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Highly Efficient Retrograde Gene Transfer for Genetic Treatment of Neurological Diseases

Shigeki Kato[1], Masahito Kuramochi[1], Kenta Kobayashi[1], Ken-ichi Inoue[2], Masahiko Takada[2] and Kazuto Kobayashi[1]

[1]*Department of Molecular Genetics, Institute of Biomedical Sciences, Fukushima Medical University School of Medicine, Fukushima,*

[2]*Systems Neuroscience Section, Primate Research Institute, Kyoto University, Inuyama,*

Japan

1. Introduction

Gene transfer vectors derived from neurotropic viruses provide a powerful tool for gene therapy of a variety of neurological diseases. The lentiviral vector system permits the efficient transfer of genes into non-dividing cells in the central nervous system and sustains long-term expression of the genes (Naldini et al., 1996; Reiser et al., 1996; Mochizuki et al., 1998; Mitrophanous et al., 1999). This vector system has been used for gene therapy trials in animal models for neurological diseases (for reviews, see Azzouz et al., 2004a; Wong et al., 2006; Lundberg et al., 2008). Axonal transport in the retrograde direction, as observed in the case of some viral vectors, has a considerable advantage for transferring genes into neuronal cell bodies situated in regions remote from the injection sites of the vectors (see Fig. 1). These viral vectors, when injected into the striatum, deliver the genes through retrograde transport into nigrostriatal dopamine neurons that are the major target for gene therapy of Parkinson's disease (Zheng et al., 2005; Barkats et al., 2006). Intramuscular injection of the vectors also delivers retrogradely the genes into motor neurons that are the target for gene therapy of motor neuron diseases (Baumgartner & Shine, 1998; Perrelet et al., 2000; Mazarakis et al., 2001; Sakamoto et al., 2003; Azzouz et al., 2004b).

To enhance the gene transfer of a human immunodeficiency virus type-1 (HIV-1)-based vector via retrograde transport, we have previously generated the HIV-1 vector pseudotyped with a selective variant of rabies virus glycoprotein (RV-G) (Kato et al., 2007). Injection of this RV-G-pseudotyped vector into the mouse striatum yields an increase in gene transfer into neuronal populations in the cerebral cortex, thalamus, and ventral midbrain, each of which innervates the striatum. Injection of the RV-G pseudotype into the monkey striatum (caudate and putamen) results in increased gene transfer into the nigrostriatal dopamine neurons. The RV-G pseudotyping of the HIV-1 vector enhances the efficiency of gene transfer through retrograde axonal transport in the rodent and nonhuman primate brains. However, because large-scale application of gene therapy trials requires

high-titer stocks of the vector, a lentiviral vector system that produces more efficient retrograde gene transfer is needed.

Recently, we developed a novel vector system for highly efficient retrograde gene transfer (designated as HiRet vector) by pseudotyping the HIV-1 vector with fusion glycoprotein B type (FuG-B), in which the cytoplasmic domain of RV-G was substituted by the corresponding part of the vesicular stomatitis virus glycoprotein (VSV-G) (Kato et al., 2011). The HiRet vector shifts the transducing property of the lentiviral vector and promotes the retrograde gene transfer into different brain regions innervating the striatum with greater efficiency than that of the RV-G pseudotype in both rodent and nonhuman primate brains. In the present chapter, we describe the development and property of the HiRet vector as well as the retrograde gene transfer of the vector into target neurons for gene therapy of neurological diseases, such as Parkinson's and motor neuron diseases.

