Published in final edited form as:

J Neurosci Methods. 2010 May 30; 189(1): 56–64. doi:10.1016/j.jneumeth.2010.03.019.

Optimal promoter usage for lentiviral vector-mediated transduction of cultured central nervous system cells

Mingjie Li^{1,2}, Nada Husic^{1,2}, Ying Lin¹, Heather Christensen³, Ibrahim Malik¹, Sally McIver¹, Christine M. LaPash Daniels¹, David A. Harris^{2,3,*}, Paul T. Kotzbauer^{1,2}, Mark P. Goldberg^{1,2}, and B. Joy Snider^{1,2}

¹Department of Neurology, Washington University School of Medicine, St Louis, MO 63110

²Hope Center for Neurological Disorders, Washington University School of Medicine, St Louis, MO 63110

³Department of Cell Biology and Physiology, Washington University School of Medicine, St Louis, MO 63110

Abstract

Lentiviral vectors transduce both dividing and non-dividing cells and can support sustained expression of transgenes. These properties make them attractive for the transduction of neurons and other neural cell types *in vitro* and *in vivo*. Lentiviral vectors can be targeted to specific cell-types by using different promoters in the lentiviral shuttle vector. Even with identical constructs, however, levels of expression can vary significantly in different types of neurons and different culture preparations; expression levels in the same neuronal subtypes can be very different in primary cell culture and *in vivo*. We systematically assessed the ability of different promoters to direct expression of foreign transgenes in primary murine neocortical neurons, cerebellar granule cells and in undifferentiated and differentiated neuroblastoma cells. In primary cortical neurons, constructs using the ubiquitin C promoter directed the highest level of transgene expression; the phosphoglycerate kinase (PGK) promoter also directed robust transgene expression, while the cytomegalovirus (CMV) and MND (a synthetic promoter that contains the U3 region of a modified MoMuLV LTR with myeloproliferative sarcoma virus enhancer) promoters resulted in the expression of the transgenes in only limited number of neurons. In contrast, in cerebellar granule cells and in differentiated SH-SY5Y neuroblastoma cultures, the CMV promoter directed the most robust transgene expression. There was similar variability in transgene expression directed by these promoters in primary cultures of oligodendrocytes and astrocytes. These findings may prove useful in the design of lentiviral vectors for use in cell culture models of the nervous system.

Keywords

Lentiviral vector; neuron; astrocyte; promoter

© 2010 Elsevier B.V. All rights reserved.

Corresponding author: Dr. Mingjie Li, Campus Box 8111, 660 S. Euclid, St. Louis, MO USA 63110. lim@neuro.wustl.edu.

*Present address: Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Most types of cultured neurons, like other post-mitotic cells, are relatively refractory to transfection. Typical transfection efficiencies for cultured neurons are 1–2% (or less) and higher transfection efficiencies are only obtained with increasing toxicity (Xia et al., 1995). Cerebellar granule cells are the most easily transfected primary neuronal cell type, but even in these cells transfection efficiencies are too low for most biochemical analyses (ranging from 0.1%–7%, Ango et al., 1999; Eldadah et al., 2000; Watson et al., 1998). Astrocytes and other dividing non-neuronal cells are slightly more amenable to transfection (Ambrosini et al., 1999), but the level of gene expression is attenuated after cell division. Viral vectors have been used to overcome these limitations with some success (Davidson and Breakefield, 2003; Mandel et al., 2006; Washbourne and McAllister, 2002; Wong et al., 2006). Lentiviral vectors offer the advantages of relatively large insert size (up to 8 kb), ability to transduce slowly dividing or nondividing cells (Blomer et al., 1997; Naldini et al., 1996a), sustained expression of transgenes, simple purification, and low toxicity. Lentiviral vectors have been used to successfully transduce many types of central and peripheral neurons (Consiglio et al., 2004; Jakobsson et al., 2003; Miletic et al., 2004; Naldini et al., 1996b). The variety of promoters available increases the utility of lentiviral vectors in neuroscience research and clinical applications (Dittgen et al., 2004; Jakobsson et al., 2003; Lai and Brady, 2002). We compared the expression of transgenes controlled by 6 different promoters carried by lentiviral vectors in primary neuronal cultures, primary astrocyte and oligodendrocyte cultures and in a neuroblastoma cell line. We show that these promoters exhibit different relative levels of expression in different types of neurons and in astrocytes. The success of future studies will depend on identification and use of the appropriate promoter for expressing genes of interest delivered by lentiviral vectors in the target cell type.

