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ARTICLE

Comparison of HIV- and EIAV-Based Vectors on Their Efficiency in Transducing Murine and Human Hematopoietic Repopulating Cells

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The use of lentiviral vectors for gene transfer into hematopoietic stem cells has raised considerable interest as these vectors can permanently integrate their genome into quiescent cells. Vectors based on alternative lentiviruses would theoretically be safer than HIV-1-based vectors and could also be used in HIV-positive patients, minimizing the risk of generating replication-competent virus. Here we report the use of third-generation equine infectious anemia virus (EIAV)- and HIV-1-based vectors with minimal viral sequences and absence of accessory proteins. We have compared their efficiency in transducing mouse and human hematopoietic stem cells both *in vitro* and *in vivo* to that of a previously documented second-generation HIV-1 vector. The third-generation EIAV- and HIV-based vectors gave comparable levels of transduction and transgene expression in both mouse and human NOD/SCID repopulating cells but were less efficient than the second-generation HIV-1 vector in human HSCs. For the EIAV vector this is possibly a reflection of the lower protein expression levels achieved in human cells, as vector copy number analysis revealed that this vector exhibited a trend to integrate equally efficiently compared to the third-generation HIV-1 vector in both mouse and human HSCs. Interestingly, the presence or absence of Tat in viral preparations did not influence the transduction efficiency of HIV-1 vectors in human HSCs.

Key Words: HSC, EIAV, HIV, gene transfer, HIV-1 Tat

INTRODUCTION

Hematopoietic stem cells (HSCs) are a prime gene therapy target for correction of various hematopoietic and other genetic disorders. Recent reports on the *trans*-differentiation potential of these cells further enhance their significance as gene therapy candidates as they may contribute to the regeneration of other nonhematopoietic tissues [1–7]. Numerous gene transfer studies have utilized oncoretroviral vectors to transduce HSCs but as these vectors require cell division for nuclear entry and integration, and HSCs are largely quiescent, their overall efficiency has been limited. In addition, recent reports from the French X-SCID clinical trial have demonstrated the potential of insertional mutagenesis using oncoretroviral vectors [8]. Lentiviral vectors on the other hand, can transduce nondividing cells [9–12] and although their integration potential is similar to that of retroviral vectors [13], no mutagenic effect has yet been reported [14].

Based on their similarity, lentiviruses have been divided into primate (HIV-1 and SIV) and nonprimate (EIAV, FIV). The majority of lentiviral vector development has focused on HIV-1-based vectors, as HIV-1 is the best characterized lentivirus. Although the majority of vectors are devoid of most of the HIV accessory genes and have further been simplified by removal of all regulatory elements except for Rev, concerns regarding their biosafety for use in human gene therapy, especially in HIV-positive patients, have prompted research into the development of alternative vectors.

Equine infectious anemia virus (EIAV) is a nonprimate lentivirus with a relatively simple genome organization,

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containing only three accessory genes, Rev, Tat, and S2. The ability of EIAV vectors to transduce cells from different species was demonstrated by Ikeda and colleagues when they successfully transduced a number of cell lines *in vitro* [15]. EIAV vectors are also capable of transducing neurons and glial cells after *in vivo* administration [16,17]. The first report on hematopoietic cell transduction using an EIAV vector was by Yamada *et al.,* who demonstrated functional correction of Fanconi anemia patient cell lines [18]. Improved EIAV vectors containing the woodchuck posttranscriptional regulatory element (WPRE) exhibited significant enhancement of gene expression in mouse muscle cells [19], while a similar vector carrying the central polypurine tract (cPPT) was capable of correcting an *in vivo* rat model of hypothalamic diabetes insipidus [20].

In this study we have compared the efficiency of EIAV and HIV vectors to transduce primary human and mouse HSCs. We designed the vectors employed for clinical application by optimizing and reducing the viral sequences present, while we studied the efficiency of transduction of HSCs both *in vitro* and *in vivo*. We demonstrate for the first time the ability of an EIAV-based vector to transduce mouse and human SCID repopulating cells efficiently.

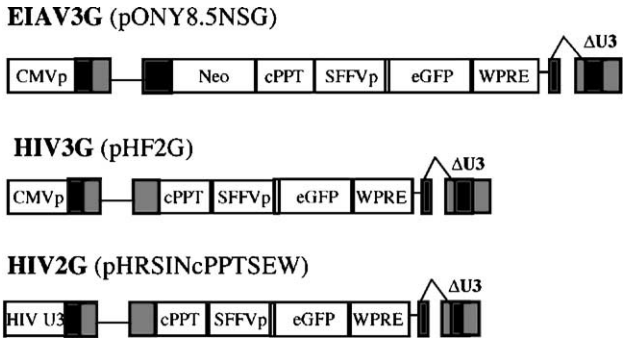

FIG. 1. Schematic overview of the vectors used in this study. pONY8.5NSG and pHF2G are third-generation lentiviral vectors that were made using a codon-optimized Gag/Pol construct in the absence of any accessory proteins except for Rev for the HIV-1 vector pHF2G. For clarification purposes these vectors were renamed EIAV3G and HIV3G, respectively, with ''3G'' denoting third-generation origin. The suffix ''2G'' was used for pHR'SINcPPTSEW, which is a second-generation HIV-1-based vector and was renamed HIV2G for simplicity. All vectors were self-inactivating and contained the eGFP reporter gene under the control of the spleen focus-forming virus (SFFV) LTR. The woodchuck posttranscriptional regulatory element (WPRE) and central polypurine tract (cPPT) sequence were also included. To transcribe RNA efficiently in the human producer cells, the 5′LTR in the EIAV3G and HIV3G vectors was replaced by a CMV promoter fused to the R-U5 region. Both HIV3G and HIV2G vectors had a Rev-response element upstream of the cPPT sequence.

