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Long-Term Transgene Expression in Mouse Neural Progenitor Cells Modified with PhiC31 Integrase

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Abstract

Stem cells can potentially be utilized in combined gene/cell therapies for neural diseases. We examined the ability of the non-viral ϕ C31 integrase system to promote stable transgene expression in mouse neural progenitor cells (mNPCs). ϕ C31 integrase catalyzes the sequence-specific integration of *attB*-containing plasmids into pseudo *attP* sites in mammalian genomes, to produce long-term transgene expression. We achieved gene transfer by co-nucleofection of a plasmid carrying the luciferase marker gene and an *attB* site and a plasmid expressing integrase in mNPCs that had been generated in a neurosphere preparation. Luciferase expression was quantified in live cells for 8 weeks, revealing persistence of gene expression. Sequence-specific integration at a preferred pseudo *attP* site in the mouse genome was detected by using PCR. Furthermore, sustained transgene expression was demonstrated in genetically modified NPCs that were cultured in conditions that promoted either growth or differentiation into neurons and astrocytes. Our results demonstrate that the ϕ C31 integrase system produces stable transgene expression in adult mNPCs and their progeny and may be useful in strategies for combating neurodegenerative disorders.

Keywords

gene/cell therapy; neural progenitor cells; nucleofection; non-viral vector; ϕ C31 integrase; prolonged gene expression; sequence-specific integration

1. INTRODUCTION

Classical pharmacotherapy for neurodegenerative diseases is restricted by the blood-brain barrier, which prevents the transport of many potentially therapeutic molecules into the brain. Recent progress in knowledge about the pathology and etiology of neurological diseases suggests that gene/cell therapy strategies for diseases such as Parkinson's, Amyotrophic Lateral Sclerosis, and possibly Alzheimer's, may present alternatives to traditional pharmacotherapy. A number of gene delivery options for the central nervous system (CNS) have been explored, including encapsulated cells (Hoffman et al., 1993) and *in vivo* delivery of therapeutic genes using viral vectors based on adenovirus (Xia et al., 2001), adeno-associated virus (Luo et al., 2002; Wang et al., 2002), herpes simplex virus (Natsume et al., 2001; Sun et al., 2003), and

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lentivirus (Azzouz et al., 2002; Palfi et al., 2002). *In vivo* gene delivery has shown promise due to the high levels of gene expression that can be achieved. However, the clinical use of viral vectors is limited by their immunogenicity, non-specific delivery of viral particles to CNS regions, and the risk of random integration at undesirable chromosomal locations, which can lead to adverse side effects (Hsich et al., 2002; Wu et al., 2002).

Neural stem/progenitor cells (NPCs) can proliferate and also produce neurons, astrocytes, and oligodendrocytes, making them attractive candidates for use in gene/cell therapies for neural disorders. It would be useful if NPCs could be stably engineered to express locally genes encoding soluble molecules that convey neuroprotective activity. *Ex vivo* gene therapy of expandable NPCs has been attempted by using viral vectors that express growth factors, followed by transplantation of the modified NPCs into the CNS (Ostenfeld et al., 2002; Ebert et al., 2005; Klein et al., 2005; Behrstock et al., 2006). The advantages of transplanting genetically modified NPCs that act as ‘biopumps’ are that these cells are of neural origin, and they can migrate diffusely in response to the cytokine cascades that accompany injury or disease. In addition, the gene product is delivered by healthy cells, reducing the burden of inducing gene expression in diseased cells, which is the basis of *in vivo* gene delivery (Klein et al., 2005). However, a major hurdle that *ex vivo* gene therapy must overcome is obtaining prolonged gene expression that is not curtailed due to down-regulation of the transgene or death of the cells modified with viral vectors (Klein et al., 2005).

Some studies have attempted to transfer plasmid-encoded genes into NPCs using non-viral methods such as liposomal reagents, cationic polymers, or naked DNA transfection (Tinsley et al., 2004; Tinsley et al., 2006). Plasmid vectors are often less complicated to design than viral vectors, having no size limit and more promoter flexibility. Furthermore, non-viral vectors have greater ease of handling, lower cost of preparation, fewer side effects from toxicity and immunogenicity, and more compatibility with clinical protocols than viruses. However, the efficacy of the non-viral approach in bringing about robust, long-term expression has been lower than that achieved using viral vectors (Burton et al., 2003) and hence has not been often pursued. We attempted to mitigate the problem of transient gene expression by employing ϕ C31 integrase-mediated transgene integration.