Materials and Methods

Plasmid constructs

The lentiviral vectors used in this study are third generation self-inactivating (SIN) vectors. These constructs are depicted in Figure 1. pRRLsinPGKGFPppt (PGK-GFP) expresses enhanced green fluorescence protein (eGFP) under the control of human phosphoglycerate kinase (PGK) promoter (Dull et al., 1998). pRRLsinCMVGFPppt (CMV-GFP) and pRRLsinMBPGFPppt (MBP-GFP) were constructed by replacing the PGK promoter in PGK-GFP with human cytomegalovirus promoter/enhancer or myelin basic protein promoter as described previously (McIver et al., 2005). In PGK-Lck-GFP, the sequence of the first 26 amino acids of human lymphocyte-specific protein tyrosine kinase (Lck) were cloned into the N-terminus of eGFP (Benediktsson et al., 2005). GFAP-GFP was constructed by excising the 2.2 kb glial fibrillary acidic protein (GFAP) promoter sequence from pGfa2 Lac-1 (provided by Jacques Perschon, University of Washington) with BglII (Klenow filled-in) and BamHI, and then inserted into PGK-GFP, in which the PGK promoter sequence was removed with SmaI and BamHI. pCCL-cppt-MNDU3-GFP (MND-GFP), in which the expression of eGFP is controlled by a modified Moloney murine leukemia virus (MoMuLV) LTR with myeloproliferative sarcoma virus enhancer and deleted negative control region, was generously provided by Donald Kohn (Childrens Hospital Los Angeles, CA). FCIV, containing a ubiquitin C promoter and internal ribosome entry site (IRES) followed by Venus (a yellow fluorescent protein), was derived from FUGW (Lois et al., 2002) and provided by Dr. Jeffrey Milbrandt (Washington University).

Lentiviral vector production

293T cells were maintained in Dulbecco's modified Eagles medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin in 37°C

incubator with 5% CO₂. The cells were plated at 30–40% confluence 24 h before transfection (70–80% confluence at time of transfection). Ten µg of lentiviral vector with the appropriate insert, 5.8 µg of pMDLgpRRE, 3.1 µg of pCMV-G, and 2.5 µg of pRSV-rev were cotransfected into 293T cells using the calcium phosphate precipitation method (Li and Rossi, 2005). Six hours after transfection, the medium was replaced with fresh medium containing 6 mM sodium butyrate. Culture supernatant was collected 42 h after transfection. The supernatant was passed through a 0.45 µm SFCA syringe filter (Corning) and ultracentrifuged through a 5 ml 20% sucrose (in PBS) cushion in 1 × 3 ½ polyallomer tube at 11000 rpm, 4°C, for 4 h with SW28 rotor (Beckman Coulter). The concentrated vector was stored at –80°C until use. To determine the vector titer, serial diluted vector was transduced to HT1080 cells in 12 well plate. Two days after transduction, the cells were trypsinized, fixed with 3.7% formaldehyde in PBS. The percentage of eGFP (or Venus) positive cells was determined by fluorescence-activated cell sorting (FACS) analysis. The titers were calculated as previously described (Li and Rossi, 2005).

Cell culture and vector transduction

Established protocols and optimal plating densities were used for each cell type (see below). Because of the inherent variability in primary culture preparations, comparisons between different promoters and viral transduction methods were made using sister cultures from the same plating.

Murine neocortical cultures-neuronal and astrocyte cultures—Neocortical cultures containing both neurons and glia were prepared from mouse cortices using a two-step plating procedure as previously described (Snider et al., 1998). Briefly, dissociated neocortices obtained from fetal mice at 14–16 days gestation were plated onto a previously established glial monolayer at a density of 3–4 hemispheres/24-well tissue culture plate. After 5 days *in vitro*, non-neuronal cell division was inhibited by treatment with 10 µM cytosine arabinoside for 2 days. Seven days after plating, lentiviral vectors were added to the cultures at an MOI (multiplicity of infection) of 5 (unless otherwise noted). Medium was replaced 24 h after transduction. Primary murine astrocyte cultures were transduced in a similar way.

Murine cerebellar granule neuronal cultures—Cerebellar granule neurons were prepared from 5-day-old wild-type C57BL/6J × CBA/J mouse pups as described previously (Miller and Johnson, 1996). Neurons were resuspended in K25+S media (Basal Media Eagle with Earle's salts without glutamine, plus 10% dialyzed FCS, 2 mM glutamine, 25 mM KCl, and 0.02 mg/ml gentamycin) and plated at a density of 560,000 cells/cm² in poly-D-lysine-coated eight-well chamber slides. After one day *in vitro*, cultures were transduced with concentrated viruses at an MOI of 1 with no media change.

