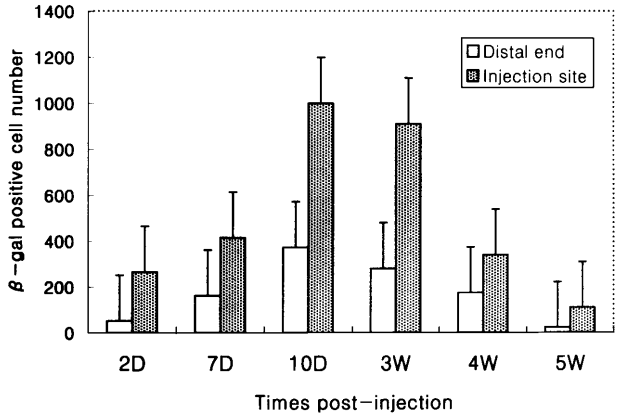

Fig. 4. Increase of Schwann cells expressing β-galactosidase at the injection site. LacZ-Ad was injected into injured sciatic nerves. During regeneration, Schwann cells expressing β-galactosidase were counted at the injection site (shaded bar) and distal stump (open bar). Error bars represent the standard deviation from the mean values.

5 weeks post-injection (Fig. 2D). Interestingly, no significant tissue degeneration was observed at this time point in the sciatic nerves receiving adenovirus, indicating that the single injection of viruses did not cause a host immune reaction. At this period the nuclei of the Schwann cells were aligned along the axons resulting from re-myelination whereas the nuclei of the proliferating Schwann cells were randomly distributed before nerve regeneration. Therefore, the results shown above indicate that replication-defective adenoviruses can infect Schwann cells at both the differentiated and proliferating state during nerve regeneration, and render the stable and safe expression of a foreign gene.

In general, the transfection efficiency of differentiated neuronal cells is very low. Thus the efficient adenoviral infection into neuronal cells promises many applications for laboratory and therapeutic studies. The present study demonstrated the potential applications of replication-defective adenovirus to deliver genes into Schwann cells in regenerating sciatic nerves after injury. In addition, the safe and efficient infection into neuronal stem cells by adenoviruses makes it possible to transfer therapeutic genes to stem cells before transplanting them to the sites of disease and injury of the brain. This could be applicable for promoting the proliferation and differentiation of the stem cells present in the injury sites.

Adenoviruses are different from retroviruses, in which the life cycle does not include a random incorporation of the viral genome into the host chromosomal DNA that may cause undesirable genetic disruption inside of virus-infected cells. Episomal gene expression driven by the adenovirus is transient because of its life cycle. This property of the adenovirus, however, has an advantage over retroviruses for the treatment of diseases such as injury and ischemia because expression of the therapeutic gene is required only for a short period. After recovery from the disease there is no need to worry about overdose and side effects of the gene product. Moreover, this study showed the low cytotoxic effect in neuronal cells by replication defective adenoviruses both *in vivo* and *in vitro*, indicating that this virus is a safe tool for delivering genes for gene therapy of these types of disease.

Axons can regenerate after injury in the PNS unlike in the central nervous system. Injury to a peripheral nerve leads a pattern of distal axonal degeneration, followed by myelin degradation and proliferation of Schwann cells induced by signal factors from degenerating axons, the so-called Wallerian degeneration (Kwon *et al.*, 1997). It has been shown that Schwann cells deprived of axonal contact produce a number of factors promoting axonal regeneration such as several well-characterized neurotrophic factors and cell adhesion molecules. These are believed to provide guidance to sprouting axons as they grow and extend toward the target in a regenerating nerve (Fawcett and Keynes, 1990).

Promotion of axonal re-growth mediated by foreign genes delivered into Schwann cells may result in efficient gene therapy for peripheral nerve regeneration. Neurotrophic factors expressed from the transferred gene in Schwann cells could be secreted and in a retrograde way affect axons to grow. These factors can be also expressed from neurons to retrogradely affect Schwann cells to differentiate and re-myelinate. Thus, delivery of genes encoding proteins such as neurotrophic factors to Schwann cells by adenoviruses in injured peripheral nerves may provide novel therapeutic tools that have wide applications for the gene therapy of peripheral neuropathies such as those secondary to diabetes, cancer chemotherapeutic agents, and other toxins.

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References

Benihoud, K., Yeh, P., and Perricaudet, M. (1999) Adenovirus vectors for gene delivery. *Curr. Opin. Biotechnol.* **10**, 440–447.

Fawcett, J. and Keynes, R. (1990) Peripheral nerve regeneration. *Annu. Rev. Neurosci.* **13**, 43–60.

Federico, M. (1999) Lentiviruses as gene delivery vectors. *Curr Opin. Biotechnol.* **10**, 448–453.

Federoff, H., Brooks, A., Muhkerjee, B., and Corden, T. (1997) Somatic gene transfer approaches to manipulate neural networks. *J. Neurosci. Methods* **71**, 133–142.

Giger, R., Ziegler, U., Hermens, W., Kunz, B., Kunz, S., and Sonderegger, P. (1997) Adenovirus-mediated gene transfer

in neurons: construction and characterization of a vector for heterologous expression of the axonal cell adhesion molecule axonin-1. *J. Neurosci. Methods* **71**, 99–111.

Haddada, H., Cordier, L., and Perricaudet, M. (1995) Gene therapy using adenovirus vectors. *Curr. Top. Microbiol. Immunol.* **199**, 297–306.

Johansson, C. B., Momma, S., Clarke, D. L., Risling, M., Lendahl, U., and Frisen J. (1999) Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* **96**, 25–34.

Kwon, Y. Kim (1997) Expression of brain-derived neutrophic factor mRNA stimulated by basic fibroblast growth factor and platelet-derived growth factor in rat hippocampal cell line. *Mol. Cells* **7**, 320–325.

Kwon, Y. Kim, Bhattacharyya, W. V., Cheon, K., Stiles, C. D., and Pomeroy, S. L. (1997) Activation of ErbB2 during wallerian degeneration of sciatic nerve. *J. Neurosci.* **17**, 8293–8299.

Renfranz, P., Cunningham, M., and RD, M. (1991) Region-specific differentiation of hippocampal stem cell line HiB5 upon implantation into the developing mammalian brain. *Cell* **66**, 713–729.

Salzer, J., and Bunge, R. (1980) Studies of Schwann cell proliferation. I. An analysis in tissue culture of proliferation during development, Wallerian degeneration, and direct injury. *J. Cell. Biol.* **84**, 739–752.

Slack, R., and Miller, F. (1996) Viral vectors for modulating gene expression in neurons. *Curr. Opin. Neurobiol.* **6**, 576–583.

Stratford-Perricaudet, L., Makeh, I., Perricaudet, M., and Briand, P. (1992) Widespread long-term gene transfer to mouse skeletal muscles and heart. *J. Clin. Invest.* **90**, 626–630.