ϕ C31 integrase is a *Streptomyces* phage-derived recombinase that catalyzes recombination between *attB* and *attP* attachment sites in the bacterial and phage genomes (Groth et al., 2000). This enzyme has been developed as a non-viral gene therapy tool, because it has the ability to integrate a transgene-containing plasmid carrying an *attB* site into pseudo *attP* sites in mammalian genomes (Thyagarajan et al., 2001; Chalberg et al., 2006). ϕ C31 integrase has previously been shown to integrate genes effectively and to prolong their expression in several mammalian cell culture systems, including human keratinocytes (Ortiz-Urda et al., 2002), muscle-derived stem cells and myoblasts (Quenneville et al., 2004), and a human T cell-line (Ishikawa et al., 2006). The ϕ C31 integrase system has also been effective in intact tissues, including mouse liver (Olivares et al., 2002) and muscle (Bertoni et al., 2006; Portlock et al., 2006), rat retina (Chalberg et al., 2005), and rabbit synovium (Keravala et al., 2006).

Here we sought to determine whether mNPCs could be transfected efficiently by using nucleofection, an electroporation-based technology, to obtain significant gene expression. We investigated the efficacy of ϕ C31 integrase to mediate sequence-specific integration and robust long-term expression of the luciferase transgene in mNPCs. Furthermore, we examined whether the modified mNPCs stably expressing the transgene after ϕ C31-mediated delivery could continue to proliferate at a rate similar to naïve mNPCs and could differentiate into neurons and astrocytes while exhibiting stable transgene expression.

2. MATERIALS AND METHODS

2.1. Plasmid construction

Plasmids pCMVInt expressing ϕ C31 integrase, and pCSmI, carrying an inactive form of the integrase that contains a single base pair change in the catalytic serine have been previously described (Groth et al., 2000; Olivares et al., 2002). The pMax vector expressing the enhanced green fluorescent protein (eGFP) reporter gene under the cytomegalovirus (CMV) promoter was obtained from Amaxa Biosystems (Cologne, Germany). pBLB contains the ϕ C31 integrase recognition site, *attB*, and the firefly luciferase gene driven by CAGGS (Niwa et al., 1991), a chimeric promoter combining the CMV immediate-early enhancer (IE) and the chicken β -actin/rabbit β -globin hybrid promoter. To create pBLB, the pNBL2 vector (Thyagarajan et al., 2006) was digested with *XhoI* to remove the CMV promoter. This linearized vector was blunt ended using the large (Klenow) fragment of *E. coli* DNA polymerase I (New England Biolabs, Ipswich, MA), followed by vector dephosphorylation using shrimp alkaline phosphatase (SAP; Roche, Palo Alto, CA). In parallel, the chimeric promoter coupling the CMV-IE and the chicken β -actin/rabbit β -globin promoter was excised from the pCAGGS vector (provided by C.H. Contag, Stanford University) with *SalI* and *EcoRI* (New England BioLabs). The resulting product was blunt ended with Klenow and ligated with T4 DNA ligase (New England Biolabs) into the *XhoI*-digested, blunt-ended, dephosphorylated pNBL2 vector. pBEB contains the ϕ C31 integrase recognition site, *attB*, and eGFP driven by CAGGS. To construct pBEB, the pDB2 vector (Keravala et al., 2006) and the pBLB vector were digested simultaneously with *AflIII/AgeI* to remove the CMV and CAGGS promoters respectively. The CAGGS promoter was ligated with T4 DNA ligase into the *AflIII/AgeI*-digested pDB2 vector.

2.2. Neurosphere harvest and culture

Neurosphere-forming cells were harvested from brains of 4 week-old C57Bl/6 mice (Taconic, Hudson, NY) as described previously (Palmer et al., 1999). These percoll-fractionated cells were plated in T-75 flasks in culture media composed of Neurobasal A Medium (Gibco, Carlsbad, CA) supplemented with 1% L-Glutamine (Gibco), 2% B-27 without retinoic acid (Gibco), and 20 ng/ml epidermal growth factor (EGF; Peprotech, Rocky Hill, NJ). Neurospheres were passaged every 7 days by dissociating them with Hank's-based Enzyme-free Cell Dissociation Buffer (Gibco) and replated at a density of 2×10^6 cells per flask in growth media.