RESULTS

Description of the EIAV- and HIV-BASED Vectors Used in This Study

We constructed the third-generation minimal HIV and EIAV vector genomes by replacing the 5′LTR with the CMV IE enhancer/promoter fused to initiate transcription at the start of the R region. We produced the EIAV vector used (EIAV3G) without all EIAV accessory genes, while the HIV-1 vector (HIV3G) required HIV Rev only in the production process. We made both these vectors using a codon-optimized Gag/Pol construct. As a comparison we used HIV2G, a second-generation HIV-1 vector that has been shown to transduce human HSCs efficiently [21]. Transcription of viral RNA for this vector in the producer cells is Tat-dependent and driven by the HIV-1 wild-type 5′LTR. We generated HIV2G virus using the pΔ8.91 packaging construct, which contains wild-type Gag/Pol and only Rev and Tat from the accessory genes [22]. All vectors had a deletion in the 3′ U3 region, rendering them self-inactivating, and were pseudotyped with the VSV-G envelope (Fig. 1). The multiplicity of infection in all our experiments was based on biological titer on HeLa cells following FACS analysis. The biological titer for each virus was also associated with the viral RNA copy number using quantitative real-time RT-PCR (Table 1). Deviations between vectors probably reflect the variability and error in efficiency within each assay. Nonetheless, the ratio of the average viral RNA copies per human transduction unit (TU) was relatively consistent among the three vectors (Table 1).

In Vitro Transduction of Human and Mouse Progenitors by an EIAV Vector

We assessed the efficiency of a minimal HIV-1- or EIAV-based vector system on human HSCs on purified $CD34^+$ cells from umbilical cord blood. We performed *ex vivo* transduction as previously described [21] by placing cord blood $CD34^+$ cells in serum-free medium supplemented with SCF, Flt3-ligand, IL-3, and IL-6 and adding viral supernatant at an m.o.i. of 35. We estimated gene transfer efficiency on human short-term hematopoietic progenitors by methylcellulose assays. We plated $CD34^+$ cells onto methylcellulose cultures immediately after the short transduction protocol and counted eGFP-positive colony-forming units (CFUs) and burst-forming units erythroid (BFU-E) by fluorescence microscopy 10–14 days later. The HIV2G vector transduced short-term hematopoietic progenitors efficiently, with 37.6% of total colonies expressing eGFP (Fig. 2A). However, HIV3G and EIAV3G vectors were significantly less efficient ($P < 0.01$), giving rise to 12.1 and 3.2% of eGFP-positive colonies, respectively. Myeloid and erythroid colonies were transduced at comparable levels with all vectors (data not shown), but the intensity of fluorescence observed in EIAV3G-transduced colonies was lower compared to that seen in HIV2G colonies or HIV3G-transduced colonies (Fig. 2B). This lower detectable gene expression may account for some of the observed differences in gene transfer efficiency among the vectors. Standard PCR for eGFP showed that >93% of $eGFP^+$ colonies were positive for the vector and little transgene silencing was observed with all vectors (>8% of

TABLE 1: Viral vector titers based on eGFP expression, RNA, or eGFP copy number

	FACS titer (TU/ml)	RNA copy number/ml	Ratio of average RNA copies per human TU[a]	eGFP copy number titer (vector copies/ml)
EIAV3G	$7.7–12 \times 10^8$	$2.7–2.9 \times 10^{12}$	2842	2.8×10^8
HIV3G	$3–8.3 \times 10^8$	$0.3–0.7 \times 10^{12}$	904	2×10^8
HIV2G	$1.5–3.5 \times 10^8$	$0.7–1.2 \times 10^{12}$	3840	5.8×10^8

[a] Mean RNA copy number divided by mean FACS titer.

eGFP$^-$ colonies were found positive for the vectors) (data not shown).

Next we tested these three vectors on lineage-depleted mouse bone marrow by overnight transduction in the presence of different cytokine combinations. We initially optimized the m.o.i. and for all reported experiments maintained it at 30 to keep the number of proviral copies per cell relatively low. Immediately after the overnight transduction period, we plated cells in methylcellulose cultures and examined them after 10 days for eGFP-positive colonies. Overall, both minimal vector systems (EIAV3G and HIV3G) proved efficient in transducing murine hematopoietic progenitors. EIAV3G gave rise to 38.9% eGFP-positive colonies, while gene transfer efficiency with the HIV3G vector was slightly lower, reaching an average of 24.2% (Fig. 2C). The HIV2G vector exhibited the most effective gene transfer, with 58.2% of murine methylcellulose colonies expressing eGFP, attaining significance at $P > 0.01$ over the HIV3G vector. Myeloid and erythroid colonies were transduced at comparable levels with all vectors and there were no obvious differences in fluorescence intensity of the colonies among different vectors (data not shown). Furthermore, eGFP expression by FACS of both human and mouse hematopoietic cells after 2 weeks of *in vitro* culture was in good agreement with the CFU data (data not shown).

Efficient Gene Transfer into NOD/SCID Repopulating Cells (SRCs) with an EIAV-Based Vector

We analyzed the bone marrow of NOD/SCID recipients 8 weeks posttransplantation of human cord blood CD34$^+$ cells for engraftment of human cells and multilineage hematopoiesis. Human cell engraftment in all transplanted recipients reached similar levels regardless of the vector used (data not shown). We observed that 21.9% of human CD45$^+$ cells expressed eGFP when transduced with the HIV3G vector, while 11.5% were eGFP positive when EIAV3G vector was used (Fig. 3A). The overall efficiency of gene transfer into CD45$^+$ cells using HIV2G proved significantly higher than that of the other two vectors ($P > 0.01$), with 50.6% of human cells expressing the reporter gene. All vectors exhibited similar efficiency in both myeloid (CD33$^+$) and lymphoid (CD19$^+$) lineages, indicating that multipotent progenitors/stem cells had been transduced (Table 2). In addition, we detected human CD34$^+$ cells expressing eGFP at comparable frequencies among vectors, indicating that transduced progenitors were maintained in the bone marrow of engrafted NOD/SCID mice.