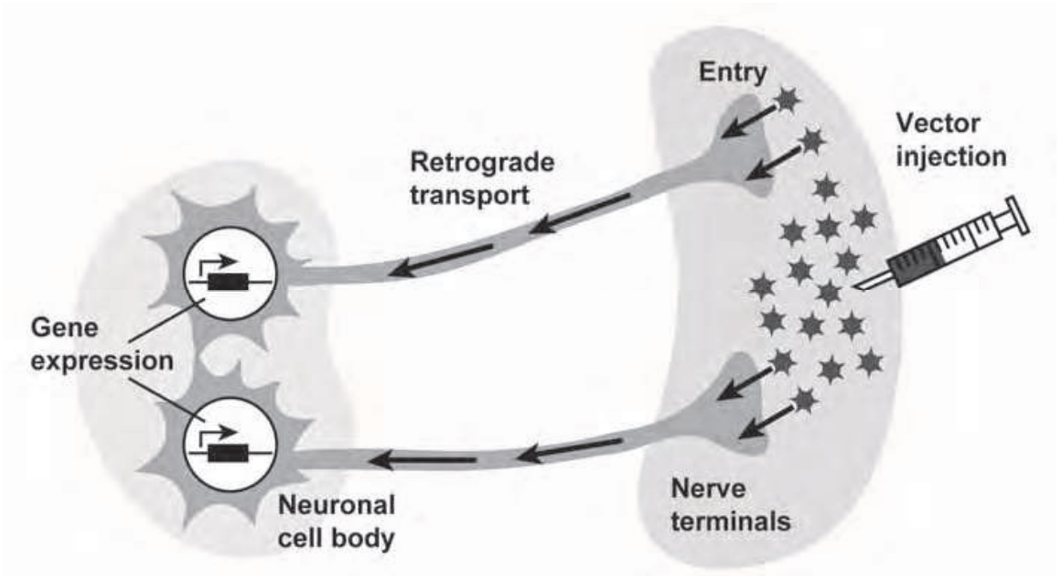

Fig. 1. **Schematic illustration of gene transfer process through retrograde axonal transport.** The viral vectors enter synaptic terminals and are transported within axons in the retrograde direction to neuronal cell bodies where transgene expression is induced.

2. Development and characterization of HiRet vector

2.1 Pseudotyping with fusion envelope glycoprotein

To develop the HiRet vector, we tested two kinds of fusion glycoprotein (termed FuG-A and FuG-B), both of which are composed of the RV-G and VSV-G segments, for the pseudotyping of the HIV-1 vector. FuG-A contains the extracellular domain of RV-G fused to the transmembrane and cytoplasmic domains of VSV-G, whereas FuG-B contains the extracellular and transmembrane domains of RV-G connected to the VSV-G cytoplasmic domain (Fig. 2A). HIV-1-based lentiviral vectors carrying transgene encoding green fluorescent protein (GFP) were produced by pseudotyping with RV-G, FuG-A, or FuG-B. When the functional titer (transducing unit) in HEK293T cells was measured by flow cytometry, the titer of the FuG-B pseudotype was 13 times greater than that of the RV-G pseudotype, whereas the titer of the FuG-A pseudotype in this cell line showed only a

moderate increase compared with the RV-G vector titer (Fig. 2B). The functional titer of the FuG-B pseudotype, when measured in neuronal cell lines (Neuro2A and N1E-115 cells), was also the highest among the three vector pseudotypes (Fig. 2B). In contrast, the concentration of physical particles (or RNA copy number) of the vectors, when estimated by quantitative reverse transcription-PCR analysis, showed no remarkable change among the three pseudotypes (data not shown). Therefore, the HIV-1 vector pseudotyped with FuG-B exhibited a greatly increased efficacy of transduction into cultured cells.