2.3. Nucleofection and selection

On the 10th passage, neurospheres were dissociated using Hank's-based Enzyme-free Cell Dissociation Buffer. Dissociated cells were washed with Hank's Balanced Salt Solution (HBSS; Gibco), and 4×10^6 cells were nucleofected (Amaxa Biosystems) in duplicate using the Mouse Neural Stem Cell (mNSC) Nucleofector kit (Amaxa Biosystems) with programs "A-31" and "A-33". 5 μ g of the pMax plasmid was used for evaluating nucleofection. After nucleofection, the cells were pelleted, resuspended in growth media, transferred to a 12-well culture plate, and incubated at 37°C in a CO₂ incubator for 24 h. For co-nucleofection, 4×10^6 mNPCs were co-nucleofected in duplicate with 2 μ g of pBEB and 2 μ g of either pCMVInt or pCSmI using the "A-33" program and the mNSC Nucleofector kit. The nucleofected cells were pelleted, resuspended, and incubated in culture medium at 37°C in a CO₂ incubator for 24 h. Similarly, 4×10^6 mNPCs were co-nucleofected in duplicate with 3 μ g of pBLB and 3 μ g of either pCMVInt or pCSmI using the "A-33" program and the mNSC Nucleofector kit. The nucleofected cells were incubated in culture medium at 37°C in a CO₂ incubator for 4 days, after which the cells were transferred to 60 mm plates and exposed to G418 selection (250 μ g/ml Geneticin; Gibco) for 8 weeks. Every 48 h, the neurospheres were fed 50:50 conditioned: fresh media containing G418.

2.4. Detection of transgene expression

To detect eGFP expression after 24 h, the nucleofected mNPCs were dissociated using Hank's-based Enzyme-free Cell Dissociation Buffer, centrifuged at 1,200 rpm for 5 min, and resuspended in HBSS. An aliquot of the cells was used for Fluorescence Activated Cell Sorting (FACS) analysis. To detect luciferase activity, the mNPCs were assayed at 1, 2, 7, 14, 21, 28, 35, 42, and 56 days after nucleofection using bioluminescence imaging as follows: 5 μ l/ml of luciferin substrate stock (30 mg/ml, Xenogen Corp., Alameda, CA, USA) was added to the cells and assayed immediately for bioluminescence activity using the IVIS 200 imaging system (Xenogen Corp.). Signal was quantified and analyzed using Living Image, version 2.20 software (Xenogen Corp.).

2.5. Detection of sequence-specific integration by polymerase chain reaction

Evidence of sequence-specific integration was obtained from genomic DNA isolated from mNPCs that were nucleofected with pBLB and pCMVInt or pCSmI and selected with G418 for 8 weeks. Genomic DNA was purified using the DNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Integration of the donor plasmid pBLB was detected by polymerase chain reaction (PCR) at the mpsL1 site in the mouse genome by using primers attBF3: 5'-CGAAGCCGCGGTGCG-3' and mpsL1-R2: 5'-GTAAATGTTATTGCGGCTCT-3' as previously described (Portlock et al, 2006). PCR products were run on an agarose gel, and the 290 bp expected band was excised, purified (QiaQuick PCR Purification Kit; Qiagen), cloned into pCR2.1-TOPO (TOPO Cloning Kit; Invitrogen, Carlsbad, CA), and sequenced.

2.6. Proliferation and differentiation analyses of mNPCs

To determine whether ϕ C31 integrase-mediated integration of the luciferase marker gene influenced mNPC proliferation and differentiation, naïve or ϕ C31-modified mNPCs were dissociated approximately 8 weeks after nucleofection in Hank's-based Enzyme-free Cell Dissociation buffer. The cells were plated at a density of 1×10^5 cells/ml growth media (Neurobasal A, 1% L-Glutamine, 2% B27 and 20 ng/ml EGF) in 8-well Lab Tek chambers (250 μ l per well; NUNC, Rochester, NY). Ninety-six hours after plating, the cells were fed with a 50:50 mix of conditioned: fresh media containing bromodeoxyuridine (BrdU; 5 μ M final concentration; Sigma Aldrich, St. Louis, MO) to label dividing cells. Exactly 12 h after the cells were fed with BrdU-supplemented media, they were either fixed with 4% paraformaldehyde for 10 min and stored in fresh Tris-buffered saline (TBS) at 4°C until immunostained, or cultured in differentiation media. In order to promote the growth of neurons in addition to astrocytes, the cells were differentiated in a cocktail of Neurobasal A Medium containing 1% FBS (Gibco), 100 ng/ml all-*trans* retinoic acid (Sigma Aldrich), 1 ng/ml FGF-2 (Peprotech), 10 ng/ml NT-3 (Peprotech), and 10 ng/ml BDNF (Peprotech) for 10 days. The differentiated cells were fixed for 10 min with 4% paraformaldehyde and stored in fresh TBS at 4°C until immunostained.

