Citation: Molecular Therapy–Nucleic Acids (2012) 1, e60; doi:10.1038/mtna.2012.53

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METHODS: ORIGINAL ARTICLE

Assessment of Integration-defective HIV-1 and EIAV Vectors *In Vitro* and *In Vivo*

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The interest in integrase-defective lentiviral vectors (IDLVs) stems from their potential advantage of large cloning capacity and broad cell tropism while avoiding the possibility of insertional mutagenesis. Here, we directly compared the transducing potential of IDLVs based on the equine infectious anemia virus (EIAV) to the more commonly described HIV-1 IDLVs. IDLVs were constructed by introducing equivalent single/triple mutations into the integrase catalytic triad. We show that both the single and the triple mutant HIV-1 IDLVs transduce the PC12 cells, but not the C2C12 cells, with similar efficiency to their parental HIV-1 vector. In contrast, the single and triple EIAV IDLVs did not efficiently transduce either differentiated cell line. Moreover, this HIV-1 IDLV-mediated expression was independent of any residual integration activity because reporter expression was lost when cell cycling was restored. Four weeks following stereotactic administration into adult rat brains, only the single HIV-1 IDLV mutant displayed a comparable transduction profile to the parental HIV-1 vector. In contrast, neither EIAV IDLV mutants showed significant reporter gene expression. This work indicates that the transducing potential of IDLVs appears to depend not only on the choice of integrase mutation and type of target cell, but also on the nature of the lentiviral vector.

Molecular Therapy–Nucleic Acids (2012) **1**, e60; doi:10.1038/mtna.2012.53; published online 11 December 2012
Subject Category: Methods section

Introduction

Lentiviral vectors represent an extremely effective way of introducing DNA into both dividing and quiescent cells[1,2] (reviewed in ref. 3,4). These vectors carry an enveloped RNA genome which is reverse transcribed into DNA upon cell entry, this resulting proviral DNA:host/viral protein complex is then shuttled to the nucleus and ultimately integrated into the host genome, allowing for long-term expression. Although integration was once thought to be a mandatory step for viral stability and expression, this has been successfully challenged by several authors who have reported efficient gene expression *in vitro* and *in vivo* using integration-defective lentiviral vectors[5–9] (IDLVs; reviewed in ref. 10). Furthermore, in some studies, IDLVs carrying a therapeutic expression cassette have shown efficacy in rodent disease models.[9,11–14] IDLVs are an attractive alternative to integrating vectors since they offer a reduced risk of insertional mutagenesis and replication-competent lentivirus generation, and additionally they may be less prone to gene silencing or chromatin-regulated expression. However, a disadvantage of using such vectors is that long-term expression may be limited to nondividing cells since expression from these vectors almost entirely originates from the unintegrated episomal forms of the virus that are rapidly lost by dilution in dividing cells.[15,16] There are two classes of integrase mutations; class I mutations specifically affect the integration process whereas class II mutations affect integration and other viral processes such as packaging.[17–19] The class I mutations can be in the coding sequence for the integrase gene, or at sites in the viral genome to which integrase attaches and mediates insertion (*att* sites).[5,20] The most effective class I mutations are those within the catalytic triad, three residues at positions D64, D116, and E152 that are conserved absolutely among all retroelements.[17] A single mutation in the D64 residue has been shown to have the largest single effect on integrase activity, and may abolish it completely.[5,19,21,22] Nevertheless, IDLVs with a single mutation in D64 still possess a low level of residual integration activity (<1%) which is thought to be due to integrase-independent cellular processes such as DNA repair.[20]

In this report, we compare the integration and expression of HIV-1 and EIAV IDLVs with matched genomes. We show that HIV-1 IDLVs are capable of long-term transgene expression that is comparable to the parental integration-competent HIV-1 vector *in vitro*, and that this expression is due specifically to unintegrated genomes. However, the level and magnitude of expression mediated by the HIV-1 IDLVs appears to be cell type-dependent. Interestingly, we show that HIV-1 IDLVs with the D64V mutation alone gives rise to strong reporter gene expression in the adult rat brain, however, additional mutations in the catalytic triad appear to abrogate expression. We also show that, in contrast to HIV-1 vectors, this phenomenon is not translatable to EIAV-based lentiviral vectors,[23] which are rendered ineffective when the integrase activity is abolished such that there is little or no reporter gene expression following administration of the single or triple mutant EIAV vectors into the adult rat brain. This study therefore highlights the importance of cell type, integrase mutation, and vector platform for IDLV efficacy both *in vivo* and *in vitro*.