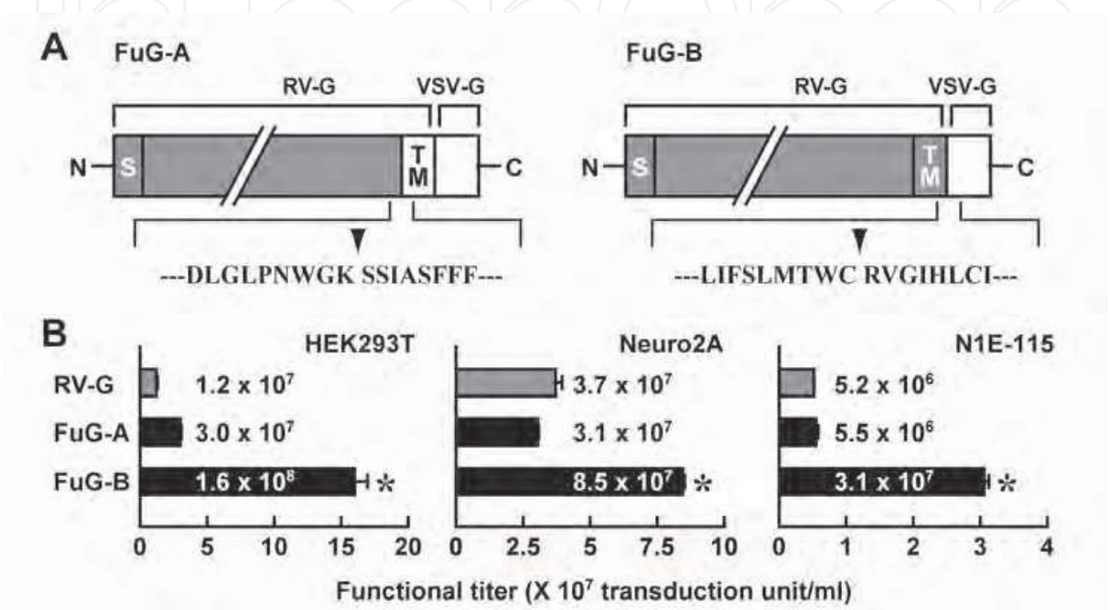

Fig. 2. **Production of vector pseudotypes with fusion glycoproteins.**
(**A**) Structure of the fusion glycoproteins FuG-A and FuG-B composed of different combinations of the N-terminal and C-terminal segments from RV-G and VSV-G. Amino acid sequences around the junction (arrowhead) between the RV-G and VSV-G segments are indicated. S, signal peptide; TM, transmembrane domain. (**B**) Functional titer of the vector pseudotypes. HEK293T cells (eighteen 10-cm-diameter tissue culture dishes) were transfected, and the conditioned medium was collected. Vector particles were pelleted by ultracentrifugation and resuspended in PBS (1 ml). HEK293T, Neuro2A, and N1E-115 cells were transduced with viral vectors, and the functional titer was determined by flow cytometry. Values were obtained from four independent experiments. *$p < 0.001$, significant difference from the titer of the RV-G or FuG-A pseudotype (ANOVA, Tukey HSD test). (Data from Kato et al., 2011)

2.2 Promoted efficiency of retrograde gene transfer

To characterize the *in vivo* gene transfer of the pseudotyped lentiviral vectors, we injected the vectors into the dorsal striatum of mice, and studied gene expression in the brain regions that innervate the striatum, including the primary motor cortex (M1), primary somatosensory cortex (S1), parafascicular thalamic nucleus (PF), and substantia nigra pars compacta (SNc). The number of GFP-positive cells in each brain region was prominently increased in the FuG-B vector-injected mice over that in the RV-G vector-injected mice (Fig. 3A); and the increases in the cell number were 12-, 12-, 8-, and 14-fold in the M1, S1, PF,

and SNc, respectively (Fig. 3B). In contrast, the cell number in each brain region was comparable between the FuG-A and RV-G vector-injected animals (Fig. 3A, B). Therefore, the FuG-B pseudotyping of the HIV-1 vector greatly enhanced the efficiency of retrograde gene transfer into the brain regions innervating the dorsal striatum. Based on these *in vivo* data, we designated the FuG-B pseudotype as the HiRet vector.

In the HiRet vector, the cytoplasmic domain of RV-G was substituted by the corresponding part of VSV-G, which resulted in the enhancement of transduction of cell lines and