2.7. Immunostaining

The paraformaldehyde-fixed cells were gently rinsed in TBS and then blocked at room temperature for 30 min in 3% normal donkey serum (Jackson ImmunoResearch, West Grove, PA). The cells were incubated overnight at 4°C in cocktails of mouse anti-luciferase to detect stable integration of pBLB (1:500; Serotec, Raleigh, NC), with goat anti-doublecortin (DCX) to detect immature neurons (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA), or guinea pig anti-glial fibrillary acidic protein (GFAP) to detect astrocytes (1:750; Santa Cruz Biotechnology, Inc.). The primary antibody-labeled cells were incubated for 2 h at room temperature in the appropriate secondary antibodies conjugated to fluorescein isothiocyanate (FITC) or cyanine 5 (Cy5), rinsed in TBS, and fixed with 4% paraformaldehyde. Next, to detect

the dividing cell population, the cells were incubated in 2 M HCl for 20 min at 37°C to denature DNA. Following DNA denaturation, the cells were incubated with rat ascites anti-BrdU (1:500; Accurate Chemical, Westbury, NY) for 24 h at 4°C and secondary antibody conjugated to cyanine 3 (Cy3) for 2 h at room temperature. After rinsing, the cells were counterstained with 10 mg/ml DAPI (Sigma Aldrich) to detect nuclear DNA and cover slipped in a solution of 50% glycerol (Sigma Aldrich), 20% polyvinyl alcohol (Sigma Aldrich), and 2.5% w/v of the antifade medium 1,4-diazobicyclo[2,2,2]-octane (DABCO; Sigma Aldrich). All secondary antibodies were purchased from Jackson Immunoresearch and were used at a concentration of 1:500. Staining sets were done in duplicate.

2.8. Imaging

Fluorescent samples were imaged using a Zeiss Meta 510 confocal laser scanning microscope using a multi-channel configuration and a 40X objective with a 2.3 electronic zoom factor, which produces an image representing a $1\ \mu\text{m} \times 1\ \mu\text{m}$ area of the sample. For each staining set (Luc/BrdU/neuron, Luc/BrdU/astrocyte) and culture condition (proliferation v/s differentiation), 3 sites were randomly chosen for z-dimension scanning. Appropriate gain and black levels were set using control slides with secondary antibody alone.

3. RESULTS

3.1. Nucleofection facilitates efficient transfection of mNPCs

Nucleofection is an electroporation-based technology that allegedly delivers DNA directly to the nucleus, thus facilitating effective delivery of plasmid DNA (Lakshmipathy et al., 2004). To determine whether mNPCs could be transfected efficiently to obtain significant gene expression, we used nucleofection with the Amaxa Nucleofector Technology. pMax was nucleofected into mNPCs using two different Nucleofector programs suggested by the manufacturer. FACS analysis conducted 24 h post-nucleofection showed that the “A-31” program produced a 32% nucleofection efficiency, whereas the “A-33” program produced an 80% nucleofection efficiency (Fig. 1b). These results demonstrated that mNPCs could be easily and efficiently transfected by using nucleofection.

Next, to determine whether mNPCs could be co-transfected effectively to obtain high levels of gene expression, we co-nucleofected pBEB (Fig. 1a) either alone or along with pCMVInt or pCSmI using the mNSC kit and the “A-33” Nucleofector program. As shown in Fig. 1b, FACS analysis after 24 h produced an ~ 23–25% co-nucleofection efficiency with pBEB and pCMVInt or pCSmI, compared to the 25% nucleofection efficiency with the pBEB plasmid alone. These results demonstrated that mNPCs can be co-transfected effectively by using nucleofection.

3.2. ϕ C31 integrase mediates prolonged transgene expression in mNPCs

We investigated the efficacy of ϕ C31 integrase to mediate integration and robust long-term expression of the luciferase transgene in mNPCs. pBLB (Fig. 2a), donor plasmid expressing the firefly luciferase gene driven by the CAGG promoter and carrying the *attB* site was co-nucleofected into the mNPCs with pCMVInt, or with pCSmI as a negative control. The nucleofected mNPCs were cultured in selective media for 8 weeks. At various time points during the course of the experiment, luciferase expression in the mNPCs was measured using bioluminescence imaging. As shown in Fig. 2b, at 8 weeks, cells co-nucleofected with pCMVInt had 39-fold higher luciferase expression than cells co-nucleofected with pCSmI. At the end of 8 weeks, G418 resistant neurospheres were also counted, revealing ~ 201 neurospheres in the pBLB + pCMVInt plates, compared to only ~ 5 neurospheres in the pBLB + pCSmI plates (data not shown). These data indicated that use of ϕ C31 integrase greatly

enhanced long-term gene expression in the nucleofected mNPCs, presumably by mediating genomic integration of pBLB.

In addition to CAGG promoter activity, we also tested the CMV promoter driving the luciferase transgene on the donor plasmid in cultured mNPCs. We noted that although the CMV promoter expressed the transgene in the mNPCs, stable luciferase expression driven by the CAGG promoter was higher *in vitro* (data not shown). Similar observations have been reported by Wu et al. (2002) in human neural progenitor cells with an AAV vector and by Liew et al. (2007) in human embryonic stem cells. Based on these observations we carried out our study with the CAGG promoter.