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Keywords: gene therapy; integration-defective; lentiviral vectors
Received 10 September 2012; accepted 16 October 2012; advance online publication 11 December 2012. doi:10.1038/mtna.2012.53

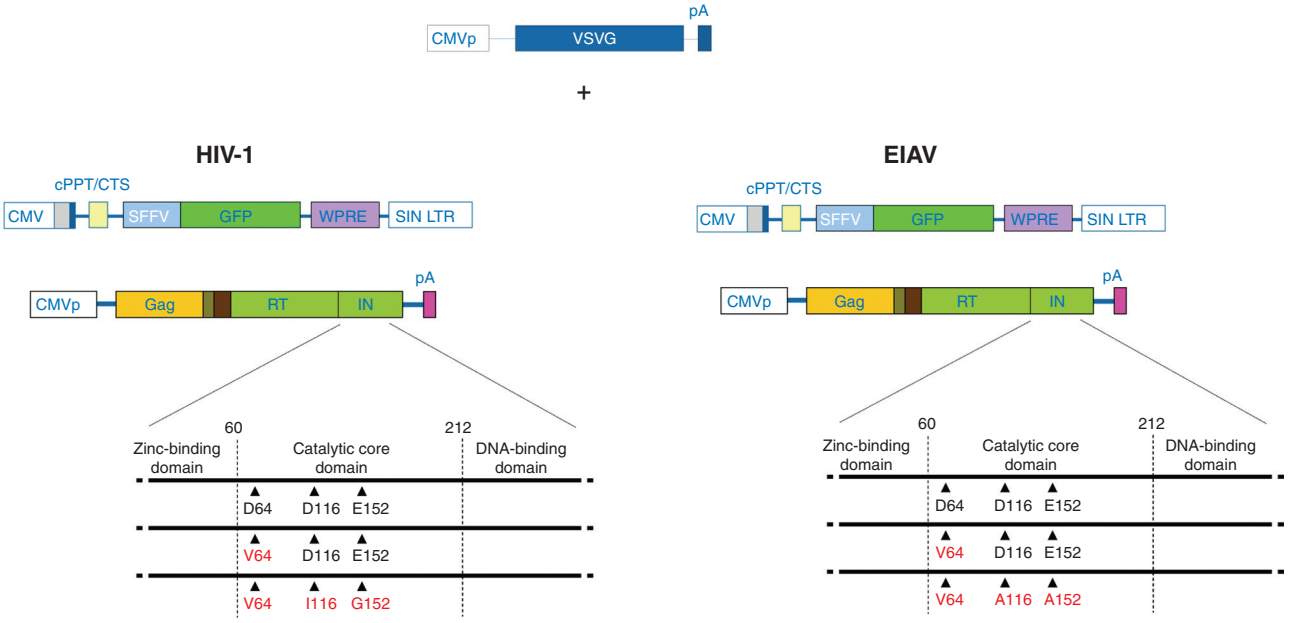

Figure 1 Transfection scheme for the production of EIAV and HIV-1 vector. Vectors were produced by triple transfection of HEK293T cells with the plasmids shown. To produce the integrase-defective lentiviral vectors (IDLVs), mutations (black arrow heads) were introduced into the GagPol plasmid to change the amino acids of the integrase protein (numbers refer to residue position). CMV, cytomegalovirus; cPPT, central polypurine tract; EIAV, equine infectious anemia virus; GFP, green fluorescent protein; LTR, long terminal repeat; SFFV, spleen focus-forming virus; SIN, self-inactivating.

Results

IDLVs are integration-defective

The aim of this study was to compare the transduction and expression characteristics of well-known HIV-1 class I integrase mutant vectors with equivalent mutations in an EIAV vector (**Figure 1**). The mutations were made in the HIV-1 and EIAV integrase genes in the so-called "catalytic triad" at amino acid positions D64, D116, and E152; these three residues are conserved absolutely across all reverse-transcribed elements, and are known to have the greatest effect on integration with no apparent effect on other viral processes.[19]

The characteristics of the HIV-1 and EIAV IDLVs with single (D64V) and triple (DDE/VIG or VAA, respectively) integrase mutations were compared with the equivalent parental integration-competent HIV-1 and EIAV vectors. The DDE/VIG HIV triple integrase mutant was constructed as these residues have previously been shown to be completely integrase-defective.[19,24] The DDE/VAA EIAV triple integrase mutant was used as this has also been shown to be integrase-defective.[25,26] All vector genomes were constructed using identical configurations (spleen focus-forming virus promoter, enhanced green fluorescent protein (eGFP) reporter gene, Woodchuck hepatitis virus post-transcriptional regulatory element, central polypurine tract (cPPT)/CTS sequences, self-inactivating long terminal repeats (LTRs)).[27] While the levels of RNA genome and reverse transcriptase (RT) in the IDLV vector preparations were similar compared with the parental vectors (**Table 1**), as expected the DNA integration titers were significantly reduced (by >190-fold) in the IDLVs compared with the parental vectors, confirming that the integration process had been attenuated (**Table 1**).

To confirm this in a biological assay, human HEK293T cells were transduced with each of the vectors and the percentage of GFP-positive cells was determined over time (**Figure 2**). While the percentage of GFP-positive cells transduced with the parental EIAV- and HIV-1–integrating vectors remained stable at 60/70% respectively for the duration of the 12-day experiment, both the single and triple mutant HIV-1 and EIAV IDLVs showed a rapid decrease in the proportion of GFP-positive cells to <12% by day 5 post-vector transduction. By day 9, the loss of GFP-positive cells transduced with HIV-1 and EIAV IDLVs appeared to stabilize (0.2–0.5%), with the proportion of cells still expressing GFP >150-fold lower than in the cells transduced with the parental-integrating vector. The decrease in GFP-positive cells in the HIV-1 and EIAV IDLV transduced population is due to loss of unintegrated viral genomes through dilution associated with HEK293T cell division, and confirms the lack of effective integrase activity in the IDLV vectors.