Rat oligodendrocyte cultures—Oligodendrocyte cultures were prepared from post-natal day 2 (P2) Sprague-Dawley rats essentially as previously described (Xu et al., 2001). Briefly, dissociated cortices were plated onto flasks in 5+5A medium (5% fetal calf serum (FCS), 5% horse serum (HS), 200 mM glutamine, and 0.05% pen/strep antibiotics). After 10–14 days *in vitro*, the flasks were shaken at 180 rpm for 5 hours to remove microglia, and then at 210 rpm for 16–20 hours for detachment of oligodendrocyte-lineage cells. Medium containing cells was collected, cells were pelleted and resuspended in low serum medium (1% FBS + 1% HS (1 + 1A)) containing platelet-derived growth factor (PDGF; 10 ng/ml) to stimulate proliferation, and plated in 24-well tissue culture plate. Twenty-four hours later, medium was exchanged with 1+1A and Triiodothyroxin (T3; 400ng/ml, Sigma) to promote differentiation of immature oligodendrocytes. On the following day, lentiviral vector was added to the media.

Neuroblastoma SH-SY5Y cultures—SH-SY5Y cells were plated at a density of 8×10^4 cells per well onto 24 well plates. Plates were coated with 10 $\mu\text{g/ml}$ poly-D-lysine (Sigma) in PBS, followed by matrigel (BD Biosciences) diluted 1:40 in DMEM. The cells were cultured in Neurobasal/B27 medium (Invitrogen) for 4 weeks in the presence of 10 μM retinoic acid and 10 ng/ml human neurturin (R&D Systems) to induce neuronal differentiation, with weekly medium changes. Lentiviral vectors were added before or after induction of neuronal differentiation

Immunostaining and cell counting

Cultures were fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100 in PBS as previously described (Lee et al., 2005). After blocking with 10% goat serum, cells were incubated with primary antibody (1:500 for mouse anti-NeuN, Chemicon; 1:3 for rabbit anti-GFAP, Immunostar), and then with Alexa 594 conjugated anti-mouse or anti-rabbit IgG. Images were obtained on a microscope equipped with epifluorescence using SPOT software with standardized exposure and camera settings. The number of GFP and NeuN/GFAP expressing cells were counted manually in images from 10 fields per experimental condition. Fields were randomly selected from throughout each culture well.

Results

Construction of lentiviral vectors containing various promoters for transgene expression

All the lentiviral vectors used in this study were third generation self-inactivating (SIN) vectors (Figure 1). The cis-regulatory sequences were completely removed from the U3 region in the 3' LTR. In 5' LTR, the U3 region was replaced with Rous sarcoma virus (RSV) or cytomegalovirus (CMV) promoter and enhancer sequence. All these vectors contain a central polypurine tract (cPPT), which facilitates nuclear entry of pre-integration complex (Follenzi et al., 2000; Zennou et al., 2000). A variety of promoters were used to control gene expression in transduced cells. These were human cytomegalovirus (CMV) promoter/enhancer, human phosphoglycerate kinase (PGK) promoter, myelin basic protein (MBP) promoter, glial fibrillary acidic protein (GFAP) promoter, MND (a modified LTR U3 region of MoMuLV, in which the enhancer sequence was replaced by myeloproliferative sarcoma virus enhancer and the negative control region was deleted), and ubiquitin C promoter. All the vectors contained enhanced green fluorescent protein (eGFP) gene, except FCIV, which contained Venus (a yellow fluorescent protein) gene under the control of ubiquitin C promoter and translated through a ribosome entry site (IRES). In PGK-Lck-GFP, the first 26 amino acids of human lymphocyte-specific protein tyrosine kinase (Lck) were inserted into the N-terminus of eGFP (Benediktsson et al., 2005; McIver et al., 2005). The vector titers were determined by fluorescence-activated cell sorting (FACS) of HT1080 cells transduced with concentrated vectors. As shown in Table 1, preparations of MND-GFP produced the highest titers, followed by CMV-GFP. PGK and FCIV vectors showed moderate titers. MBP and GFAP had low titer. The MND vector may have produced the highest titer in part because a CMV promoter, rather than RSV, is used to direct transcription of full length vector RNA, resulting in higher copy number of the vector in the producer cells. Although the FCIV vector contains the same CMV promoter as in MND-GFP for full length vector transcription, the large size of the FCIV plasmid may have contributed to the reduced vector titer. In addition, the Venus reporter gene is translated through IRES, which would further reduce the reporter gene expression (Mizuguchi et al., 2000). The intensity of eGFP expression in CMV-GFP transduced HT1080 cells was much higher than that seen following transduction with PGK-GFP (not shown). This may in part mediate the difference in observed titers between these two vectors. MBP and GFAP promoters may have very low activity in non-target cell types, rendering the low titers determined by HT1080 cells. The overall titers for all these vectors were high enough for detectable transduction of appropriate target cells (see below). In our preliminary experiments,

we tested all vectors in neocortical cultures and found adequate levels of reporter gene expression with no detectable cytotoxicity at a multiplicity of infection (MOI) of 5. We assessed cytotoxicity using several different methods, including visual assessment by light microscopy, measurement of lactate dehydrogenase efflux and propidium iodide staining and did not detect any increase in cell death over background with any of the vectors and cell types used in this study (data not shown). Therefore, we chose MOI of 5 (unless otherwise noted) for all transductions.