3.3. ϕ C31 integrase catalyzes sequence-specific integration

Preferential ϕ C31 integrase mediated integration at a “hot-spot” in the mouse genome called mpsL1 has been documented in several studies (Olivares et al., 2002; Bertoni et al., 2006; Portlock et al., 2006), and integration at this site can be used to verify that sequence-specific integration occurred. To detect site-specific integration in the mNPCs, genomic DNA was isolated from cells that had been nucleofected with pBLB and either pCMVInt or pCSmI and then subjected to G418 selection. The genomic DNA was analyzed by using PCR, utilizing probes that detect a band that would result after integration of pBLB at mpsL1. In cells that were nucleofected with pCMVInt, the PCR analysis revealed a band of 290 bp, as expected. No band was seen after PCR analysis of genomic DNA derived from cells that received pBLB and pCSmI (Fig. 2c). The 290 bp band was sequenced, and the resulting sequence verified that integration of pBLB occurred at the mpsL1 site. This experiment confirmed that ϕ C31 integrase catalyzed sequence-specific genomic integration in the nucleofected mNPCs.

3.4. ϕ C31-modified mNPCs retain their ability to proliferate and differentiate

In order to examine whether proliferation of mNPCs *in vitro* was influenced by nucleofection and luciferase expression, the ϕ C31-modified and naïve mNPCs were cultured in growth medium containing bromodeoxyuridine (BrdU). BrdU labels actively dividing cells. It was used for 12 h to determine the number of proliferating cells, followed by fixing the cells. We observed no significant difference between the numbers of dividing, unmodified or ϕ C31-modified mNPCs (Fig. 3a). Next, we addressed whether nucleofection with ϕ C31 integrase and stable luciferase expression altered the ability of mNPCs to differentiate into neurons and astrocytes. Naïve and luciferase-expressing mNPCs were cultured for 12 h in growth media containing BrdU and then cultured for 10 days in differentiation media containing retinoic acid and low concentrations of growth factors. The mNPCs that divided while exposed to BrdU differentiated normally into neurons (DCX positive cells) and astrocytes (GFAP positive cells), regardless of modification with ϕ C31 integrase (Fig. 3b). Also, as shown in Table 1, the percentages of DCX-positive cells and GFAP-positive cells did not vary between the naïve-differentiated and the modified-differentiated groups. These experiments indicated that nucleofection and integration with ϕ C31 integrase did not affect the ability of mNPCs to proliferate and to differentiate, while stably expressing the delivered luciferase transgene.

4. DISCUSSION

Our study is the first to demonstrate that ϕ C31 integrase functions in cultured mNPCs. We established that nucleofection and ϕ C31-mediated non-viral delivery provides prolonged, robust levels of transgene expression in cultured mNPCs. More importantly, we determined that the modified mNPCs retained their ability to proliferate and to differentiate into their neural lineages.

Recent advances in the field of neurobiology have shown that neural stem cells are an excellent source for *ex vivo* gene therapy. However, the efficiency of gene transfer to neurospheres using non-viral vectors had to date been well below that achieved with viral vectors (Burton et al., 2003). Here, we examined the efficacy of nucleofection to deliver genes to mNPCs and showed transfection levels nearly comparable to viral infection. In a similar, recent study by Cesnulevicius et al. (2006), the authors compared various non-viral transfection methods, including nucleofection in rat neuronal stem cells derived from ventral mesencephali and obtained the highest rate of transfection with nucleofection. We then performed co-nucleofection with a donor plasmid and a ϕ C31-integrase plasmid and attained good transfection levels. We addressed the limitations associated with risky *in vivo* viral delivery and transient gene expression with non-viral vectors. We demonstrated robust levels of long-term gene expression in cultured mNPCs using the non-viral ϕ C31 integrase system. We observed nearly 40-fold higher levels of luciferase expression in the mNPCs modified with ϕ C31 integrase as compared to those without at 8 weeks after nucleofection. The stable expression of the transgene has been attributed to the ability of ϕ C31 integrase to mediate integration of the gene into host chromosomes (Groth et al., 2000; Thyagarajan et al., 2001; Olivares et al., 2002; Ortiz-Urda et al., 2002; Quenneville et al., 2004; Chalberg et al., 2005; Ishikawa et al., 2006; Bertoni et al., 2006; Keravala et al., 2006; Portlock et al., 2006). We obtained PCR evidence for integration of the luciferase plasmid at mpsL1, a known genomic hot-spot utilized by ϕ C31 integrase (Olivares et al., 2002; Bertoni et al., 2006; Portlock et al., 2006). Furthermore, we determined that nucleofection, followed by ϕ C31-mediated integration and long-term expression of luciferase, did not affect proliferation of neurosphere-derived mNPCs. This result is in contrast to studies in hNPCs with AAV and retroviral vectors using eGFP as the marker gene. These studies reported a slowing, followed by a cessation, of growth of the transgene-expressing hNPCs (Martinez-Serrano et al., 2000; Wu et al., 2002). One possible reason for this dissimilarity could be that we used luciferase as our marker gene for long-term studies, and luciferase may be less toxic than eGFP. Also, we obtained genomic integration with ϕ C31 integrase and therefore continuous expression of the transgene, whereas AAV-mediated transduction would lead to loss of the AAV genomes carrying the transgene which tend to remain episomal and be lost during subsequent cell divisions over time. Finally, we demonstrated that our ϕ C31-modified, stable luciferase-expressing mNPCs retained the capability to differentiate into neurons and astrocytes. Similar observations of hNPCs transduced with AAV vectors were reported by Wu et al. (2002).

