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RESEARCH ARTICLE

Optimization of equine infectious anemia derived vectors for hematopoietic cell lineage gene transfer

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Gene transfer into hematopoietic cells may allow correction of a variety of hematopoietic and metabolic disorders. Optimized HIV-1 based lentiviral vectors have been developed for improved gene transfer and transgene expression into hematopoietic cells. However, the use of HIV-1 based vectors for human gene therapy may be limited due to ethical and biosafety issues. We report that vectors based on the non-primate equine infectious anemia virus (EIAV) transduce a variety of human hematopoietic cell lines and primary blood cells. To investigate optimization of gene expression in hematopoietic cells, we compared a variety of post-transcriptional elements and promoters in the context of EIAV vectors. We observed cell specific increase in the number of transgene expressing cells with the different post-transcriptional elements, whereas the use of elongation factor alpha 1 (EFα1) promoter resulted in significant increases in both the number of transgene expressing cells and the level of transgene protein in all cell types tested. We then demonstrate increased transduction of hematopoietic cells using a second-generation EIAV vector containing a self-inactivating EIAV LTR and the EIAV central polypurine tract (cppt). These data suggest that optimized EIAV vectors may be a suitable alternative to HIV-1 vectors for use in hematopoietic gene therapy.

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Introduction

Ex vivo gene transfer into differentiated human hematopoietic cells and hematopoietic stem cells offer the possibility of treating a variety immunodeficiencies, hemoglobinpathies, and metabolic diseases.[1,2] However, due to the quiescent nature of these cells, gene transfer using oncoretrovirus vectors has been disappointingly low.[3] Furthermore, the long cytokine stimulation protocol necessary for the efficient transduction of hematopoietic progenitor and stem cells with oncoretrovirus vectors may alter their homing and differentiation potential.[4] The development of lentivirus vectors has resulted in great improvements in the transduction of human hematopoietic cells due to cell cycle independent integration provided by lentivirus vectors. Vectors based on the lentivirus HIV-1 are effective in transducing human lymphocytes,[5] myeloid cells,[6] hematopoietic progenitor,[7] and NOD/SCID repopulating cells.[8] Optimization of HIV-1 vectors using self-inactivating LTRs, a variety of viral, cellular and hematopoietic cell type specific promoters, post-transcriptional elements, and insulator sequences have further improved transduction and expression of these vectors in hematopoietic cells (reviewed in Hawley[9]).

While HIV-1 lentivirus vectors are efficient in transducing hematopoietic cells, ethical and biosafety issues linger. Development of vectors based on non-human lentiviruses, which may have a reduced potential for human pathogenesis, may be an important alternative to HIV-1 based vectors (reviewed in Curran and Nolan[10]). The two most studied non-primate lentiviral vectors are based on feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV).[11,12] FIV vectors are efficient in transducing a variety of human cells.[13] However, expression from FIV vectors is defective in many human hematopoietic cell lines and in primary human hematopoietic cells and, therefore, FIV is not suitable for hematopoietic gene therapy with current vector technology.[14]

Wild-type EIAV cannot replicate in human cells, potentially making vectors based on EIAV safer than HIV-1 vectors.[15] EIAV lentivirus vectors have been investigated in a variety of cell and tissue types including rodent skeletal muscle and central nervous system, fetal mice, and a variety of growing and growth arrested cell lines.[16–19] We recently demonstrated similar gene transfer into human cell lines between EIAV and HIV-1 vectors, suggesting that EIAV vectors may be clinically relevant for human gene transfer.[19] In this report, we demonstrate that EIAV vectors, unlike FIV

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vectors, transduce a variety of human hematopoietic cell lines and primary human hematopoietic cells. Furthermore, we begin the optimization of EIAV vectors for use in hematopoietic cells by assessing a variety of post-transcriptional elements and different viral and cellular promoters. These data suggest that optimized EIAV vectors may be a suitable alternative to HIV-1 vectors for the treatment of hematopoietic disorders.

Results

EIAV derived lentivirus vectors

Recently, we demonstrated that EIAV vectors transduced human cell lines. In this report, optimized EIAV derived vectors for gene transfer in human hematopoietic cells. We tested first-generation EIAV vectors alone (PEC-EIAV) or with various post-transcriptional elements (EIAV-PTE). We also investigated second-generation self-inactivating EIAV vectors (E-SIN) and compared various promoters in the SIN backbone (E-SIN Promoter). The various EIAV transfer vectors used in this study are found in Figure 1.