Table 1 HIV-1 and EIAV vector characterization: quantitation of RT activity (PERT), integration titer, and genomic RNA copies

Vector	Integrase mutation	RT activity/ml[a]	RNA copy number (copies/ml)	Integration titer (TU/ml)	Reduction in titer compared with parental
HIV-1	Parental	2.4×10^7	4.3×10^{12}	6.2×10^9	—
	D64V	4.6×10^7	3.2×10^{12}	2.3×10^7	270-fold
	DDE/VIG	3.0×10^7	3.1×10^{12}	1.6×10^7	390-fold
EIAV	Parental	6.8×10^7	2.2×10^{12}	9.3×10^8	—
	D64V	7.5×10^7	2.4×10^{12}	3.2×10^6	290-fold
	DDE/VAA	9.8×10^7	3.2×10^{12}	4.8×10^6	190-fold

EIAV, equine infectious anemia virus; PERT, performance-enhanced reverse transcriptase; RT, reverse transcriptase; TU, transducing units.
[a]PERT assay.

Evaluating IDLV-mediated expression in differentiated cells *in vitro*

The transduction and expression capabilities of the HIV-1 and EIAV IDLVs were compared with the parental vectors in differentiated nondividing skeletal muscle (C2C12) and neuronal (PC12) cells *in vitro*. Transduced differentiated cells were assayed for GFP expression by fluorescence-activated cell sorting analysis. The percentage of GFP-positive cells and the mean fluorescence intensity (MFI) increased and subsequently stabilized at around 2 weeks for all vectors (**Figures 3** and **4**). In the C2C12 differentiated cell line, the HIV-1 and EIAV IDLVs gave rise to a lower percentage of GFP-positive cells with a lower MFI compared with the parental-integrating vector (**Figure 3**). In particular, the triple mutant EIAV IDLVs gave rise to a lower percentage of GFP-positive cells with a lower MFI compared with the single mutant, whereas there was no significant difference between the HIV-1 single and triple IDLV mutants. Interestingly, in the differentiated PC12 cells the level of gene expression mediated by the HIV-1 IDLVs was comparable to the parental HIV-1 vector, although transduction efficiency was still reduced. In contrast, both transduction efficiency and expression level with both the single and triple EIAV IDLVs remained poor compared with the parental vector (**Figure 4**).

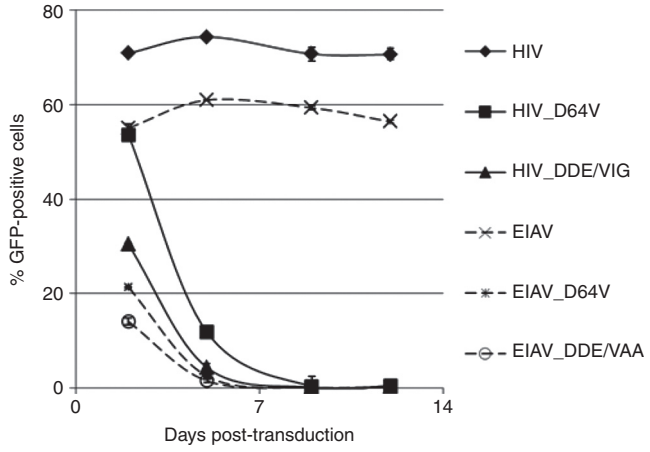

Figure 2 Percentage of GFP-expressing cells after transduction of proliferating human HEK293T cells. HEK293T cells were transduced with vector at a multiplicity of infection of 0.6. The number of GFP-expressing cells was measured by flow cytometry over 2 weeks. The EIAV vectors are shown by broken lines and the HIV-1 vectors by solid lines. Error bars are SD from infections. EIAV, equine infectious anemia virus; GFP, green fluorescent protein.

Effect of dedifferentiation on HIV-1 IDLV-mediated expression in PC12 cells

Since GFP expression in differentiated PC12 cells mediated by the single and triple mutant HIV-1 IDLVs was comparable to the parental HIV-1 vector we wanted to confirm that this was not due to residual integration activity. Therefore, differentiated PC12 cells transduced with either the HIV-1 single or triple mutant IDLVs or the parental HIV-1 vector were dedifferentiated at 2 weeks post-transduction (the time of maximal transduction and MFI). Removal of nerve growth factor (NGF)