Our report establishes, for the first time, efficient and stable gene expression with a non-viral vector in cultured mNPCs. Long-term transplantation of these ϕ C31-modified cells in disease model animals will be needed to fully establish the potential use of the ϕ C31 integrase system in neural stem cells. Neurospheres can be expanded in culture, are very plastic, and have the ability to undergo differentiation into multiple lineages even after transplantation in the CNS (Suzuki et al., 2007). Growth factors such as glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) have neuroprotective and trophic effects on neurons (Henderson et al., 1994; Sofroniew et al., 2001). Genetically modifying NPCs by delivering genes of such therapeutic growth factors with the simple ϕ C31 integrase system could potentially provide powerful new tools to study and treat neurodegenerative diseases.

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REFERENCES

- Azzouz M, Martin-Rendon E, Barber RD, Mitrophanous KA, Carter EE, Rohll JB, et al. Multicistronic lentiviral vector-mediated striatal gene transfer of aromatic L-amino acid decarboxylase, tyrosine hydroxylase, and GTP cyclohydrolase I induces sustained transgene expression, dopamine production, and functional improvement in a rat model of Parkinson's disease. *J Neurosci* 2002;22:10302–10312. [PubMed: 12451130]
- Behrstock S, Ebert A, McHugh J, Vosberg S, Moore J, Schneider B, et al. Human neural progenitors deliver glial cell line-derived neurotrophic factor to parkinsonian rodents and aged primates. *Gene Ther* 2006;13:379–388. [PubMed: 16355116]
- Bertoni C, Jarrahan S, Wheeler TM, Li Y, Olivares EC, Calos MP, Rando TA. Enhancement of plasmid-mediated gene therapy for muscular dystrophy by directed plasmid integration. *Proc Natl Acad Sci U S A* 2006;103:419–424. [PubMed: 16387861]
- Burton EA, Glorioso JC, Fink DJ. Gene therapy progress and prospects: Parkinson's disease. *Gene Ther* 2003;10:1721–1727. [PubMed: 12939638]
- Cesnulevicius K, Timmer M, Wesemann M, Thomas T, Barkhausen T, Grothe C. Nucleofection is the most efficient nonviral transfection method for neuronal stem cells derived from ventral mesencephali with no changes in cell composition or dopaminergic fate. *Stem Cells* 2006;24:2776–2791. [PubMed: 16902196]
- Chalberg TW, Genise HL, Vollrath D, Calos MP. PhiC31 integrase confers genomic integration and long-term transgene expression in rat retina. *Invest Ophthalmol Vis Sci* 2005;46:2140–2146. [PubMed: 15914635]
- Chalberg TW, Portlock JL, Olivares EC, Thyagarajan B, Kirby PJ, Hillman RT, et al. Integration specificity of phage ϕ C31 integrase in the human genome. *J Mol Biol* 2006;357:28–48. [PubMed: 16414067]
- Ebert A, Svendsen CN. A new tool in the battle against Alzheimer's disease and aging: ex vivo gene therapy. *Rejuvenation Res* 2005;8:131–134. [PubMed: 16144466]
- Groth AC, Olivares EC, Thyagarajan B, Calos MP. A phage integrase directs efficient site-specific integration in human cells. *Proc Natl Acad Sci USA* 2000;97:5995–6000. [PubMed: 10801973]
- Henderson CE, Phillips HS, Pollack RA, Davies AM, Lemeulle C, Armanini M, et al. GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. *Science* 1994;266:1062–1064. [PubMed: 7973664]
- Hoffman D, Breakefield XO, Short MP, Aebischer P. Transplantation of a polymer-encapsulated cell line genetically engineered to release NGF. *Exp Neurol* 1993;122:100–106. [PubMed: 8339781]
- Hsich G, Sena-Esteves M, Breakefield XO. Critical issues in gene therapy for neurologic disease. *Hum Gene Ther* 2002;13:579–604. [PubMed: 11916483]
- Ishikawa Y, Tanaka N, Murakami K, Uchiyama T, Kumaki S, Tsuchiya S, et al. Phage phiC31 integrase-mediated genomic integration of the common cytokine receptor gamma chain in human T-cell lines. *J Gene Med* 2006;8:646–653. [PubMed: 16508910]
- Keravala A, Portlock JL, Nash JA, Vitrant DG, Robbins PD, Calos MP. PhiC31 integrase mediates integration in cultured synovial cells and enhances gene expression in rabbit joints. *J Gene Med* 2006;8:1008–1017. [PubMed: 16779871]
- Klein SM, Behrstock S, McHugh J, Hoffmann K, Wallace K, Suzuki M, et al. GDNF delivery using human neural progenitor cells in a rat model of ALS. *Hum Gene Ther* 2005;16:509–521. [PubMed: 15871682]
- Lakshmipathy U, Pelacho B, Sudo K, Linehan JL, Coucouvanis E, Kaufman DS, Verfaillie CM. Efficient transfection of embryonic and adult stem cells. *Stem Cells* 2004;22:531–543. [PubMed: 15277699]
- Liew CG, Draper JS, Walsh J, Moore H, Andrews PW. Transient and stable transgene expression in human embryonic stem cells. *Stem Cells* 2007;25:1521–1528. [PubMed: 17379764]
- Luo J, Kaplitt MG, Fitzsimons HL, Zuzga DS, Liu Y, Oshinsky ML, During MJ. Subthalamic GAD gene therapy in a Parkinson's disease rat model. *Science* 2002;298:425–429. [PubMed: 12376704]
- Martinez-Serrano A, Villa A, Navarro B, Rubio FJ, Beuno C. Human neural progenitor cells: better blue than green? *Nat Med* 2000;6:483–484. [PubMed: 10802677]