EIAV vectors transduce a variety of hematopoietic cell lines

To begin investigating the effectiveness of EIAV derived vectors for use in hematopoietic stem cell gene therapy, we transduced a variety of human hematopoietic cell lines. SupT1 and Jurkat (T-cell), THP-1 (Myeloid cell), and K562 (leukemia) were transduced at MOI of 0.5, 5, and 50 and analyzed for EGFP expression 4 and 14 days post-transduction. The percentage of transduced cells increased in all cell lines with increasing MOI, with levels between 50 and 99% when analyzed by flow cytometry 4 days post-transduction (Figure 2). However, a reduction in the number of transduced cells between days 4 and 14 is observed with the PEC-EIAV vectors. The loss in EGFP positive cells is not associated with any observed cell death, suggesting that vector toxicity is not a major cause for the loss in transduced cells. In samples grown to day 24, little differences were observed in the

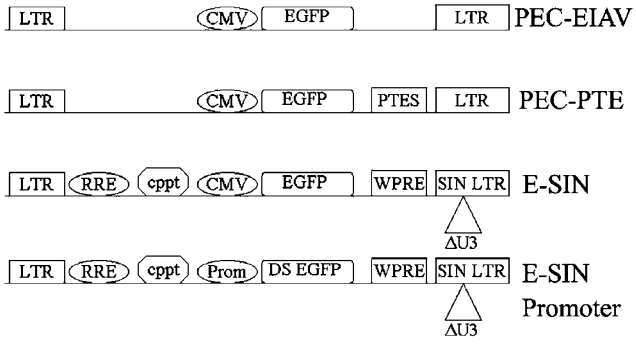

Figure 1 *The different EIAV transfer vectors used in the study. Four basic vector designs were used in this study: first-generation EIAV (EIAV PEC), first generation with either the WPRE, CTE or DR-1 element (PEC-PTE), second-generation self-inactivating vector (E-SIN), and SIN vector containing the various promoters (E-SIN Promoter). Abbreviations used: Post-transcriptional elements (PTES), rev responsive element (RRE), woodchuck hepatitis post-transcriptional element (WPRE), central polypurine tract (cppt), 2-h destabilized EGFP (DS EGFP).*

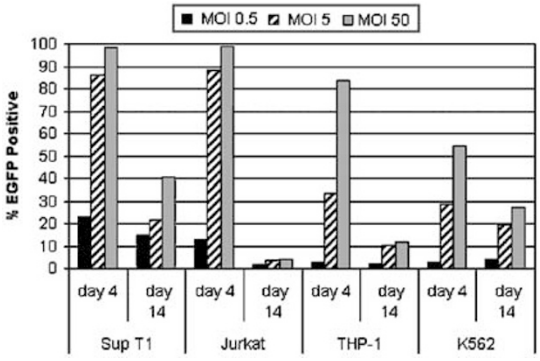

Figure 2 *Transduction of hematopoietic cells at increasing MOI with the PEC-EIAV vector. Hematopoietic cell lines were transduced with the PEC-EIAV vector and analyzed at the indicated times for EGFP expression. Data presented are representative of an experiment performed three times.*

number of EGFP positive cells between day 14 and day 24 (data not shown). Hass *et al*[20] showed that transduction with HIV-1 vectors, the contribution of episomal, nonintegrated vector forms and pseudotransduction on EGFP positivity was eliminated by 14 days post-transduction. Therefore, to reduce the possible contribution of EGFP expression from episomal forms and pseudotransduction, data were analyzed 14 days post-transduction in all subsequent studies.