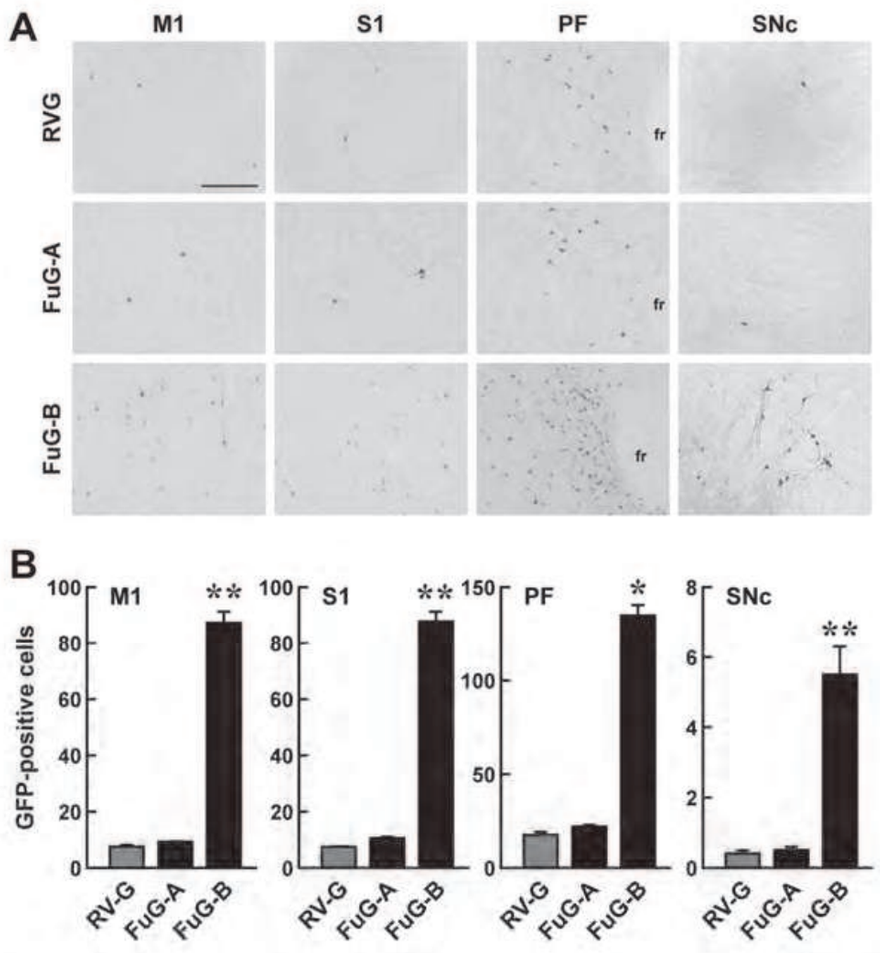

Fig. 3. **Efficiency of retrograde gene transfer by the pseudotyped vectors.**
Lentiviral vectors pseudotyped with RV-G, FuG-A or FuG-B with equivalent copy numbers of viral RNA (1.2 x 10^{10} copies/ml, 1.0 µl x 2 sites) were injected into the mouse striatum, and four weeks later sections were used for GFP immunostaining. (**A**) Transgene expression through retrograde transport in the primary motor cortex (M1), primary somatosensory cortex (S1), parafascicular thalamic nucleus (PF), and substantia nigra pars compacta (SNc). fr, fasciculus retroflexus. Scale bars: 200 µm. (**B**) The cell number in the various brain regions. The number of GFP-positive cells per section was counted (n = 4). $^{*}p < 0.01$, $^{**}p < 0.001$, significant differences from the RV-G or FuG-A pseudotype (ANOVA/Tukey HSD test). (Data from Kato et al., 2011)

retrograde gene transfer. The cytoplasmic domain differs in length between RV-G (44 amino acids) and VSV-G (29 amino acids), and their amino acid sequences do not show any particular homology (Rose et al., 1982). To test whether the length of the cytoplasmic sequence may be involved in the enhancement of retrograde gene transfer, we constructed deletion mutants of the RV-G cytoplasmic domain consisting of 10 or 20 amino acids, and used them for the pseudotyping of the HIV-1 vector. Analysis of the *in vivo* gene transfer indicated that shorter cytoplasmic domain of RV-G increased the efficiency of gene transfer through retrograde transport, but that the VSV-G cytoplasmic domain induced most efficiently the retrograde gene transfer (data not shown). The results suggest the importance of both the length and sequence of the glycoprotein cytoplasmic domain in the retrograde gene transfer of the HiRet vector. Based on our titration experiments using the pseudotyped vectors, the viral RNA titration displayed no significant change in the yield of physical particles between the pseudotyped vectors. Modification of the cytoplasmic domain of a viral glycoprotein thus seemed to shift the property of gene transduction of the pseudotyped vectors into the host cells, although the efficacy of formation or budding of the particles appeared to be unaffected.