Transgene expression in primary cortical mixed culture

We transduced mouse neocortical cultures containing both neurons and astrocytes with CMV-GFP, PGK-GFP, MND-GFP, and FCIV at an MOI of 5. Four days after transduction, we counted neurons (NeuN positive) and scored reporter gene positive cells in this population. We found that 85% of neurons expressed Venus in FCIV transduced cultures and 55.2% of neurons expressed eGFP in PGK-GFP transduced cultures (Table 2). In CMV-GFP and MND-GFP transduced cultures, only 4.1% and 0.7% neurons expressed eGFP. Most reporter positive cells colocalized with NeuN in FCIV and PGK-GFP transductions (Figure 2A), while almost no NeuN stained cells were eGFP positive in CMV-GFP and MND-GFP transductions. Fourteen days after transduction with FCIV at MOI of 1.5, the percentage of Venus positive cells were close to that observed with an MOI of 5, indicating that the lower dose was already saturated for FCIV transduction. At day 14, PGK-GFP positive neurons increased to 81.9%, approaching the level of FCIV. Although eGFP expressing neurons in CMV-GFP and MND-GFP transductions also increased with time (Table 2 and Figure 2B), there was no further increase in the number of eGFP positive neurons beyond 14 days after transduction (data not shown), suggesting that only a fraction (or subtypes) of the cortical neurons were able to express the transgene under the CMV and MND promoters. We also transduced murine neocortical cultures with PGK-Lck-GFP, in which a membrane-targeting Lck sequence is fused to N terminus of GFP. Unmodified eGFP was primarily detected in neuronal cell bodies, while the Lck-GFP fusion protein was almost exclusively localized in processes (Figure 2C). This is consistent with expression of this construct in mouse brain (McIver et al., 2005). Although CMV and MND promoters showed much lower activity compared with ubiquitin C and PGK promoters in cortical neurons, all 4 of these promoters efficiently directed gene expression in astrocytes as confirmed by immunostaining with anti-GFAP antibody (Figure 3A). Note that eGFP expression is most robust in nuclei, while GFAP immunostaining is largely excluded from the nuclei. We also transduced murine neocortical cultures with lentiviral vector containing GFAP promoter and immunostained with either anti-GFAP or anti-NeuN antibody. EGFP was expressed only in astrocytes as confirmed by exclusive colocalization with the GFAP immunostaining (Figure 3B), indicating the GFAP promoter is astrocyte-specific in murine neocortical cultures containing both neurons and astrocytes.

Reporter gene expression in cerebellar granule neurons

We compared activity of the CMV and PGK promoters in cerebellar granule cells by transducing these cells with CMV-GFP and PGK-GFP lentiviral vectors. Most cells expressed eGFP (Figure 4) 5 days after transduction with either vector. The CMV promoter supported stronger gene expression than the PGK promoter in this cell type.

MBP promoter in oligodendrocytes

We have reported that lentiviral vectors using the MBP promoter direct oligodendrocyte specific gene expression in mouse brain (McIver et al., 2005). We tested the ability of this vector to transduce primary cultured oligodendrocytes. As shown in Figure 5, the MBP promoter directed robust eGFP expression in cultured oligodendrocytes, consistent with the *in vivo* results.

Transgene expression in cultured neuroblastoma cells

We transduced undifferentiated cells from the SH-SY5Y neuroblastoma line with CMV-GFP, PGK-GFP, and FCIV vectors. The majority of cells expressed eGFP or Venus in all transductions (Figure 6A). We used the same vectors to transduce SH-SY5Y cells that had been cultured in serum-free medium with retinoic acid to promote neuronal differentiation. Three days after transduction, the differentiated neurons all expressed the reporter genes with the most robust eGFP expression seen with the CMV promoter (Figure 6B). Similar levels of expression were observed 7 days after transduction (not shown). These results indicate that CMV, PGK and ubiquitin C promoters (FCIV) are all active in both undifferentiated and differentiated neuroblastoma cells. It is noteworthy that even though the CMV promoter results in lower eGFP expression than ubiquitin C and PGK promoters in primary cortical neurons, it is more active than the others in neurons differentiated from neuroblastoma cells.