- Natsume A, Mata M, Goss J, Huang S, Wolfe D, Oligino T, Glorioso JC, Fink DJ. Bcl-2 and GDNF delivered by HSV-mediated gene transfer act additively to protect dopaminergic neurons from 6-OHDA-induced degeneration. *Exp Neurol* 2001;169:231–238. [PubMed: 11358438]
- Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991;108:193–199. [PubMed: 1660837]
- Olivares EC, Hollis RP, Chalberg TW, Meuse L, Kay MA, Calos MP. Site-specific genomic integration produces therapeutic Factor IX levels in mice. *Nat Biotechnol* 2002;20:1124–1128. [PubMed: 12379870]
- Ortiz-Urda S, Thyagarajan B, Keene DR, Lin Q, Fang M, Calos MP, Khavari PA. Stable nonviral genetic correction of inherited human skin disease. *Nat Med* 2002;8:1166–1170. [PubMed: 12244305]
- Ostenfeld T, Tai YT, Martin P, Deglon N, Aebischer P, Svendsen CN. Neurospheres modified to produce glial cell line-derived neurotrophic factor increase the survival of transplanted dopamine neurons. *J Neurosci Res* 2002;69:955–965. [PubMed: 12205689]
- Palfi S, Leventhal L, Chu Y, Ma SY, Emborg M, Bakay R, et al. Lentivirally delivered glial cell line-derived neurotrophic factor increases the number of striatal dopaminergic neurons in primate models of nigrostriatal degeneration. *J Neurosci* 2002;22:4942–4954. [PubMed: 12077191]
- Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH. Fibroblast Growth Factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J Neurosci* 1999;19:8487–8497. [PubMed: 10493749]
- Portlock JL, Keravala A, Bertoni C, Lee S, Rando TA, Calos MP. Long-term increase in mVEGF164 in mouse hindlimb muscle mediated by phage phiC31 integrase after nonviral DNA delivery. *Hum Gene Ther* 2006;17:871–876. [PubMed: 16942446]
- Quenneville SP, Chapdelaine P, Rousseau J, Beaulieu J, Caron NJ, Skuk D, et al. Nucleofection of muscle-derived stem cells and myoblasts with phiC31 integrase: stable expression of a full-length-dystrophin fusion gene by human myoblasts. *Mol Ther* 2004;10:679–687. [PubMed: 15451452]
- Sofroniew MV, Howe CL, Mobley WC. Nerve growth factor signaling, neuroprotection and neural repair. *Annu Rev Neurosci* 2001;24:1217–1281. [PubMed: 11520933]
- Sun M, Zhang GR, Kong L, Holmes C, Wang X, Zhang W, et al. Correction of a rat model of Parkinson's disease by co-expression of tyrosine hydroxylase and aromatic amino acid decarboxylase from a helper virus-free herpes simplex virus type 1 vector. *Hum Gene Ther* 2003;14:415–424. [PubMed: 12691607]
- Suzuki M, McHugh J, Tork C, Shelley B, Klein S, Aebischer P, et al. GDNF secreting human neural progenitor cells protect dying motor neurons, but not their projection to muscle, in a rat model of familial ALS. *PloS One* 2007;2:e689. [PubMed: 17668067]
- Thyagarajan, B.; Calos, MP. *Methods in Molecular Biology, Therapeutic Proteins: Methods and Protocols*. Volume 308. Totowa, NJ: Humana Press; 2005. Site-specific integration mediated by phiC31 integrase for high level protein production; p. 99-106.
- Thyagarajan B, Olivares EC, Hollis RP, Ginsburg DS, Calos MP. Site-specific genomic integration in mammalian cells mediated by phage phiC31 integrase. *Mol Cell Biol* 2001;21:3926–3934. [PubMed: 11359900]
- Tinsley RB, Faijerson J, Eriksson PS. Efficient non-viral transfection of adult neural stem/progenitor cells, without affecting viability, proliferation or differentiation. *J Gene Med* 2006;8:72–81. [PubMed: 16097040]
- Tinsley RB, Vesey MJ, Barati S, Rush RA, Ferguson IA. Improved non-viral transfection of glial and adult neural stem cell lines and of primary astrocytes by combining agents with complementary modes of action. *J Gene Med* 2004;6:1023–1032. [PubMed: 15352075]
- Wang L, Muramatsu S, Lu Y, Ikeguchi K, Fujimoto K, Okada T, et al. Delayed delivery of AAV-GDNF prevents nigral neurodegeneration and promotes functional recovery in a rat model of Parkinson's disease. *Gene Therapy* 2002;9:381–389. [PubMed: 11960314]
- Wu P, Ye Y, Svendsen CN. Transduction of human neural progenitor cells using recombinant adeno-associated viral vectors. *Gene Ther* 2002;9:245–255. [PubMed: 11896463]
- Xia XG, Harding T, Weller M, Bieneman A, Uney JB, Schulz JB. Gene transfer of the JNK interacting protein-1 protects dopaminergic neurons in the MPTP model of Parkinson's disease. *Proc Natl Acad Sci* 2001;98:10433–10438. [PubMed: 11504916]