Next, to compare the levels of reporter gene expression achieved with each vector, we compared the mean fluorescence intensity (MFI) of eGFP in the human CD45$^+$ population of the bone marrow of transplanted animals. We calculated the eGFP MFI values as the geometric mean of the eGFP-positive population and normalized them to those of the eGFP-negative population in the same CD45$^+$ gate. Despite variation in the eGFP expression among individual animals and different experiments, we found a significant difference in the eGFP MFI in the bone marrow of animals engrafted with cells transduced with the HIV2G vector (590) compared to the EIAV3G vector (154) ($P > 0.01$) (Fig. 3B). Although eGFP expression with HIV3G was lower (239) than with HIV2G, no statistically significant difference was attained at $P = 0.05$. The lower eGFP MFI suggested that maybe fewer copies of HIV3G or EIAV3G vectors were present in these cells, thus giving rise to lower protein expression levels (see below and Fig. 3C). Alternatively, differences in the integration sites or the expression cassette may explain the lower expression observed with EIAV3G.

An EIAV-Based Vector Is Capable of Transducing Mouse Repopulating Cells

We assessed gene transfer efficiency of each vector into mouse repopulating stem cells by transplantation of 1×10^4 Lin$^-$ cells into lethally irradiated recipients. After 6 weeks, we analyzed the bone marrow of recipient animals by FACS to determine the levels of eGFP expression in the CD45$^+$ population. Overall, the percentage of eGFP-positive cells was variable among individual mice, probably due to random expansion of stem cell clones in the chimeric animals. We tested different combinations of cytokines but no significant differences in transduction efficiency were observed (data not shown). On the whole, HIV2G and HIV3G vectors showed similar efficiency in transducing mouse repopulating cells, with mean values of 20.6 and 19.7%, respectively (Fig. 4A). Animals engrafted with EIAV3G-transduced cells had an average 8% eGFP$^+$ hematopoietic cells. This difference was not statistically significant relative to the other vectors.

The SFFV 3′LTR has been shown to drive expression in most hematopoietic lineages after transplantation of human CD34$^+$ cells in NOD/SCID recipients [21]. To test

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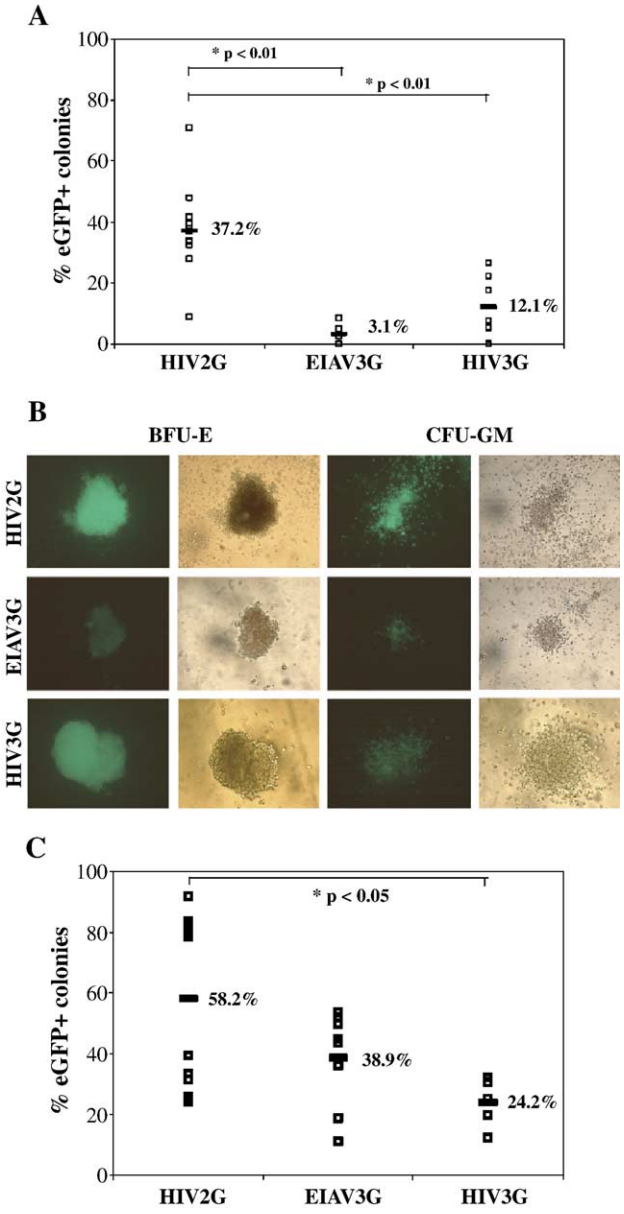


FIG. 2. *In vitro* transduction of mouse and human progenitor cells. (A) Methylcellulose cultures that support the growth of human hematopoietic progenitors were seeded with 1×10^3 CB $CD34^+$ cells/ml after overnight transduction. CFU and BFU-E were counted after 14 days and the percentage of eGFP-expressing colonies from three separate experiments is presented. (B) Fluorescent and phase-contrast microscopy photos of human BFU-E and CFU-GM colonies taken 10 days after transduction with the different vectors (original magnification ×400). (C) Mouse Lin^- cells were plated onto methylcellulose cultures after overnight transduction with EIAV3G, HIV3G, or HIV2G vectors in the presence of different cytokine combinations. The presence of eGFP-positive colonies was determined by fluorescence microscopy 10 days later and expressed as the percentage of total colonies present on each plate. Duplicate plates were seeded per transduction experiment and data plotted are from three separate experiments.

its efficiency in the murine system and also examine the potential of each virus to transduce multipotent progenitors equally, we analyzed the degree of transduction in bone marrow leukocyte populations expressing B220, CD4 and CD8, or Gr-1 markers. Gene transfer into lymphoid and monocytic/myeloid progenitors was achieved with comparable efficiency with either HIV-1- or EIAV-based vectors. Sixteen to twenty-four percent of either $B220^+$ or $Gr\text{-}1^+$ cells expressed eGFP when HIV2G or HIV3G was used to transduce the initial Lin^- cell transplant (Table 2). Transduction efficiency of the same subpopulations was lower with EIAV3G—approximately 8% of $B220^+$ and $Gr\text{-}1^+$ populations expressed eGFP—but nevertheless the gene transfer efficiencies in these two leukocyte subsets were similar. Albeit slightly lower, the percentage of eGFP-expressing cells within the T cell population ($CD4^+$ and $CD8^+$) of engrafted bone marrow was not significantly different from that seen in $B220^+$ or $Gr\text{-}1^+$ cells with the same virus, indicating that multipotent hematopoietic progenitors/stem cells had been transduced.