Discussion

We show here that lentiviral vectors containing either ubiquitous or cell type-specific promoters can direct robust expression of transgenes in cell cultures derived from the central nervous system (CNS). There are two main approaches for achieving targeted expression of genes delivered by lentiviral vectors. The first is to use envelope proteins that bind to specific receptors only on the desired cell type. Several envelope proteins have been explored to target different cell types in CNS (Kang et al., 2002; Watson et al., 2002; Wong et al., 2004). However, the choice of envelopes from naturally occurring viruses is limited and engineered viral glycoproteins often lose transducibility (Verhoeyen and Cosset, 2004). The second approach is to use cell-specific promoters that direct expression of genes only in target cells. Many promoters have been tested in the CNS, including CMV, PGK, neuron specific enolase (NSE), human synapsin (SYN), GFAP and elongation factor 1- α (EF-1 α) (Baekelandt et al., 2002; Gascon et al., 2008; Jakobsson et al., 2003; Kordower et al., 1999; Naldini et al., 1996a). Ubiquitous promoters have been used widely in neuroscience owing to their ability to drive robust gene expression in many cell types. Most of these promoters (e.g. CMV, PGK) are relatively small, allowing large or multiple genes of interest to be expressed in a lentiviral vector. In the cases when gene expression in nontarget cells is a concern, a cell-specific promoters will be desired. Several neuron-specific promoters have been developed. For example, the following injection of lentiviral vectors using the NSE promoter into rat brain, 98% of transduced cells express a neuronal marker; no transgene expression is seen in astrocyte marker-positive cells (Jakobsson et al., 2003). In rat primary cortical cultures transduced with a SYN promoter construct, 94% of EGFP transgene expressing cells are neurons as compared to transduction with a vector driven by the SV40 promoter where only 8.4% EGFP transgene positive cells are neurons (Gascon et al., 2008). Hioki and collaborators (Hioki et al., 2007) have reported that among 5 neuron-specific promoters SYN displays the highest specificity for neuronal expression in all regions of rat brain examined (more than 96%). When using cell-specific promoters, the target cells have to be efficiently transduced with a lentiviral vector pseudotyped with a ubiquitously recognized envelope protein. Vesicular stomatitis virus glycoprotein (VSV-G) has a widely distributed receptor, a lipid component of the plasma membrane (Seganti et al., 1986). Lentiviral vectors pseudotyped with VSV-G are able to transduce virtually all cell types, making VSV-G an ideal envelope protein to use for screening target specificity of different promoters.

We tested 6 different promoters, including both ubiquitous and cell-specific promoters in lentiviral vectors pseudotyped with VSV-G for gene expression in different CNS derived cells in culture and in a neuron-like cell line. In murine neocortical cultures containing both neurons and astrocytes, both ubiquitin C promoter and PGK promoters showed high activity in neurons. Expression driven by the ubiquitin C promoter peaked earlier than that of the PGK promoter.

In contrast with *in vivo* studies of lentiviral vectors (Blomer et al., 1997; Jakobsson et al., 2003) and adenoviral vectors (Smith et al., 2000), the CMV promoter showed much lower activity than other ubiquitous promoters in cultured mouse cortical neurons. The MND promoter led to the least reporter gene-positive neurons, although this promoter has been reported to be very active in embryonic stem cells (Haas et al., 2003) and in *ex vivo* gene therapy in a mouse model of glycogen storage disease type II (Douillard-Guilloux et al., 2009). In spite of the differences observed in neuronal expression, all these vectors supported robust expression in astrocytes in these cultures. The CMV promoter was as active as ubiquitin C promoter in astrocytes with detectable expression of reporter genes as early as 24 h after transduction (not shown), while there were 20-fold fewer reporter gene-positive neurons following transduction with CMV as compared to ubiquitin C vector. Furthermore, although the CMV promoter underperformed in cortical neurons, it was more active than other promoters in cerebellar granule neurons and in neurons differentiated from neuroblastoma cells.

In summary, for studies involving primary cortical neuronal cultures, ubiquitin C and PGK promoters would likely be the best choices for the most robust gene expression. Ubiquitin C, PGK, CMV, and MND promoters all are appropriate for transducing primary astrocyte cultures. Lentiviral vectors with a GFAP promoter drive astrocyte-specific expression in cultures containing both neurons and astrocytes. The MBP promoter did not support eGFP expression in neurons (not shown), but strongly directed eGFP expression in cultured oligodendocytes. A version of PGK-GFP containing Lck sequence fused with GFP (PGK-Lck-GFP) also efficiently transduced neurons; the Lck sequence resulted in expression localized to the cell surface and neurites. This makes the PGK-Lck-GFP vector useful for studies that require visualizing neuronal processes. These vectors containing cell type specific promoters may be useful tools for the studies requiring transduction of neuronal cell types in cell culture and *in vivo*.

Acknowledgments

This work was supported by the NIH Neuroscience Blueprint Core grant (P30 NS057105, BJS) to Washington University, Program Project Grant NS032636 (BJS) and by the Hope Center for Neurological Disorders. We thank Drs. Mark Sands and Jeffrey Milbrandt for their advice and support and Dr. Donald Kohn for providing the MND vector.