Fig. 1. Efficient transfection of mNPCs by nucleofection

(a) Donor plasmid pBEB. (b) Comparison between two mNPC nucleofection programs. FACS analysis demonstrated that nucleofection was more efficient in mNPCs using the A-33 program relative to the A-31 program. Asterisks denote values of percent eGFP positive cells between the A-31 and A-33 programs that differ at $p < 0.05$ (Student's t test). (c) Co-nucleofection of mNPCs. FACS analysis demonstrated that co-nucleofection was effective in mNPCs with the A-33 program.

Fig. 2. Prolonged luciferase expression in mNPCs with ϕ C31 integrase

(a) Donor plasmid pBLB. (b) Co-nucleofection of mNPCs with ϕ C31 integrase. Shown is the luciferase activity in the co-nucleofected cells assayed at several time points after nucleofection, using bioluminescent imaging. A significant increase in luciferase expression with ϕ C31 integrase (pBLB + pCMVInt) over no integrase (pBLB + pCSmI) was observed. Asterisks denote values between cells nucleofected with pCMVInt and pCSmI that differ at $p < 0.05$ (Student's t test). (c) Sequence-specific integration. Lane 1 is the 100 bp DNA Ladder. Lanes 1 and 2 are PCR products from mNPCs that were co-nucleofected with pBLB along with the plasmid indicated. This PCR revealed evidence of plasmid integration at mpsL1 in cells that received pBLB in addition to the pCMVInt plasmid and not in cells that received pBLB and pCSmI.

Fig. 3. Growth and differentiation of modified mNPCs

(a) Proliferation. BrdU-labeled cells were counted to compare the rate of proliferation between the non-nucleofected and nucleofected, luciferase-expressing cells. No significant difference in proliferation was observed between the two conditions. (b) Differentiation. The non-nucleofected, naïve cells as well as the nucleofected, ϕ C31-modified cells proliferated (Panel 2) and differentiated into neurons and astrocytes (Panel 3). Cells nucleofected and modified with pCMVInt revealed sustained luciferase expression even after the differentiation process (Panel 4).

Table 1Differentiation of mNPCs is unaffected by modification with ϕ C31 integrase

mNPC Sample	Number of Positive Cells			% Differentiated mNPC
	DAPI	DCX	GFAP	
Non-nucleofected cells	61	12	N/A	Immature Neuron 19.7
Nucleofected cells	41	10	N/A	24.4
Non-nucleofected cells	68	N/A	14	Astrocyte 20.6
Nucleofected cells	39	N/A	9	23.1