Addition of post-transcriptional elements into EIAV vectors improves transduction of hematopoietic cells

Use of posttranscriptional elements affect transduction from lentivirus vectors in a cell type dependent manner.[21] Therefore, we investigated the viral constitutive transport elements (CTE) from the Mason-Pfizer monkey virus; the direct repeat 1 element (DR1) from the Rous sarcoma virus and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) in the context of EIAV vectors. Transduction of the T-cell lines with either the CTE or DR1 containing vectors increased the number of EGFP positive cells between three- and approximately five-fold (Table 1). However, in the myeloid derived cells, no increase in EGFP expressing cells was observed with THP-1 cells while a modest two-fold increase was seen in MPD cells. Similar to the myeloid cell lines, transduction of K562 cells with CTE or DR1 containing vectors resulted in a modest two-fold increase in EGFP expressing cells. In contrast to the T-cell specific increases in EGFP positive cells with the CTE and DR1 vectors, the inclusion of the WPRE element increased the number of transgene expressing cells in SupT1 (six-fold), Jurkat (four-fold), THP-1 (five-fold), and K562 (four-fold) (Table 1). However, no increase in EGFP expressing cells was observed in the MPD cell line.

Next, we compared the effect of transgene protein levels in hematopoietic cells with vectors containing the various post-transcriptional elements. Transduction of the various hematopoietic cells with vectors containing either the CTE or DR1 element resulted in no detectable increase in the levels of EGFP protein in any of the cell lines tested (Table 1). In contrast, WPRE containing EIAV vectors resulted in increased EGFP protein levels in SupT1 (two-fold), Jurkat (eight-fold), THP-1 (3.5-fold), MPD (five-fold), and K562 (2.5-fold). From these data, we

Table 1 Analysis of hematopoietic cells transduced with EIAV vectors

	SupT1	*Jurkat*	*THP-1*	*MPD*	*K562*
Percentage of EGFP positive cells day 14 (average±s.d.)					
PEC	10.7±5.8	3.5±2.7	3±1.4	24.3±18.7	12.7±3.4
WPRE	59.6±12	13.5±2.5	16.4±6	6.9±5	58.8±18.6
CTE	39±20.6	59.9±0.8	2.1±2.8	40.2±24.9	6.7±4.9
DR	31.1±11.9	47.4±12.7	1.5±1.4	47.9±23.9	6.3±3.9
Relative mean intensity day 14 (average±s.d.)					
PEC	7.12±1.3	1.6±0.7	8.6±3.1	1.9±0.8	3.1±2.2
WPRE	17.1±1.7	9.2±3.3	30.9±13.1	6.5±1.9	8.1±3.6
CTE	4.7±3.2	1.6±0.5	2.5±0.6	1.5±0.6	1.5±0.4
DR	4.5±3.9	2.0±0.7	2.6±1.8	2.3±1.5	2.3±0.6

Table 2 Comparison of WPRE containing first- and second-generation EIAV vectors

	SupT1	*Jurkat*	*THP-1*	*MPD*	*K562*
Percentage of EGFP positive cells day 14 (average±s.d.)					
WPRE	59.6±12	13.5±2.5	16.4±6	6.9±5	58.8±18.6
E-SIN	64±16.3	21.5±1.5	24.4±7.7	20.5±21.7	71.6±12
Relative mean intensity day 14 (average±s.d.)					
WPRE	17.1±1.7	9.2±3.3	30.9±13.1	6.5±1.9	8.1±3.6
E-SIN	14±3.9	9.3±3.6	36.2±13	14.4±3.2	11.2±1.6

conclude that, while all of the post-transcriptional elements resulted in increased transgene expressing cells, only the WPRE element increased both the number of EGFP positive cells and the levels of EGFP protein. Therefore, we incorporated the WPRE element in next generation EIAV vectors.

Comparison of first-generation EIAV vectors and second-generation self-inactivating EIAV vectors in hematopoietic cells

Addition of the HIV polypurine tract/central flap (cppt) and self-inactivating LTR (SIN LTR) into HIV-derived vectors resulted in increased transduction of hematopoietic cells.[22] To investigate if inclusion of the EIAV cppt and EIAV SIN LTR into EIAV vectors would improve transduction of human hematopoietic cells, we compared first- and second-generation vectors in the various hematopoietic cell lines. The design of the second-generation EIAV vector (E-SIN) is found in Figure 1. We first investigated the transduction levels of the various hematopoietic cell lines with increasing MOI and analyzed EGFP at day 4 and 14 post-transduction (Figure 3). There was a substantial increase in the percentage of EGFP positive cell with increasing MOI at day 4. However, a plateau effect was seen when analyzed at day 14 with slightly lower levels of EGFP positive cells observed with MOI of 50 compared to 5 in nearly all cell lines examined. When we compared the MOI curve of E-SIN with PEC-EIAV, the significant reduction in EGFP positive cells between day 4 and day 14 observed with the first-generation PEC-EIAV vector was blunted with the E-SIN vector (compare Figures 2 and 3).