References

- Ambrosini E, Ceccherini-Silberstein F, Erfle V, Aloisi F, Levi G. Gene transfer in astrocytes: comparison between different delivering methods and expression of the HIV-1 protein Nef. *J Neurosci Res* 1999;55:569–577. [PubMed: 10082079]
- Ango F, Albani-Torregrossa S, Joly C, Robbe D, Michel JM, Pin JP, Bockaert J, Fagni L. A simple method to transfer plasmid DNA into neuronal primary cultures: functional expression of the mGlu5 receptor in cerebellar granule cells. *Neuropharmacology* 1999;38:793–803. [PubMed: 10465683]
- Baekelandt V, Claeys A, Eggermont K, Lauwers E, De Strooper B, Nuttin B, Debyser Z. Characterization of lentiviral vector-mediated gene transfer in adult mouse brain. *Hum Gene Ther* 2002;13:841–853. [PubMed: 11975850]
- Benediktsson AM, Schachtele SJ, Green SH, Dailey ME. Ballistic labeling and dynamic imaging of astrocytes in organotypic hippocampal slice cultures. *J Neurosci Methods* 2005;141:41–53. [PubMed: 15585287]
- Blomer U, Naldini L, Kafri T, Trono D, Verma IM, Gage FH. Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *J Virol* 1997;71:6641–6649. [PubMed: 9261386]
- Consiglio A, Gritti A, Dolcetta D, Follenzi A, Bordignon C, Gage FH, Vescovi AL, Naldini L. Robust *in vivo* gene transfer into adult mammalian neural stem cells by lentiviral vectors. *Proc Natl Acad Sci U S A* 2004;101:14835–14840. [PubMed: 15466696]

- Davidson BL, Breakefield XO. Viral vectors for gene delivery to the nervous system. *Nat Rev Neurosci* 2003;4:353–364. [PubMed: 12728263]
- Dittgen T, Nimmerjahn A, Komai S, Licznarski P, Waters J, Margrie TW, Helmchen F, Denk W, Brecht M, Osten P. Lentivirus-based genetic manipulations of cortical neurons and their optical and electrophysiological monitoring in vivo. *Proc Natl Acad Sci U S A* 2004;101:18206–18211. [PubMed: 15608064]
- Douillard-Guilloux G, Richard E, Batista L, Caillaud C. Partial phenotypic correction and immune tolerance induction to enzyme replacement therapy after hematopoietic stem cell gene transfer of alpha-glucosidase in Pompe disease. *J Gene Med* 2009;11:279–287. [PubMed: 19263466]
- Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L. A third-generation lentivirus vector with a conditional packaging system. *J Virol* 1998;72:8463–8471. [PubMed: 9765382]
- Eldadah BA, Ren RF, Faden AI. Ribozyme-mediated inhibition of caspase-3 protects cerebellar granule cells from apoptosis induced by serum-potassium deprivation. *J Neurosci* 2000;20:179–186. [PubMed: 10627595]
- Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L. Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat Genet* 2000;25:217–222. [PubMed: 10835641]
- Gascon S, Paez-Gomez JA, Diaz-Guerra M, Scheiffele P, Scholl FG. Dual-promoter lentiviral vectors for constitutive and regulated gene expression in neurons. *J Neurosci Methods* 2008;168:104–112. [PubMed: 17983662]
- Haas DL, Lutzko C, Logan AC, Cho GJ, Skelton D, Jin Yu X, Pepper KA, Kohn DB. The Moloney murine leukemia virus repressor binding site represses expression in murine and human hematopoietic stem cells. *J Virol* 2003;77:9439–9450. [PubMed: 12915559]
- Hioki H, Kameda H, Nakamura H, Okunomiya T, Ohira K, Nakamura K, Kuroda M, Furuta T, Kaneko T. Efficient gene transduction of neurons by lentivirus with enhanced neuron-specific promoters. *Gene Ther* 2007;14:872–882. [PubMed: 17361216]
- Jakobsson J, Ericson C, Jansson M, Bjork E, Lundberg C. Targeted transgene expression in rat brain using lentiviral vectors. *J Neurosci Res* 2003;73:876–885. [PubMed: 12949915]
- Kang Y, Stein CS, Heth JA, Sinn PL, Penisten AK, Staber PD, Ratliff KL, Shen H, Barker CK, Martins I, Sharkey CM, Sanders DA, McCray PB Jr, Davidson BL. In vivo gene transfer using a nonprimate lentiviral vector pseudotyped with Ross River Virus glycoproteins. *J Virol* 2002;76:9378–9388. [PubMed: 12186920]
- Kordower JH, Bloch J, Ma SY, Chu Y, Palfi S, Roitberg BZ, Emborg M, Hantraye P, Deglon N, Aebischer P. Lentiviral gene transfer to the nonhuman primate brain. *Exp Neurol* 1999;160:1–16. [PubMed: 10630186]
- Lai Z, Brady RO. Gene transfer into the central nervous system in vivo using a recombinant lentivirus vector. *J Neurosci Res* 2002;67:363–371. [PubMed: 11813241]
- Lee CS, Tee LY, Dusenbery S, Takata T, Golden JP, Pierchala BA, Gottlieb DI, Johnson EM Jr, Choi DW, Snider BJ. Neurotrophin and GDNF family ligands promote survival and alter excitotoxic vulnerability of neurons derived from murine embryonic stem cells. *Exp Neurol* 2005;191:65–76. [PubMed: 15589513]
- Li M, Rossi JJ. Lentiviral vector delivery of siRNA and shRNA encoding genes into cultured and primary hematopoietic cells. *Methods Mol Biol* 2005;309:261–272. [PubMed: 15990406]
- Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 2002;295:868–872. [PubMed: 11786607]
- Mandel RJ, Manfredsson FP, Foust KD, Rising A, Reimsnider S, Nash K, Burger C. Recombinant adeno-associated viral vectors as therapeutic agents to treat neurological disorders. *Mol Ther* 2006;13:463–483. [PubMed: 16412695]
- McIver SR, Lee CS, Lee JM, Green SH, Sands MS, Snider BJ, Goldberg MP. Lentiviral transduction of murine oligodendrocytes in vivo. *J Neurosci Res* 2005;82:397–403. [PubMed: 16158420]
- Miletic H, Fischer YH, Neumann H, Hans V, Stenzel W, Giroglou T, Hermann M, Deckert M, Von Laer D. Selective transduction of malignant glioma by lentiviral vectors pseudotyped with lymphocytic choriomeningitis virus glycoproteins. *Hum Gene Ther* 2004;15:1091–1100. [PubMed: 15610609]