We observed great variability in the eGFP expression levels among different animals as calculated by the MFI, which did not correlate with the eGFP levels in the bone marrow. There was a significant ($P > 0.05$) fourfold difference in the eGFP MFI between HIV2G and EIAV3G vectors. However, the two third-generation vectors exhibited comparable ($P > 0.05$) eGFP expression levels (117 versus 128) (Fig. 4B), indicating that transgene expression by an EIAV-based vector system in mouse repopulating cells is similar to that by HIV-1.

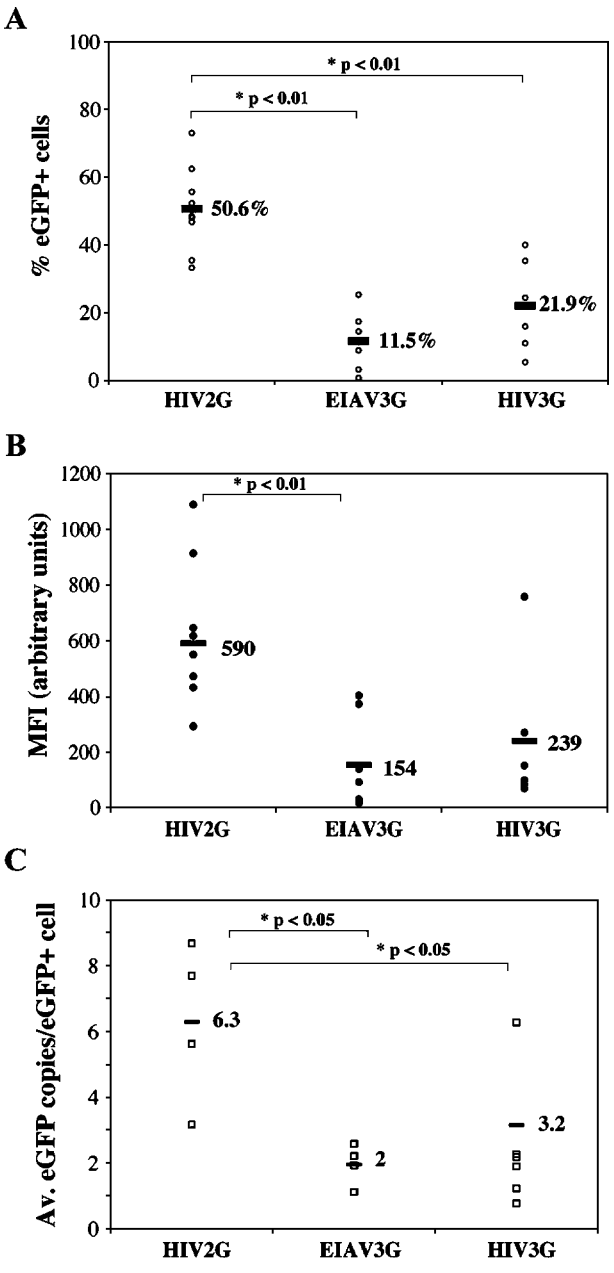
Integration Analysis of HIV- and EIAV-Based Vectors in Mouse and Human HSCS

We wanted to investigate the possible cause for the observed difference in eGFP MFI in human SRCs between HIV2G and EIAV3G vectors. Previous reports had attributed the low expression levels by EIAV vectors to reduced RNA transcript half-life due to promoter interference [23]. Nonetheless our vectors had minimal wild-type viral sequences since they were self-inactivating (SIN) by deletion of the U3 region of the 3′LTR. Although we did not rule out the possibility that other vector sequences may be interfering with transcription from EIAV3G vector, we analyzed the provirus copy number per eGFP-expressing cell by real-time PCR. We prepared DNA from total bone marrow of engrafted animals and analyzed it for eGFP copy number standardized to copies of RNase P for human cells or rodent GAPDH for murine cells. All values were corrected for the percentage of eGFP-expressing cells in the bone marrow. This correction was necessary for mouse HSCs to determine the proportion of donor-derived bone marrow cells, but in the case of human HSCs it may represent an overestimation of copy number especially for EIAV3G, which generated lower transgene expression. Human methy-

lcellulose colonies from the bone marrow of each animal were also analyzed for eGFP copy number and were generally in good agreement with the values obtained for the total bone marrow (data not shown).

For the HIV2G vector, we observed great variability in the average vector copy number per human cell expressing eGFP regardless of the fact that the same viral preparation was used across experiments (Fig. 3C). The two minimal vectors (EIAV3G and HIV3G) resulted in significantly fewer average vector integrations per cell (2 for EIAV3G and 3.2 for HIV3G) ($P > 0.05$) and exhibited a tighter clustering around the mean compared to HIV2G. Although the ratio of RNA copies per TU for EIAV3G was similar to that for HIV2G (Table 1), the former resulted in lower average copy number per human cell, indicating that a smaller proportion of the EIAV vector genomes had integrated into the human genome. This finding confirms published reports on EIAV restriction in human cells and suggests that it influences subsequent vector integration.

In the case of mouse repopulating stem cells, we observed no significant differences in copy number among vectors. HIV2G exhibited a variable integration pattern similar that that in human SRCs, with an average of 5.4 integrations per cell (Fig. 4C). EIAV3G, on the other hand, showed increased variability compared to its integration in human cells and reached an average of 4 copies per mouse genome. Although a small number of animals was used for the comparison, EIAV3G integration in mouse cells appeared to be higher than in human cells.