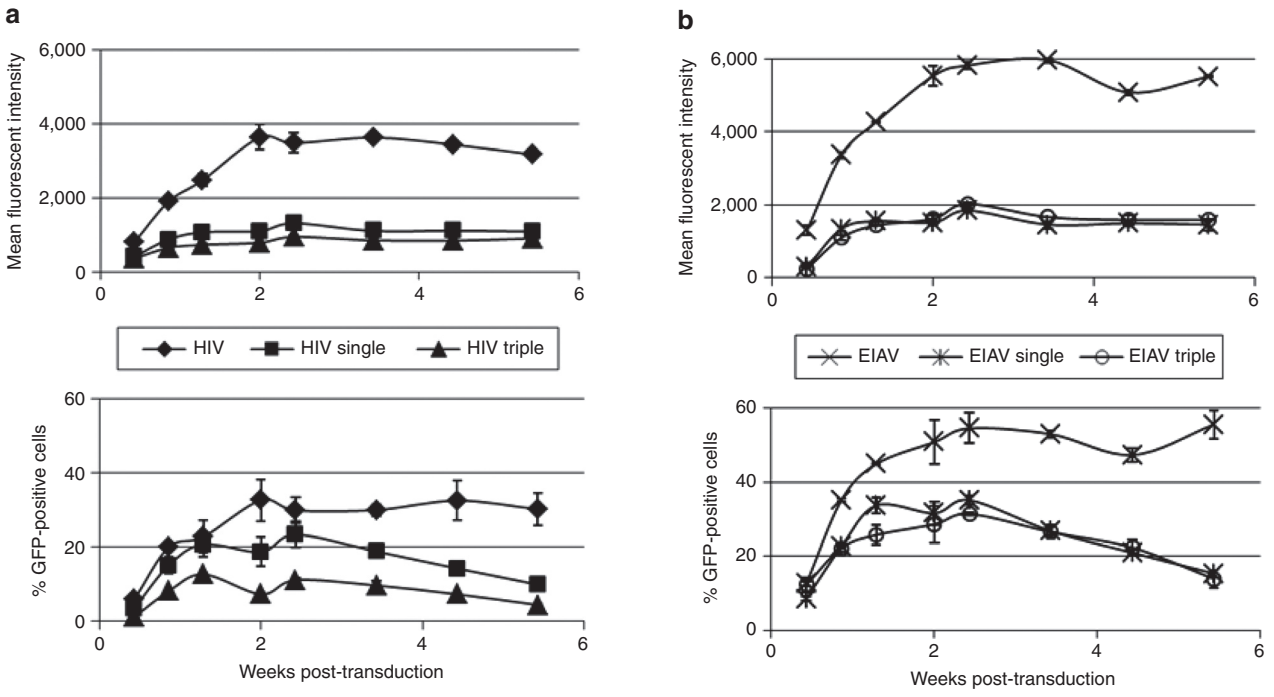

Figure 3 Mean fluorescent intensity (MFI) and percentage of GFP-positive cells in HIV-1 vector- and EIAV vector-transduced differentiated mouse C2C12 myoblast cells. (**a**) Cells transduced with HIV-1 vector; (**b**) cells transduced with EIAV vector. Cells were differentiated by culturing in 2% horse serum and transduced with vector at a multiplicity of infection of 30. The number of GFP-expressing cells was measured over time by flow cytometry. Graphs on the top show the mean GFP level in GFP-expressing cells, and graphs on the bottom show the percentage of GFP-positive cells. For clarity, results for HIV-1 and EIAV vector have been plotted separately. Error bars are SD from infections. EIAV, equine infectious anemia virus; GFP, green fluorescent protein.