- Miller TM, Johnson EM Jr. Metabolic and genetic analyses of apoptosis in potassium/serum-deprived rat cerebellar granule cells. *J Neurosci* 1996;16:7487–7495. [PubMed: 8922404]
- Mizuguchi H, Xu Z, Ishii-Watabe A, Uchida E, Hayakawa T. IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. *Mol Ther* 2000;1:376–382. [PubMed: 10933956]
- Naldini L, Blomer U, Gage FH, Trono D, Verma IM. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci U S A* 1996a;93:11382–11388. [PubMed: 8876144]
- Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 1996b;272:263–267. [PubMed: 8602510]
- Seganti L, Superti F, Girmenia C, Melucci L, Orsi N. Study of receptors for vesicular stomatitis virus in vertebrate and invertebrate cells. *Microbiologica* 1986;9:259–267. [PubMed: 3018449]
- Smith RL, Traul DL, Schaack J, Clayton GH, Staley KJ, Wilcox CL. Characterization of promoter function and cell-type-specific expression from viral vectors in the nervous system. *J Virol* 2000;74:11254–11261. [PubMed: 11070024]
- Snider BJ, Lobner D, Yamada KA, Choi DW. Conditioning heat stress reduces excitotoxic and apoptotic components of oxygen-glucose deprivation-induced neuronal death in vitro. *J Neurochem* 1998;70:120–129. [PubMed: 9422354]
- Verhoeven E, Cosset FL. Surface-engineering of lentiviral vectors. *J Gene Med* 2004;6:S83–S94. [PubMed: 14978753]
- Washbourne P, McAllister AK. Techniques for gene transfer into neurons. *Curr Opin Neurobiol* 2002;12:566–573. [PubMed: 12367637]
- Watson A, Eilers A, Lallemand D, Kyriakis J, Rubin LL, Ham J. Phosphorylation of c-Jun is necessary for apoptosis induced by survival signal withdrawal in cerebellar granule neurons. *J Neurosci* 1998;18:751–762. [PubMed: 9425017]
- Watson DJ, Kobinger GP, Passini MA, Wilson JM, Wolfe JH. Targeted transduction patterns in the mouse brain by lentivirus vectors pseudotyped with VSV, Ebola, Mokola, LCMV, or MuLV envelope proteins. *Mol Ther* 2002;5:528–537. [PubMed: 11991743]
- Wong LF, Azzouz M, Walmsley LE, Askham Z, Wilkes FJ, Mitrophanous KA, Kingsman SM, Mazarakis ND. Transduction patterns of pseudotyped lentiviral vectors in the nervous system. *Mol Ther* 2004;9:101–111. [PubMed: 14741783]
- Wong LF, Goodhead L, Prat C, Mitrophanous KA, Kingsman SM, Mazarakis ND. Lentivirus-mediated gene transfer to the central nervous system: therapeutic and research applications. *Hum Gene Ther* 2006;17:1–9. [PubMed: 16409120]
- Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 1995;270:1326–1331. [PubMed: 7481820]
- Xu J, Chen S, Ahmed SH, Chen H, Ku G, Goldberg MP, Hsu CY. Amyloid-beta peptides are cytotoxic to oligodendrocytes. *J Neurosci* 2001;21:RC118. [PubMed: 11150354]
- Zennou V, Petit C, Guetard D, Nerhbass U, Montagnier L, Charneau P. HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* 2000;101:173–185. [PubMed: 10786833]