HIV-Tat Does Not Influence Transduction Efficiency of HSCs by Lentivirus Vectors

Tat has been implicated in HIV-1 reverse transcription [24,25] as well as in KSHV infectivity [26] and may therefore play a crucial part in the transduction efficiency of lentivirus vectors. To investigate the reason for the observed difference in efficiency between the two HIV-1 vectors and also examine the role of Tat in the transduction efficiency of human HSCs by lentivirus vectors, we produced a second-generation lentivirus vector using the genome of HIV3G (pHF2G) and the packaging constructs used for producing second-generation virus HIV2G. This second-generation lentivirus was named HIV*2G and contained Tat and Rev of the HIV-1 accessory proteins. We then transduced human cord blood $CD34^+$ cells *in vitro* and set up methylcellulose cultures. Interestingly, analysis of the colonies 2 weeks

FIG. 3. *In vivo* transduction efficiency of human SCID-repopulating cells. (A) Analysis of SCID-repopulating cell transduction efficiency was performed after transplantation of 1–2 × 10^5 CB $CD34^+$ cells immediately after overnight transduction into sublethally irradiated NOD/SCID recipients. Eight weeks after transplantation, the bone marrow of chimeric mice was analyzed for the presence of eGFP-expressing human $CD45^+$ cells. The scatter plot represents data from four separate experiments ($n = 9$ for HIV2G; $n = 7$ for EIAV3G; $n = 6$ for HIV3G) and the mean percentage of $eGFP^+$ cells for each vector is shown. (B) The mean fluorescence intensity of eGFP in the bone marrow of engrafted NOD/SCID animals is shown after normalization to the $CD45^+$ $eGFP^-$ population. The scatter plot shows arbitrary units of fluorescence intensity from individual animals. (C) Vector copy number in human SCID-repopulating cells. The average vector copy number per human cell expressing eGFP in the bone marrow of NOD/SCID animals was estimated by real-time TaqMan PCR for eGFP normalized to human genome copies of RNase P (Applied Biosystems). DNA from total bone marrow was analyzed on three separate occasions and the mean value for each individual animal is presented (HIV2G, 2842 RNA copies/TU; EIAV3G, 3840 RNA copies/TU; HIV3G, 905 RNA copies/TU).

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TABLE 2: eGFP expression in various mouse and human hematopoietic cell ($CD45^+$) subpopulations in the bone marrow of C57BL/6J and NOD/SCID recipients, respectively

	Mouse ($CD45^+$ $eGFP^+$)			Human ($CD45^+$ $eGFP^+$)		
	% B220	% CD4/CD8	% Gr–1	% CD19	% CD33	% CD34
EIAV3G	8.3 ± 10.5	1.9 ± 2.2	8.5 ± 14.1	9.5 ± 6	15.7 ± 16.1	17.7 ± 17.9
HIV3G	24.5 ± 31	3.7 ± 3.3	16.3 ± 19.5	20.1 ± 15.2	12.1 ± 9.2	18.7 ± 10.6
HIV2G	20.6 ± 23.3	5.3 ± 4.2	21.6 ± 25.3	43.3 ± 17.3	41.3 ± 18.7	31 ± 14.1

posttransduction did not reveal any significant differences in the transduction efficiency between HIV*2G and HIV3G vectors (data not shown). This suggested that Tat was not responsible for the lower percentage of $eGFP^+$ colonies seen with HIV3G compared to HIV2G vector and that this must be due to inherent vector differences.

DISCUSSION

In this study we have compared EIAV- and HIV-1-based vectors for their efficiency to transduce murine and human hematopoietic progenitors as well as mouse and human SRCs. Third-generation minimal lentiviral vector systems (HIV3G, EIAV3G) lacking all accessory proteins apart from Rev for the former were compared to a well-documented pHR′-based second-generation HIV-1 vector (HIV2G) [21]. We observed no significant difference in the transduction efficiency of mouse hematopoietic progenitors among EIAV- and HIV-1-based vectors, while HIV2G resulted in a significantly higher number of eGFP-positive methylcellulose colonies compared to either minimal vector system. A similar pattern was observed for human SRCs with HIV2G vector giving higher transduction compared to either HIV3G or EIAV3G. Again the latter two vectors exhibited similar proportions of transduced human cells in the bone marrow of engrafted NOD/SCID animals and also had comparable protein expression levels. This difference was less evident in mouse repopulating cells in which we observed no significant variation among the different vectors in the percentage of eGFP expression in bone marrow cells 6 weeks after transplantation. Overall, no differences were observed between the two minimal vector systems in either mouse or human HSCs.

Surprisingly, SRCs were more efficiently transduced compared to short-term hematopoietic progenitors assessed by methylcellulose cultures. This phenomenon was observed with all vectors tested and could be due to an underestimation of the *in vitro* gene transfer efficiency due to low undetectable eGFP levels in some colonies by fluorescence microscopy. This may well have been the case with EIAV3G vector for which the intensity of eGFP fluorescence was significantly reduced compared to the HIV-1-based vectors tested. Alternatively, low level eGFP expression in hematopoietic progenitors or increased numbers of transduced progenitors in the bone marrow compared to the proportion of transduced hematopoietic cells of all types may account for this observation [27]. We observed the reverse phenomenon with mouse HSCs in which higher gene transfer efficiency was achieved *in vitro* rather than *in vivo*. Mouse colonies expressing eGFP had similar fluorescence intensity with all vectors so detection errors were relatively minimized. The reduced *in vivo* eGFP expression is likely due to arbitrary engraftment and amplification of nontransduced stem cells rather than lower gene transfer efficiency [28]. However, we cannot rule out the possibility that transduced clones have reduced viability compared to untransduced clones or that low-eGFP-expressing cells are cleared by host immune responses [29].

Transcription from the EIAV LTR is inefficient in human cells, therefore an internal promoter is required for expression from EIAV vectors. In addition, the levels of steady-state RNA generated by an internal promoter are influenced by possible endogenous sequence elements found in the vector backbone [23]. Lower transcription levels by an EIAV vector in FANCC cell lines was speculated to be due to LTR promoter interference with the internal CMV promoter [18]. However, the EIAV vector used in this study contains a SIN LTR and considerably reduced viral sequences compared to the vector reported previously [23]. The minimal vectors described in this study are devoid of any accessory genes, except for Rev in the case of HIV3G, and the 3′LTR has a deletion in the U3 region (SIN), which should eliminate promoter interference. In mouse Lin^- cell engraftment experiments, the eGFP MFI in hematopoietic cell clones generated by EIAV3G or HIV3G vectors was the same 6 weeks after transplantation. In contrast, HIV2G-engrafted NOD/SCID bone marrow exhibited higher eGFP mean fluorescence intensity compared to their EIAV3G-transduced counterparts. These lower eGFP protein levels are consistent with previous reports on human cell lines in which transcription from an EIAV-based vector resulted in lower RNA and protein levels compared to an HIV-based vector, probably due to shorter RNA half-life. However, the difference in the eGFP intensity between the two comparable EIAV3G and HIV3G vectors that we have observed is only 1.6, much lower than previously reported values [23].