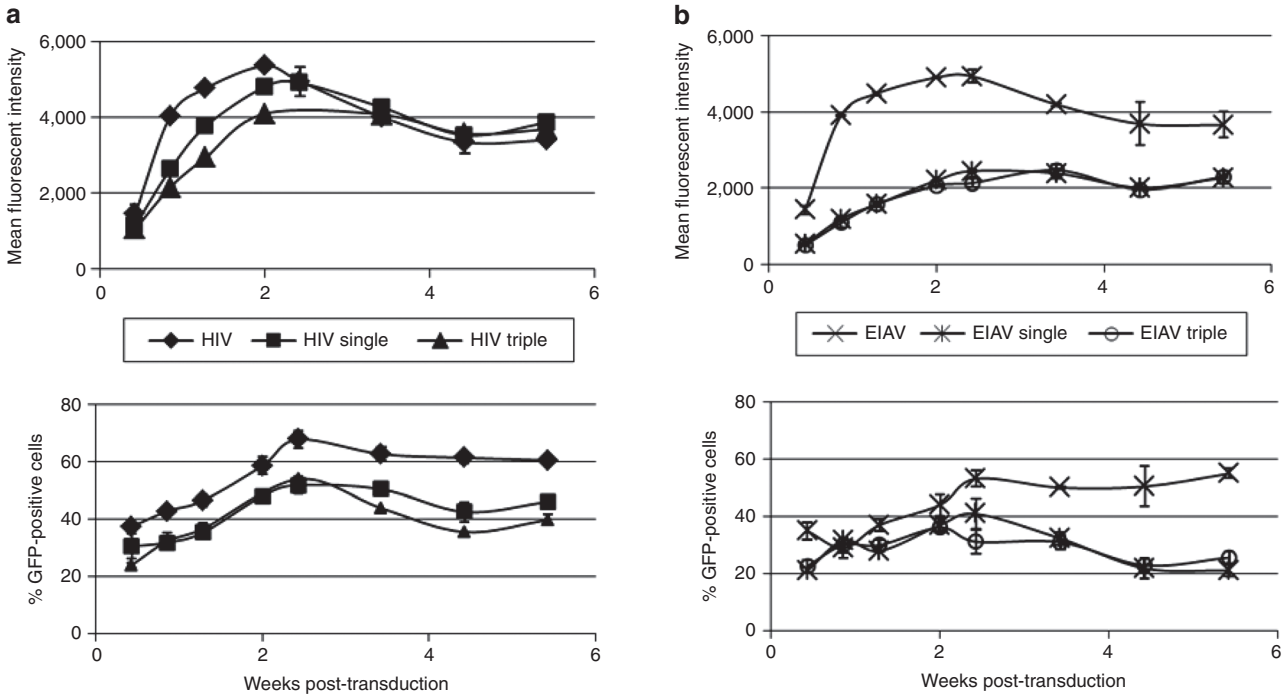

Figure 4 Mean fluorescent intensity (MFI) and percentage of GFP-positive cells in HIV-1 vector- and EIAV vector-transduced differentiated rat PC12 neuronal cells. (**a**) Cells transduced with HIV-1 vector; (**b**) Cells transduced with EIAV vector. Cells were differentiated in collagen IV-coated 24-well plates by culturing in 100 ng/ml Nerve growth factor for 7 days, then adding fluorodeoxyuridine to 20 µmol/l for 3 days to eliminate undifferentiated cells. These were transduced with vector at a multiplicity of infection of 30. The number of GFP-expressing cells was measured over time by flow cytometry. Graphs on the top show the mean GFP level in GFP-expressing cells, and graphs on the bottom show the percentage of GFP-positive cells. For clarity, results for HIV-1 and EIAV vector have been plotted separately. Error bars are SD from duplicate transductions. EIAV, equine infectious anemia virus; GFP, green fluorescent protein.

was followed by an increase in the proportion of cells that were GFP-positive (**Figure 5**); the exact cause of this is not known, but may be due to these cells becoming more metabolically active upon cell cycle re-entry. As the cells resumed division (*i.e.,* only after the cells were first passaged, not upon removal of NGF), there was a concurrent loss of GFP expression in cells transduced with both HIV-1 IDLVs, whereas the GFP expression in cells transduced with parental vector remained stable. The loss of GFP expression in dedifferentiated PC12 cells transduced with the HIV-1 IDLVs confirms that expression was exclusively due to unintegrated IDLVgenomes. We attempted to confirm this by integration-specific PCR to vector sequences but unfortunately this assay was not sufficiently sensitive or robust for this (data not shown).

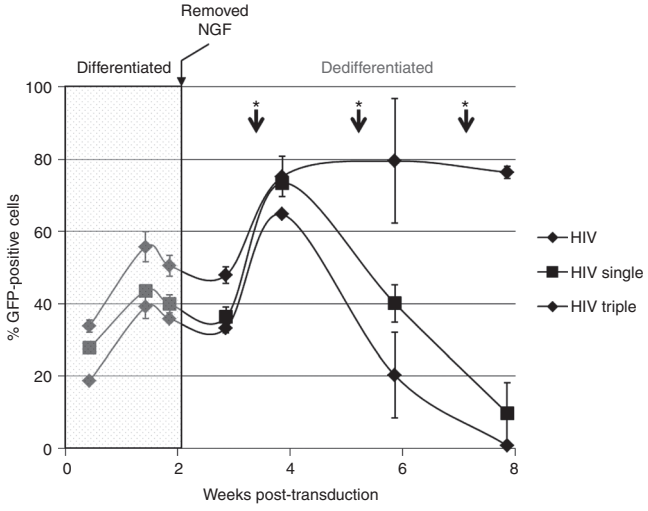

Figure 5 Percentage of GFP-expressing cells after transduction of differentiated (nondividing) rat PC12 neuronal cells that have been induced to dedifferentiate and resume cell division. Cells were differentiated in collagen IV-coated 24-well plates by culturing in 100 ng/ml nerve growth factor (NGF) for 7 days, then adding fluorodeoxyuridine to 20 µmol/l for 3 days to eliminate undifferentiated cells. The differentiated PC12 cells were transduced with the HIV-1 vectors at a multiplicity of infection of 25 and 2 weeks later, when GFP expression was maximal, NGF was removed to dedifferentiate. Following dedifferentiation, the cells were split regularly (*on figure) to restore cell cycling. Error bars are SD from duplicate transductions. GFP, green fluorescent protein.

Evaluating EIAV- and HIV-1 IDLV-mediated expression *in vivo*

Each of the EIAV and HIV-1 single and triple integrase mutants and parental vectors shown in **Table 1** were stereotactically injected at a volume of 5 µl into the striatum of the adult rat brain. One month later, the brains were removed for the evaluation of eGFP expression. Although the HIV-1 parental vector had approximately sevenfold higher DNA integration titer than the EIAV parental vector (**Table 1**), the parental EIAV vector had significantly greater mean striatal fluorescence (**Figure 6**). The expression of the GFP reporter gene was detected to some degree in both the EIAV- and HIV-1 IDLV-treated brains. However, the mean striatal fluorescence in the