Figure 1. Schematic representation of lentiviral vector constructs

In all vectors, the cis regulatory sequences in U3 region of 3'LTR are completely removed. In 5'LTR, the HIV-1 U3 region is replaced with a promoter from a different virus. RSV: Rous sarcoma virus promoter; Ψ : packaging signal; RRE: REV responsive element; cPPT: central polypurine tract; CMV: cytomegalovirus promoter; EGFP: enhanced green fluorescence protein; PGK: phosphoglycerate kinase promoter; Lck: the first 26 amino acids of human lymphocyte-specific protein tyrosine kinase; MBP: myelin basic protein promoter; GFAP: glial fibrillary acidic protein promoter; MNDU3: modified MoMuLV LTR containing myeloproliferative sarcoma virus enhancer; Ubiquitin C: Ubiquitin C promoter; IRES: internal ribosome entry site; Venus: a variant of yellow fluorescent protein; WPRE: woodchuck posttranscriptional regulatory element.

Figure 2. Expression of reporter genes in neurons from mouse cortical mixed cultures transduced with lentiviral vectors under various promoters

Eight days after plating, murine neocortical cultures were transduced with the indicated vectors at an MOI of 5. A. Four days after transduction, the cells were fixed with 4% paraformaldehyde and stained with anti-NeuN antibody. Top panel shows GFP or YFP expression, middle panel shows NeuN immunostaining, bottom panel shows overlay of both images (GFP/YFP green, NeuN shown as red). B. Same except 14 days after transduction; only GFP/YFP expression is shown. C. Comparison of neurons transduced with PGK-Lck-GFP and PGK-GFP. Images were taken 14 days after transduction. Images shown are GFP fluorescence (left), phase contrast image of the same field (middle) and overlay of GFP and phase contrast images (right).

Figure 3. Transgene expression in astrocytes in murine neocortical cultures transduced with lentiviral vectors under various promoters

Eight days after plating, cultures were transduced with the indicated vector at an MOI of 5. A. Four days after transduction, cultures were immunostained with GFAP antibody. Upper panels show GFP fluorescence, middle panels show GFAP immunostaining and lower panel is merged image (GFP-green, GFAP-red). B. Seven days after transduction with GFP-GFP, cultures were immunostained with anti-GFAP (upper panels) or anti-NeuN (lower panels) antibodies. Left panel shows GFP fluorescence, middle panel shows immunostaining, and right panel shows GFP (green) and GFAP or NeuN immunostaining (red).

Figure 4. Expression of transgenes in rat cerebellar granule cells transduced with lentiviral vectors under CMV or PGK promoters
The cultures were transduced one day after plating and the images were obtained 4 days later.

A **B**

Figure 5. Expression of transgenes in oligodendrocyte cultures transduced with lentiviral vector under MBP promoter

A. Rat oligodendrocyte cultures were transduced with MBP-GFP and the images were obtained 4 days after transduction. **B.** Same as A, except the cultures were immunostained with anti-MBP antibody.

A. Undifferentiated

B. Differentiated

Figure 6. Transgene expression in SH-SY5Y neuroblastoma cell line transduced with lentiviral vectors under various promoters

SH-SY5Y cells were transduced with the indicated lentiviral vectors at an MOI of 5.

Photomicrographs were taken 3 days after transduction. A. Undifferentiated cells. Upper panels show GFP fluorescence and lower panels show phase contrast photomicrograph of the same field. B. Cells were differentiated into neurons before transduction. Only GFP expression is shown.

Table 1

Titers of lentiviral vectors containing various promoters after transduction in HT1080 cells

Vector	CMV-GFP	PGK-GFP	PGK-Lck-GFP	MBP-GFP	GFAP-GFP	MND-GFP	FCIV
Titer (×10 ⁸)	9.73±3.21	2.57±1.07	1.63±0.15	0.28±0.03	0.22±0.10	20.7±10.87	1.05±0.18

HT1080 cells were transduced with various vectors as indicated. Forty-eight hours after transduction, the cells were analyzed by FACS for eGFP (or Venus in FCIV) expression. The titers were represented as the number of eGFP (or Venus)⁺ cells per milliliter of concentrated vectors. Values shown are means ± SD from three independent experiments.

Table 2

Transduction of neurons with lentiviral vectors containing different promoters

Days after transduction	Vector			
	CMV	PGK	MND	FCIV
4 day	4.1±3.1	55.2±13.5	0.7±0.6	85.0±9.2
7 day	33.8±5.8	63.4±6.4	4.6±2.2	88.4±5.3
14 day	52.6±9.1	81.9±5.5	9.2±4.2	89.2±7.5

Murine neocortical cultures were transduced with various vectors at MOI of 5 (except otherwise noted). EGFP or Venus positive neurons were scored at different days after transduction and the values represent the percentage of neurons (NeuN+ cells) expressing the reporter gene. Values shown are means ± SD from three independent experiments.