Analysis of the integration profile of each vector in human HSCs indicated that the lower transgene expres-

sion levels seen using HIV3G compared to HIV2G vector are probably due to greater vector copy numbers per cell observed for the latter. Although the m.o.i. for each experiment was based on HeLa titers for all viruses, the RNA copy number per human TU for HIV2G was higher than that for HIV3G. Since these two viruses were prepared in different ways it is difficult to draw any absolute conclusions regarding their integration pattern. Nonetheless, one may speculate that inherent vector differences such as the use of wild-type Gag/Pol in the producer cells may account for the lower percentage of transduction and reduced average copy number per cell observed with the HIV3G vector. We were able to show that second- or third-generation HIV-1 virus produced using the HIV3G genome (pHF2G) in the presence or absence of Tat, respectively, attained identical transduction efficiency in human $CD34^+$ cells *in vitro*. This indicated that although Tat has been implicated in reverse transcription of HIV-1, it does not influence the efficiency of transduction of human HSCs by VSV-G-pseudotyped lentivirus vectors [24,25].

Interestingly, EIAV3G was comparable to the third-generation HIV-1 vector in human HSCs. Direct comparison with HIV2G, however, showed that although the ratio of RNA genome copies per TU was similar between the two vectors, EIAV3G integrated significantly less efficiently. This finding supports the well-documented restriction to EIAV transduction of human cells [30] and may account for some of the observed differences compared to HIV-1 vectors. In addition, this indicates that restriction affects integration of EIAV vectors into the human genome.

Such a phenomenon was not observed in mouse cells as all vectors resulted in comparable copy numbers per cell. The lower expression levels seen with EIAV3G compared to HIV2G may be due to some transcriptional inhibition from the former vector [23] without, of course, excluding the possibility that the genomic locations of EIAV3G integration may differ from those of HIV3G vector, should integration show specific-site bias [31].

Depending on the viral preparation we observed differences in the biological and RNA titers of the vectors and their integration pattern. This suggested that although these titers are valuable estimates of the infectivity of a viral vector, preliminary experiments in the target cell population should be performed to estimate precisely the

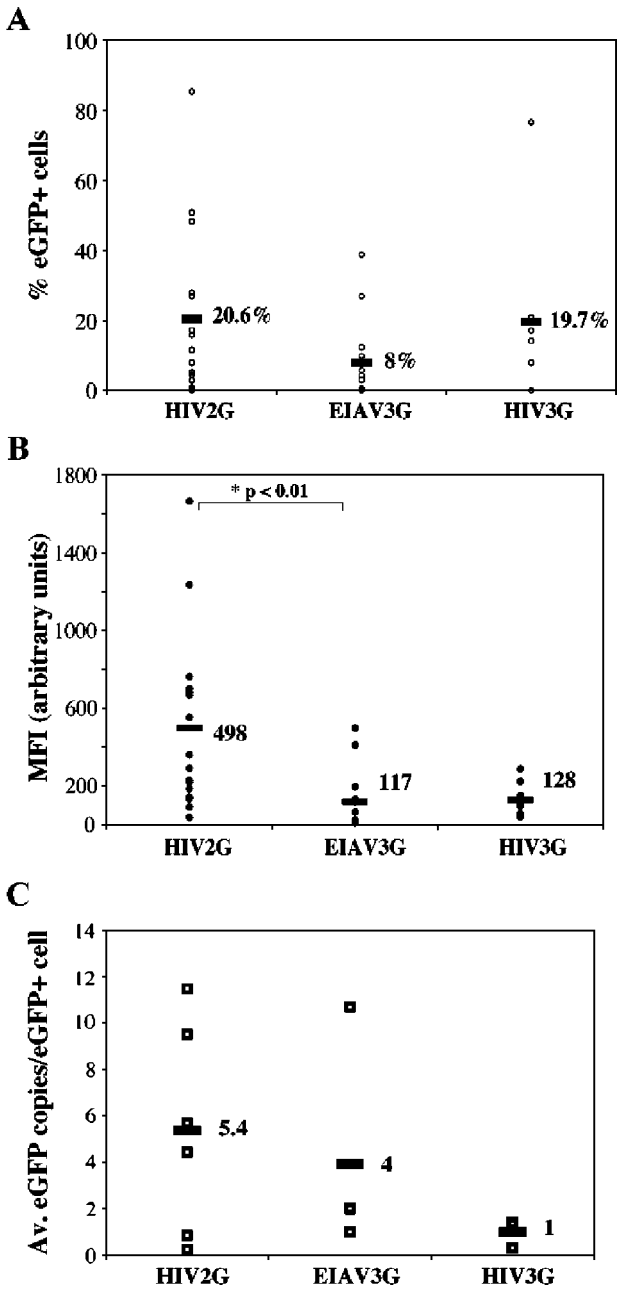

FIG. 4. Efficient gene transfer into mouse repopulating cells with an EIAV vector. (A) Lineage-depleted mouse bone marrow was transduced overnight with different vectors in the presence of various cytokine combinations (SCF, Flt3, IL-6; SCF, LIF, IL-6; or SCF, TPO, Flt3). Intravenous administration of 1×10^4 Lin^- cells into lethally irradiated animals was performed the following day and bone marrow engraftment was assessed 6 weeks later by eGFP expression. Values for individual mice ($n = 16$ for HIV2G; $n = 13$ for EIAV3G; $n = 7$ for HIV3G) from three separate experiments are plotted and the mean percentage of eGFP+ cells is shown. (B) Transgene expression levels in the bone marrow of engrafted C57BL/6J recipients were quantified by the mean fluorescence intensity of eGFP after normalization to the mouse $CD45^+$ $eGFP^-$ population. Data from three separate experiments are presented and the mean value for each vector is shown. (C) Vector copy number in mouse repopulating cells after engraftment. Analysis of the number of proviral copies per eGFP-expressing cell in the bone marrow of C57BL/6J recipients was estimated by TaqMan PCR using primers/probes for eGFP normalized to number of mouse cells (rodent GAPDH). Samples from individual animals were assessed in three different experiments and the mean values are presented as a scatter plot. Numbers shown correspond to the mean of all animals for each vector.