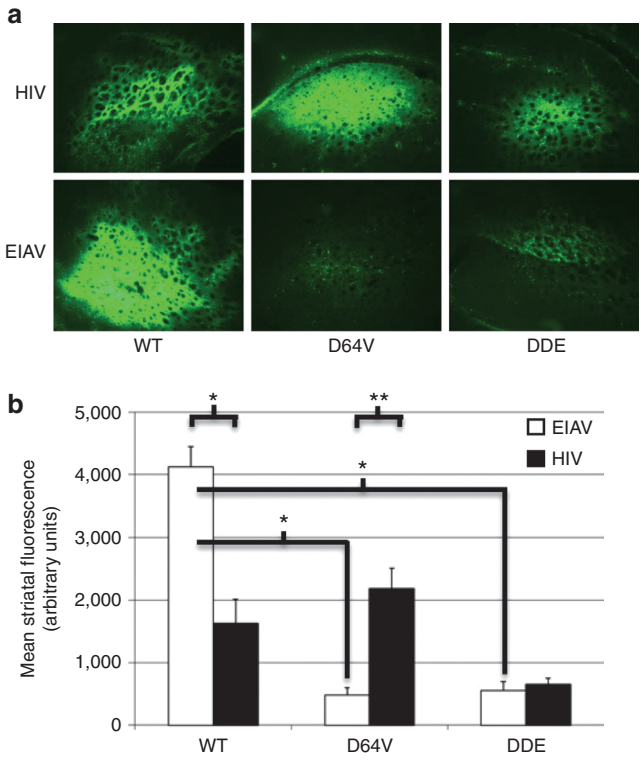

Figure 6 GFP expression in adult rat brains transduced with different HIV-1 and EIAV vectors expressing the eGFP reporter gene. Neat vector (5 µl) was injected into the striatum of adult Wistar rats ($n = 3$) and after 1 month the animals were killed, the brains fixed, removed, and sectioned. (**a**) Sections were photographed under a fluorescence microscope. (**b**) Graph to show the total GFP fluorescence in each rat brain striatum treated with the different HIV and EIAV vectors. Error bars are SD from three striata (except for EIAV WT, which is from two striata). Statistical analysis was performed using the Student's *t*-test (two-tailed, unpaired; $*P < 0.05$, $**P < 0.01$). D64V, single integrase mutant; DDE, triple integrase mutant; eGFP, enhanced green fluorescent protein; EIAV, equine infectious anemia virus; WT, wild-type.

single and triple mutant EIAV IDLV-treated brains was poor and statistically lower than the fluorescence in the parental EIAV vector-treated brain (**Figure 6**). In contrast, the HIV-1 single integrase mutant IDLV-treated brain showed a mean striatal fluorescence level that was comparable to the level observed with the parental HIV-1 vector. However, the triple integrase mutant HIV-1 IDLV showed a relatively poor mean striatal fluorescence level that had a similar intensity to both of the EIAV IDLVs (**Figure 6b**).

Discussion

There continues to be considerable interest in IDLVs which is unsurprising given their potential advantages for gene therapy for significantly reducing the risk of insertional mutagenesis and the potential to generate replication-competent lentivirus. Interestingly, early reports using IDLVs failed to show any significant associated expression suggesting that transgene expression from the episomal genome was extremely inefficient.[28–30] However, more recently, unequivocal gene expression from cells transduced with HIV-1 IDLVs has been demonstrated (reviewed in ref. 10,31). The reasons for the early failure of HIV-1 IDLVs to produce significant gene expression point to design differences in the first generation vectors, including lack of cPPT, self-inactivating LTRs or Woodchuck hepatitis virus post-transcriptional regulatory element and the more recent use of stronger constitutive promoters or non-HIV vectors (such as feline immunodeficiency virus (FIV) and EIAV), or combinations of these.[6,7,32]

In this study, we compared the gene transfer and expression characteristics of matched HIV-1 and EIAV IDLVs.[27] As expected, in dividing HEK293T cells transduced with the HIV-1 or EIAV IDLVs, the number of GFP-positive cells was rapidly reduced as the nonintegrated genome was lost by dilution as the cell divides, confirming that integrase activity was attenuated in the IDLV vectors.

Both HIV-1 and EIAV single and triple mutant IDLVs gave rise to a relatively lower percentage of GFP-positive cells (*i.e.,* transduction efficiency) with lower mean eGFP levels compared with the parental vectors in differentiated C2C12 cells. A previous study has shown that the HIV-1 single mutant (D64V) IDLV gave a better transduction efficiency in this cell type than the parental HIV-1 vector.[5] The discrepancy with our results may simply reflect the differences in the vector titration methods between these studies, however, as only the transduction efficiency (and not the mean fluorescence) was previously reported, this remains to be determined. Interestingly, both the single and triple mutant HIV-1 IDLVs-transduced differentiated PC12 neuronal cells to give an expression level that was comparable to the parental HIV-1 vector, albeit at a slightly lower transduction efficiency. Moreover, when these cells were dedifferentiated and cell division restored, GFP fluorescence was lost over time, confirming that expression was due to episomally maintained nonintegrated genome.[7] In the case of the single mutant HIV-1 IDLV, this result was replicated *in vivo* in neurons following a stereotactic injection into the rat striatum with strong eGFP expression that was equivalent to that seen in the rat brain treated with the parental HIV-1 vector, which has been observed previously for this[9,33] and other integrase mutations.[6] However, stereotactic injection of the triple mutant HIV-1 IDLV that contains a completely mutated integrase catalytic triad did not lead to significant eGFP expression in the rat brain. Therefore, although both the single and triple HIV-1 IDLVs expressed efficiently in nondividing neuronal PC12 cells *in vitro*, it appears that the additional mutations (DDE→VIG) in the catalytic triad prohibit effective eGFP gene expression in the rat brain, suggesting that results obtained with IDLVs *in vitro* should be treated with caution, as they may not be replicated *in vivo*.