The host range of lentiviral vectors is altered by pseudotyping with distinct envelope glycoproteins (for review, see Cronin et al., 2005). RV-G interacts with certain neuronal receptors, such as the nicotinic acetylcholine receptor α-subunit, low-affinity nerve growth factor receptor, and neural cell adhesion molecule (Hanham et al., 1993; Gastka et al., 1996; Thoulouze et al., 1998; Tuffereau et al., 1998). Substitution of the cytoplasmic domain of a viral glycoprotein may influence incorporation of the glycoprotein into vector particles or cause conformational changes in the glycoprotein structure involved in binding to receptor molecules or membrane fusion of the pseudotyped vector. These changes may affect the mechanism involved in vector entry into synaptic terminals or the transduction level of the vector, resulting in the enhanced retrograde gene transfer.

3. Retrograde gene delivery by HiRet vector into target neurons for gene therapy

3.1 Nigrostriatal dopamine neurons

We investigated the capability of the HiRet vector to introduce retrograde gene transfer into target neurons for gene therapy of neurological diseases. Nigrostriatal dopamine neurons are the major target for the therapy of Parkinson's disease (Zheng et al., 2005; Barkats et al., 2006). Retrograde gene transfer of the HiRet vector into the dopamine neurons in the mouse SNc was less efficient, although transfer of the vector into cortical and thalamic neurons was greatly increased (see Fig. 3). We thus tested the retrograde gene transfer of the HiRet vector into the SNc of the monkey. The pseudotyped vectors were injected into the striatum (caudate nucleus and putamen) of crab-eating monkeys, and sections through the SNc were stained immunohistochemically for GFP. Injection of the HiRet vector (FuG-B pseudotype) resulted in the appearance of a larger number of GFP-positive cells in the SNc as compared with that of the RV-G pseudotype (Fig. 4A), and the increase was 10-fold of the RV-G pseudotype control (Fig. 4B). The GFP transgene was expressed in a majority of the dopamine neurons (74.8%, n = 2), which were identified by immunostaining for tyrosine hydroxylase (TH), a key enzyme of dopamine biosynthesis (Fig. 4C). The HiRet vector thus achieved highly efficient delivery of genes through retrograde transport into the nigrostriatal dopamine system in the nonhuman primate brain.