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vector copy number per cell and adjust the multiplicity of infection accordingly. This observation highlights some of limitations of using an artificial *in vitro* system to estimate the *in vivo* potency of viral vectors. Furthermore, recent insertional leukemogenesis reports [8] have emphasized the importance of consistent vector copy number over transduction efficiency especially for HSC therapy.

In this study we report the use of third-generation minimal EIAV and HIV vector systems for the transduction of mouse and human SRCs. Transduction of both HSC populations as well as short-term hematopoietic progenitors has been demonstrated at significant levels. Both minimal vector systems were capable of transducing SRCs, albeit at a lower efficiency than a previously reported HIV-based vector, HIV2G. Furthermore, we demonstrate that Tat does not play a role in the transduction efficiency of HIV-1-based lentivirus vectors on human HSCs. Both minimal systems also demonstrated relatively low and stable integration patterns. Transduction of human primary HSCs at this level could provide a basis for human gene therapy of single gene hematologic disorders that may require lower transduction efficiency of HSCs or other diseases that offer a selective advantage of transduced clones.

MATERIALS AND METHODS

Lentivirus Vectors

All vectors used in this study were self-inactivating, were pseudotyped with the VSV-G envelope, and contained the eGFP reporter gene under the control of the spleen focus-forming virus (SFFV) 3′LTR. The cPPT and WPRE were also included in these vectors.

HIV3G (pHF2G). The HIV vector genome used to generate third-generation virus in this study was pHF2G. The precursor plasmid pH7G was constructed by a fragment from pH3G [16] that contained the eGFP gene and the HIV cPPT from pGP-RRE1. The SFFV-eGFP-WPRE cassette was excised from pBSSFFV-GFP-WPRE [21] and cloned into blunt-ended pH7G, creating pH7SFGW. Vector pHF1G was created by inserting an *Xho*I site downstream of the HIV-1 packaging signal followed by cloning of the cPPT, SFFV-eGFP-WPRE, CMV-R-U5, the packaging region, and the Rev-responsive element, and the resultant plasmid was designated pHF1GA. The HIV 3′LTR was cloned into pBluescript (Stratagene, The Netherlands) and converted to SIN configuration by removal of the majority of the U3 region (37 bp downstream of the start of the HIV U3 region to 5 bp upstream of the HIV R region). Finally, the SIN LTR was inserted into pHF1GA creating pHF2G, which for simplicity is referred to as HIV3G in this article. Viral vector was produced by cotransfection of HEK293T cells with pSYNGP (codon-optimized HIV Gag/Pol expression construct), pHIVRev (an HIV Rev expression construct), and pHCMVG (a VSV-G expression construct) using the Fugene6 transfection reagent (Roche). For second-generation pHF2G virus, which is referred to as HIV*2G, pΔ8.91 and pMDG plasmids were used as described for HIV2G below.

EIAV3G (pONY8.5NSG). pSmart2G is an EIAV vector that has been previously described [20]. The SFFV-eGFP-WPRE [21] cassette was inserted into pSmart2G together with a multiple cloning site, to form pSmart1-linker. This was further modified initially by removing the SFFV-eGFP and cloning the Neo gene (pCIneo, Promega, Southampton, UK) and the cPPT sequence. The SFFV-eGFP cassette was then re-introduced and the 3′LTR was replaced with a SIN LTR [32] to form pONY8.5NSG. For simplicity this is referred to as EIAV3G in this article. Viral vector was produced by cotransfection of HEK293T cells with pESYNGP (codon-optimized EIAV Gag/Pol expression construct) and pHCMVG (a VSV-G expression construct) using the Fugene6 transfection reagent (Roche).

HIV2G (pHR′SINcPPTSEW). The pHR′SINcPPTSEW (HIV2G) vector was constructed as described previously [21]. Viral supernatant was produced by transient transfection of 293T cells with this construct, pΔ8.91 (Gag/Pol packaging construct) [22], and pMDG (VSV-G-encoding plasmid) using polyethylenimine (Sigma, Dorset, UK). Concentrated viral vector was produced by ultracentrifugation of cell culture supernatant. Titration of all viral vectors was performed on HeLa cells by FACS analysis. The titers of the concentrated lentivirus vectors were 1–6 $\times$ 10^8 TU/ml for HIV2G and HIV*2G and 10^9 for EIAV3G and HIV3G.

Gene Transfer into Murine or Human Hematopoietic Stem Cells

Mouse total bone marrow mononuclear cells were isolated from tibias and femurs of C57BL/6J animals and Lin^- cells purified using the Murine Hematopoietic Progenitor Enrichment Cocktail (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Cells were resuspended at 0.7–1 $\times$ 10^6 cells/ml in X-VIVO-10 (BioWhittaker) serum-free medium containing murine SCF, Flt3-ligand (both at 50 ng/ml), and IL-6 (10 ng/ml) or SCF, TPO, and Flt3-ligand (all at 50 ng/ml) or SCF, LIF (both at 50 ng/ml), and IL-6 (10 ng/ml) (all from Peprotech, London, UK). Viral supernatants were added at an m.o.i. of 30 (based on FACS titer) and cells were transduced over a 20-h period. Using the eGFP copy number titer we also calculated the number of viral vector copies expected on average per cell by the transduction of a similar number of HeLa cell equivalents. These were found to be 12–20 copies/cell for EIAV3G and HIV3G and 90 for HIV2G.

Human cord blood $CD34^+$ cells were isolated from total mononuclear cells after gradient centrifugation of cord blood on Ficoll–Paque (Amersham Biosciences, Buckinghamshire, UK) and hypotonic lysis of red blood cells. Positive selection was performed using StemSep human $CD34^+$ positive selection cocktail (Stem Cell Technologies) according to the manufacturer's recommendations. CD34-enriched cells were transduced in X-VIVO-10 medium at 0.7–1 $\times$ 10^6 cells/ml containing SCF, Flt3-ligand (both at 100 ng/ml), and IL-3 and IL-6 (both at 20 ng/ml) at an m.o.i. 35 (eGFP FACS titer) for 18–20 h. The expected numbers of vector copies per cell based on eGFP copy number titer were determined to be 11–13 for EIAV3G and HIV3G and 60 for HIV2G.