In contrast to the HIV-1 IDLVs, both the single and the triple mutant EIAV IDLVs led to poor eGFP expression *in vitro* and *in vivo* in nondividing neuronal PC12 cells and in the rat brain. This is in agreement with previous studies using these EIAV integrase mutations[25,34] and other studies using nonhuman/primate lentivirus-based vectors[7,28] (reviewed in ref. 31), and is in stark contrast to the robust expression and therapeutic efficacy demonstrated with HIV-1 and simian immunodeficiency virus (SIV) IDLVs.[5,6,9,11–14,35] This raises the possibility that efficient expression from IDLVs may be restricted to human and primate lentivirus-based vectors, *in vivo* at least. Moreover, there seem to be differences in

the properties between different nonhuman/primate lentiviral IDLVs; some are ineffective both *in vitro* and *in vivo*, such as the EIAV IDLVs shown here, whereas others such as FIV IDLVs have been shown to function well *in vitro* but failed to show expression *in vivo* following subretinal injection in the neonate rat.[5,7] These observations may be highlighting differences in key components of these different lentiviral vectors that are involved in nonintegrative lentiviral expression, and comparison of the similarities/differences of these vector systems with HIV-1/SIV IDLVs may shed light on the mechanism behind this phenomenon. Only when this mechanism has been elucidated will it be possible to ascertain whether other gene therapy platforms can be modified to function in a nonintegrative form.

So what could be behind the mechanism involved in nonintegrative expression from HIV-1/SIV vectors that is not present with other lentiviral vectors? It could be that there are higher levels of episomal viral DNA formed with HIV-1/SIV vectors compared with other lentiviral vectors, and/or it could be due to some interaction between a cellular factor and the HIV-1/SIV viral genome/protein complex specifically. In support of this, one study has implicated the cPPT in the production of the HIV-1 circular vDNA,[36] and interestingly, there are differences between HIV-1/SIV and both EIAV and FIV PPT elements (the cPPT and PPT sequences are identical in HIV-1 and some SIV subtypes, but are different in EIAV and FIV).[37–39] However, when the cPPT was removed from the HIV-1 vectors in the current studies, there was no significant reduction in expression in the PC12 cells *in vitro* (data not shown), this has also been demonstrated *in vivo*.[9]

Although IDLV reduce the potential risk of insertional mutagenesis, the magnitude of this benefit is difficult to determine because it is difficult to accurately calculate the level of residual integration, with studies ranging from 1:100 to 1:10,000 compared with the parental vector.[40] The reason for this range is most likely due to the methods used to determine integration. PCR-based methods to quantitate the level of integrated virus tend to show the residual activity at the lower end of this scale (as our measurements of 200- to 400-fold also show) whereas measurements based on selection for a biological phenotype (such as an antibiotic marker or reporter gene) are at the upper end. This is perhaps unsurprising, since assays based on selection will be subject to a basal expression level (below which selection/detection will fail), so quantitation by this method will be an underestimate of the true integration rate. This is true in the current study if we used the FACS data instead (**Figure 2**) to calculate titer based on GFP expression. The titer of the IDLVs then falls from 200- to 400-fold less than the parental-integrating vector based on integration titer to 500- to 1,000-fold less based on biological titer, presumably due to very low expressing integrants being below the level of detection.

These data add to the growing body of literature that shows HIV-1 IDLV is a viable alternative to the integrating vector in certain nondividing cells, at least in the short term. Consistent with other studies using HIV-1 vectors with similar components, we have shown that the HIV-1 D64V mutant IDLV produces comparable transgene levels to the parental vector *in vitro* and *in vivo*. The only apparent "penalty" for the loss of integrase activity seems to be a modest reduction in transduction efficiency, which might be overcome practically by simply increasing dose. However, the triple integrase mutant HIV-1 IDLV is not effective *in vivo* which is surprising and, to our knowledge, this has been shown for the first time. Conversely, although the parental EIAV vector is more effective than the HIV-1 vector at transducing the rat striatum *in vivo*, the EIAV IDLVs, like FIV, are relatively ineffective. In fact, only human and primate lentiviral vector platforms have been shown so far to be amenable to these mutations *in vivo* to date, and this raises the possibility that this phenomenon may be restricted to these groups of lentiviruses. Furthermore, of the *in vivo* studies using IDLVs that have shown good expression or efficacy, only one used a non-HIV-1 IDLV vector. Michelini *et al.* used SIV IDLVs to show comparable immunization to the parental vector;[35] however, as immune response only (and not transgene level) was measured, this study did not show that SIV IDLV produced equivalent levels of transgene expression. It is therefore possible, that efficient IDLV expression is a property of HIV-1 vectors exclusively, although this remains to be determined.

In summary, we have shown that effective expression from IDLVs is dependent on the type of tissue targeted, the integrase mutation(s), and the nature of lentiviral vector platform. Further work into the precise mechanisms involved will be important to determine whether the properties of the HIV-1 IDLV could be expanded to other gene therapy vector platforms in the future.

Materials And Methods

Plasmids. The HIV-1 and EIAV vector genome plasmids, pHF2g and pONY8.5NSG, both contain an internal spleen focus-forming virus LTR promoter driving *eGFP* expression, a Woodchuck hepatitis virus post-transcriptional regulatory element, a cPPT, and self-inactivating LTR. Both plasmids have been described previously.[27]

Mutations in the EIAV and HIV integrase gene to generate the D64V single residue change and the DDE→VAA and DDE→VIG triple residue changes were introduced into the pESYNGP and pSYNGPgag-pol plasmids, respectively by site-directed mutagenesis. Full cloning details can be made available on request.