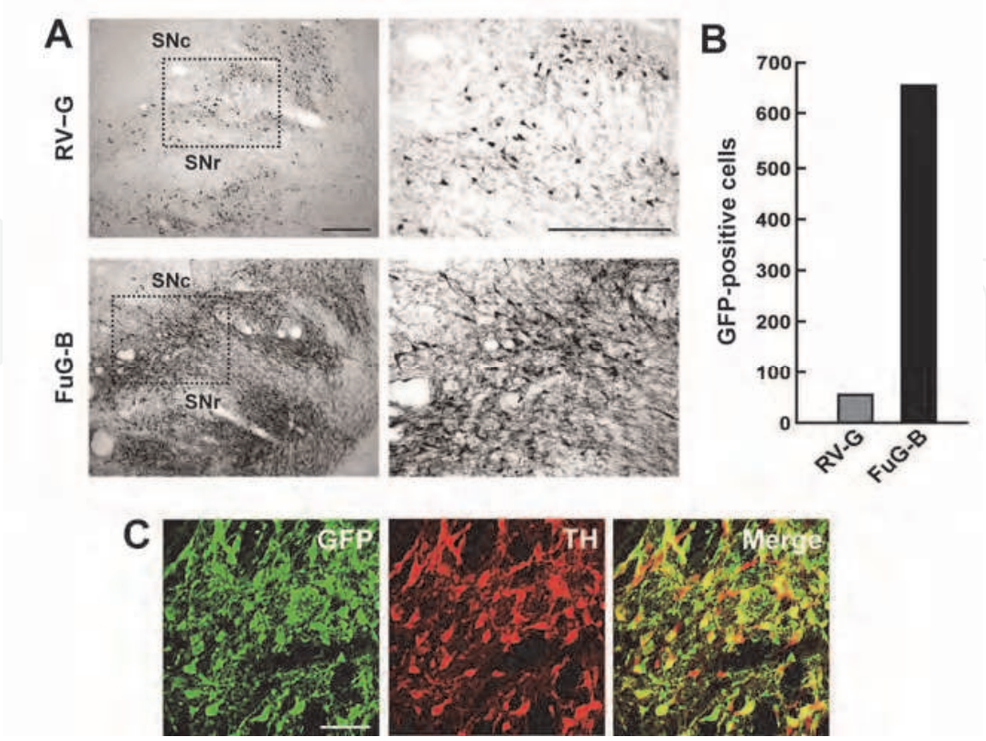

Fig. 4. **Retrograde gene transfer into nigrostriatal dopamine neurons.**
Lentiviral vectors pseudotyped with RV-G or FuG-B with equivalent copy numbers of viral RNA (8.5 x 10^9 copies/ml, 7.5 µl x 14 sites) were stereotaxically injected into the caudate and putamen, and four weeks later a series of sections were used for histological analysis. (**A**) GFP staining of sections through the SNc. Photos at the right denote magnified views of the rectangular boxes in the left ones. (**B**) Average number of GFP-positive cells per SNc section (n = 2). (**C**) Double immunofluorescence staining for GFP and TH in the SNc. GFP-positive signals, TH-positive signals, and merged images are presented in green, red, and yellow, respectively. SNr, substantia nigra pars reticulata. Scale bars, 1 mm (**A**) and 50 µm (**C**). (Data from Kato et al., 2011)

3.2 Hindbrain/spinal motor neurons

Motor neurons are the target for gene therapy of motor neuron diseases (Baumgartner & Shine, 1998; Perrelet et al., 2000; Mazarakis et al., 2001; Sakamoto et al., 2003; Azzouz et al., 2004b). Therefore, we studied retrograde gene transfer of the HiRet vector into motor neurons in the hindbrain and spinal cord. The vector was injected into the tongue or hindlimb muscles in mice, and sections through the hindbrain (hypoglossal nerve) or spinal cord (lumbar level) were stained for GFP immunohistochemistry. Such injections labeled cells in the hypoglossal nucleus of the posterior hindbrain and in the ventral horn of the spinal cord at the lumbar level (Fig. 5A). Double immunofluorescence histochemistry for GFP and the motor neuronal marker choline acetyltransferase confirmed GFP expression in the hypoglossal and spinal motor neurons that innervate the injected muscles (Fig. 5B). The HiRet vector thus enabled us to induce retrograde gene delivery into motor neurons. The extent of motor neuron transfer of the HiRet vector will be compared with that of the RV-G pseudotype elsewhere.

4. Conclusion

The lentiviral vector system based on HIV-1 has been extensively applied to gene therapy trials for neurological diseases. Retrograde axonal transport of viral vectors offers a great advantage to the delivery of genes into neuronal cell bodies that are located in brain areas distant from the injection site. The pseudotyping of HIV-1-based vectors with selective variants of RV-G increases gene transfer via retrograde transport into the central nervous system. Since the large-scale application for gene therapy trials requires high-titer stocks of the vector, the pseudotyping of a lentiviral vector that generates a greater efficiency of retrograde transport was needed. Therefore, we developed the HiRet vector for highly efficient retrograde gene transfer by pseudotyping an HIV-1 vector with a fusion envelope glycoprotein FuG-B, in which the cytoplasmic domain of RV-G was substituted by the corresponding part of VSV-G. The HiRet vector shifted the transducing property of the