Hematopoietic Progenitor Assays

After transduction, mouse Lin^- cells were seeded in MethoCultGF M3434 medium containing SCF, IL-3, IL-6, and EPO (Stem Cell Technologies) at 10^4 cells/ml/35-mm^2 petri dish. eGFP-positive colonies were scored after 10 days by fluorescence microscopy. Human CB $CD34^+$ cells were plated at 10^3 cells/ml/35-mm^2 petri dish of MehoCultH4434 methylcellulose medium (Stem Cell Technologies) containing SCF, GM-CSF, IL-3, and EPO, and eGFP colonies were counted after 14 days of culture. EGFP-positive colonies were scored under an inverted Leica DMIL microscope with a UV lamp and images were captured using a Leica DC300 digital camera (Leica Microsystems, Heerbrugg, Switzerland). To assess transgene silencing, individual $eGFP^+$ and $eGFP^-$ (by fluorescence microscopy) colonies were picked and analyzed by quantitative TaqMan PCR for eGFP and RNase P after lysis in 10 mM Tris, 0.5% Tween 20 (Sigma), 0.5% Nonidet P-40 (BDH), and 0.9 mg/ml proteinase K (Sigma) for 1 h at 60°C followed by 5 min at 95°C.

Animals

NOD/SCID immunodeficient mice were originally purchased from The Jackson Laboratory and bred at Charles Rivers Laboratories, kept in microisolators, and handled under sterile conditions. Prior to transplantation, 8- to 12-week-old mice were irradiated with 375 cGy from a Cs source and conditioned intraperitoneally with 10 μl carbon tetrachloride in corn oil [33]. Transplantation of 1–2 $\times$ 10^5 untransduced or lentivirus-transduced human CB $CD34^+$ cells was done intravenously the same day. In addition, 10^6 $CD34^-$ cells irradiated at 1500 cGy were also co-injected. Mouse-to-mouse engraftment experiments were performed in C57BL/6J animals after 1000 cGy total body irradiation (2$\times$ 500 cGy, 4 h apart).

Mouse Lin$^-$ cells transduced with different vectors were administered intravenously at 1–2 $\times$ 10^4 cells per animal.

Flow Cytometry

Bone marrow engraftment of animals was analyzed 6–8 weeks posttransplant by flow cytometry. The presence of human cells was detected using mouse anti-human CD45–Cy-Chrome, and eGFP expression in mature hematopoietic lineages was assessed by staining with anti-human CD33–PE, anti-human CD19–PE, or anti-human CD34–PE (all antibodies from BD Pharmingen, Oxford, UK). In mouse-to-mouse engraftment experiments, staining with rat anti-mouse B220–PE, CD4–PE and CD8–PE, or Gr-1–PE was used to define the lineages expressing eGFP in the bone marrow. Mouse and rat isotype controls (all from BD Pharmingen) were also used. Dead cell exclusion was performed using DAPI and data were acquired on an LSR (Becton–Dickinson, Oxford, UK). FACS analysis was performed using Flo Jo software (Tree Star, OR, USA).

Real-Time PCR (TaqMan)

The eGFP copy number in bone marrow samples and methylcellulose colonies was analyzed by Quantitative TaqMan PCR analysis performed on an ABI Prism 7700 sequence detector (Applied Biosystems). Primers for eGFP amplification were as follows: (300 nM) 5′-CAACAGCCACAACGTCTATATCATG-3′ and (900 nM) 5′-ATGTTGTGGCGGATCTTGAAG-3′; probe (150 nM) 5′-(FAM)-CCGACAAGCAGAAGAACGGCATCAA-(TAMRA)-3′. A primer/probe (VIC dye) for RNase P was used to adjust for the amount of human genomic DNA loaded per reaction and primers/probe for rodent GAPDH (VIC dye) were used for mouse cells (Applied Biosystems). All samples were analyzed in duplicate 25-μl reactions using the cycling parameters 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Standards based on different amounts of pHR′SINcPPTSEW plasmid (range 2–20 $\times$ 105 vector copies) or human (2–150 ng) or mouse DNA were also analyzed and their threshold cycle values were plotted in standard curves. These were then used to extrapolate the absolute eGFP copy number and number of cells per reaction, respectively. The eGFP copy number detection limit was 20 copies/reaction while the lower limit of RNase P or rodent GAPDH detection was in the region of 2 ng of human or mouse DNA per reaction.

The eGFP copy number of our viral stocks was determined after transduction of 5 $\times$ 104 HeLa cells using different dilutions of virus and subsequent quantitative TaqMan PCR for eGFP, standardized to the number of human genomes by RNase P analysis (as above). The titers were expressed as vector copies per eGFP+ HeLa cell equivalent after correcting for the dilution of virus and the number of HeLa cells used in the original transduction. The RNA copy number of viral stocks was determined by quantitative RT-PCR for eGFP with the same primers and probes as above. RT-minus control reactions were run for each sample as previously described [15]. RNA was extracted from virus-like particles using the QIAmp Viral RNA Mini Kit (Qiagen, Crawley, UK) and subjected to RNase-free DNase I treatment (Ambion, Huntington, UK). Standards for the RNA copy number TaqMan assay were based on in vitro transcripts of linearized pCRBluntII-TOPO-eGFP plasmid using the MEGAscript T7 high-yield transcription kit (Ambion) according to the manufacturer's recommendations.

Statistical Analysis

Data were assessed for their suitability to be analyzed using parametric statistical tests as these are considerably more powerful than nonparametric methods. Criteria for inclusion included similarity in variances, assessed by the Kruskal–Wallis test, and normality. Data sets that failed (MFI graphs) were log(10) transformed to satisfy these requirements. A one-way analysis of variance using Tukey's multiple comparisons test was used to assess the significance of differences between means with an individual error rate of $P > 0.05$ or $P > 0.01$ as noted on graphs.

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