Cell lines. The human embryonic kidney cell line HEK293T (ATCC, Manassas, VA) and the mouse muscle myoblast cell line C2C12 (ECACC, Porton Down, UK) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mmol/l glutamine and penicillin/streptomycin. The rat adrenal pheochromocytoma cell line PC12 (ECACC) was cultured in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin, and the cells were adhered to collagen IV-coated plasticware before transduction.

We decided to arrest cell growth by differentiation rather than by chemical induction as this more closely mirrors nondividing cells *in vivo*. C2C12 cells were irreversibly differentiated by culturing in low serum medium containing 2% horse serum for 7 days, and PC12 cells were reversibly differentiated by supplementing media with 100 ng/ml NGF for 7 days (which induces a neuronal phonotype), then

adding fluorodeoxyuridine to 20 µmol/l for a further 3 days to eliminate undifferentiated cells. Differentiation was reversed by removing NGF for 1 week before splitting to induce cell cycling.

HEK293T, C2C12, and PC12 were seeded at 9, 2, and 3 × 10^4 cells/well respectively in 12-well plates. Cells were transduced in duplicate in the presence of 8 µg/ml polybrene for 6 hours at multiplicity of infections of 0.6 (for dividing HEK293T) and 30 (for C2C12 and PC12 cells). For the IDLVs, vector volumes for transduction were calculated by using the parental integration titer normalized to the RNA copy number of the respective IDLV.

Vector production. HIV-1 and EIAV vectors were produced by transient transfection of vector genome plasmid, gag-pol packaging plasmid, and envelope plasmid (and for HIV-1 vector only, pREV plasmid) into HEK293T using Fugene-6 (Roche Diagnostics, Burgess Hill, UK) in 10-layer cell factories (Nunc, Rochester, NY) as described previously (Wong *et al.*, 2005). Virus was pseudotyped with the G glycoprotein of vesicular stomatitis virus envelope and concentrated by double centrifugation (6,000*g* for 18–24 hours, then 20,000*g* for 1.5 hours).

Vector characterization. All of the vectors were characterized with respect to integration (strength titer) in HEK293T cells, RNA copy number, and RT activity.

To determine the integration titer in HEK293T cells, DNA was extracted from the transduced cells after >12 population doublings to ensure dilution of unintegrated copies to undetectable levels. Integrated vector genomes were quantified by quantitative PCR using a GFP-specific primers and probe set.[24]

To determine the RNA copy number, RNA was extracted from neat virus using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA). RNA copy number was quantified by quantitative RT-PCR using the same GFP-specific primers and probe set as above.

The RT activity in each vector was determined using the performance-enhanced RT assay. RT was released from vector particles with a mild detergent, and used to synthesize cDNA using MS2 bacteriophage RNA as a template. This cDNA product was then quantified by quantitative PCR using an MS2-specific primers and probe set. The product was normalized to an internal control vector sample of known integration titer to generate a "titer" based on RT activity.

FACS analysis. Duplicate wells were trypsinized and cells measured for GFP expression using flow cytometry at regular timepoints. Dead cell exclusion was performed using TO-PRO-3 and data was acquired on a FACScalibur (Becton–Dickinson, Oxford, UK). FACS analysis was performed using WinList software (Verity Software House, Topsham, ME).

Animal work. The adult Wistar rats were handled according to procedures approved by the UK Home Office and the University of Bristol Research Ethics Committee. A total volume of 5 µl of each vector was injected into the striata of the adult rat brain using a Hamilton syringe fitted with a 33 gauge needle at 0.2 µl/minute. Coordinates used were 0 mm anterior-posterior, –3.5 mm lateral, and –4.75 mm dorsoventral. After 1 month, all rats were killed and perfused with 400 ml 4% paraformaldehyde in 0.1 mol/l phosphate-buffered saline before removing the brains into 4% paraformaldehyde for 16–24 hours and cryoprotecting in 30% sucrose/phosphate-buffered saline. The brains were mounted in OCT (Sakura, Alphen aan den Rijn, Netherlands) and into 40 µmol/l sections using a Leica 3050 cryostat (Leica Microsystems, Milton Keynes, UK). Every fifth 40 µmol/l section was assessed for GFP fluorescence, and all positive sections were photographed using identical image-capturing parameters. Total GFP fluorescence in each section was then measured using Adobe Photoshop CS3 following a published methodology,[41] from the section in which it was first detected to the section when the signal was lost; these measurements were plotted against section number, and the area-under-the-curve (subtracting background fluorescence) was then calculated in order to quantify total fluorescence in each striatum.

Statistical analysis. Statistical differences between mean total fluorescence measurement for each vector group was determined using the Student's *t*-test (unpaired, two-tailed).

Acknowledgments. S.E. is grateful to James Miskin and Pippa Radcliffe for the kind gift of the EIAV and HIV triple mutant integrase constructs, respectively. This work was funded by Oxford BioMedica (UK) Ltd. This work was done in Oxford, Oxfordshire, and Bristol, Avonshire (UK). The authors declared no conflict of interest.

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