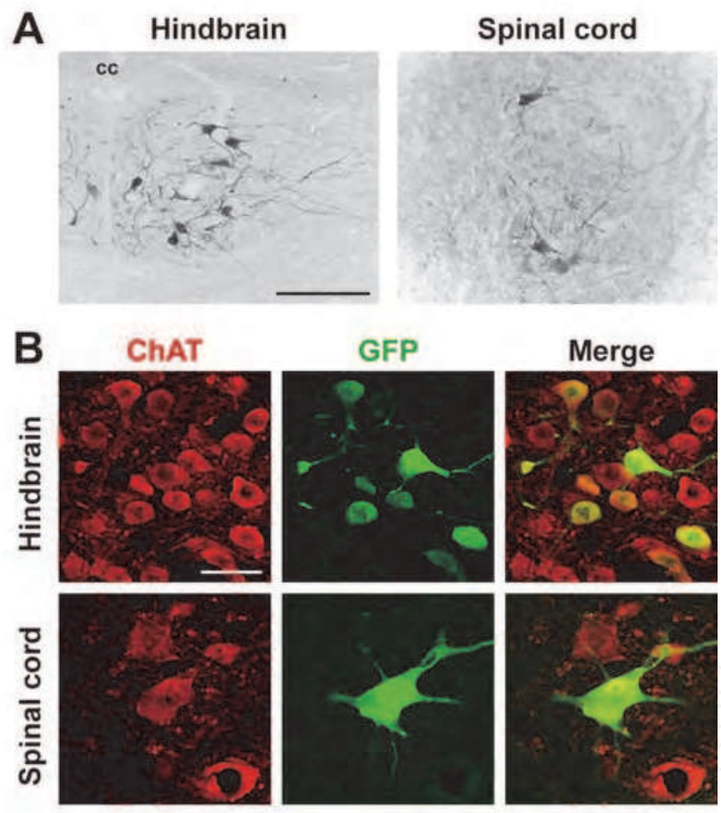

Fig. 5. **Retrograde gene transfer into motor neurons.**
(**A**) GFP expression in the hindbrain and spinal cord after the FuG-B vector injection (2.4 X 10^9 transduction units/ml). Sections through the posterior hindbrain containing the hypoglossal nucleus and the lumbar spinal cord were prepared from the injected animals and stained by using GFP immunohistochemistry. (**B**) GFP expression in motor neurons. The sections were used for double immunofluorescence staining for GFP and choline acetyltransferase (ChAT), a marker of motor neurons. GFP-positive signals, ChAT-positive signals, and merged images are shown in green, red, and yellow, respectively. cc, central canal. Scale bar: 50 μm.

lentiviral vector and enhanced the retrograde transport-mediated gene transfer into different brain regions. In particular, its transfer efficiency into the brain regions innervating the striatum attained 8- to 14-fold increases over the efficiency of the RV-G pseudotype. In addition, intrastriatal injection of the HiRet vector in the monkey brain resulted in gene transfer into a large number of nigrostriatal dopamine neurons that are the major target for gene therapy of Parkinson' disease. Furthermore, intramuscular injection of the HiRet vector achieved gene transfer into motor neurons in the hindbrain and spinal cord that are the target for gene therapy of motor neuron diseases. Our strategy with the HiRet vector provides a powerful tool for the treatment of certain neurological diseases by promoting retrograde gene delivery of this viral vector.

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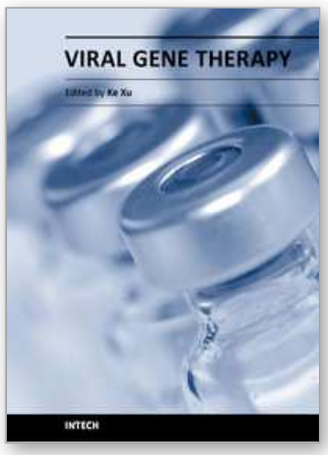

Viral Gene Therapy

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The development of technologies that allow targeting of specific cells has progressed substantially in recent years for several types of vectors, particularly viral vectors, which have been used in 70% of gene therapy clinical trials. Particular viruses have been selected as gene delivery vehicles because of their capacities to carry foreign genes and their ability to efficiently deliver these genes associated with efficient gene expression. This book is designed to present the most recent advances in viral gene therapy

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