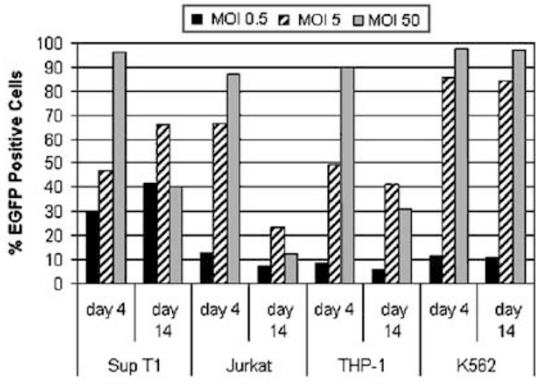

Figure 3 *Transduction of hematopoietic cells at increasing MOI with a second-generation self-inactivating EIAV vector. Hematopoietic cell lines were transduced with the E-SIN vector and analyzed at the indicated times for EGFP expression. Data presented are representative of an experiment performed three times.*

The data in Figure 3 suggest that E-SIN vectors are much more efficient than first generation in transducing hematopoietic cell lines. However, we showed a marked increase in the percentage of EGFP expressing cell with WPRE containing first-generation vectors. In order to more fairly compare the addition of the EIAV cppt and SIN LTR into EIAV vectors, we compared the E-SIN vectors (which already contains the WPRE element) with first-generation WPRE vectors. Only modest increases in the percentage of EGFP expressing cells were observed in the SupT1, THP-1, and K562 cell lines (Table 2). However, in the Jurkat and MPD cell lines, transduction with E-SIN vectors resulted in an approximately two- to

three-fold increase in EGFP expressing cells 14 days post-transduction. We measured the levels of EGFP protein in hematopoietic cells transduced with E-SIN vectors. No difference was observed in the EGFP protein levels in E-SIN transduced cells compared to first-generation WPRE EIAV vectors, suggesting that increases in transgene expression levels are solely due to the presence of the WPRE element.

Analysis of promoter strength in EIAV derived vectors

In hematopoietic cells, the CMV promoter is relatively weak and very sensitive to silencing mechanisms. Therefore, we investigated a variety of promoters in the context of the E-SIN vectors on both the number of transgene expressing cells and transgene protein levels. Due to the extended protein half-life of EGFP, we used a destabilized version of EGFP (dsGFP; 2 h half-life) in order to more accurately compare the relative activity of the various promoters.[23] We compared a 2.3 kb fragment of the elongation factor 1α promoter (EF1α), an 800 bp fragment of the phosphoglycerol kinase gene (PGK), the long terminal repeat of the myeloid proliferative sarcoma virus (MPSV), and the modified murine leukemia virus LTR (MND), with the immediate early CMV promoter. Hematopoietic cells were transduced with the various E-SIN promoter vectors and dsGFP and were analyzed 14 days later by flow cytometry. In the T-cell lines and the MPD myeloid cell line, the numbers of dsGFP expressing cells were markedly lower with E-SIN vectors containing the viral LTRs as promoters compared to the CMV promoter (MPSV; three- to 5.5-fold lower; MND 1.5- to three-fold lower) (Table 3). The number of dsGFP expressing THP-1 and K562 cell lines transduced with the E-SIN vectors containing the viral LTRs cells were more similar to levels achieved with CMV vectors. Transduction of the various cells lines with the PGK promoter vector resulted in significantly lower levels of dsGFP expressing cells in all cell lines tested (1.5- to 20-fold). In contrast to the viral LTRs and the PGK promoter vectors, E-SIN vectors containing the EF1α promoter significantly increased the number of dsGFP expressing cells in all cell lines examined (~two- to five-fold).

The level of dsGFP protein measured in the various hematopoietic cells was investigated next. Cell lines transduced with vectors containing the MPSV LTR contained similar or increased (up to five-fold) levels of dsGFP protein compared to CMV promoter vectors in all cell lines examined except for the MPD cell line (Table 3). The levels of dsGFP protein achieved in cells transduced with MND LTR was similar to or better than CMV vectors in some cell lines (SupT1, THP-1 and K562) and lower in the Jurkat and MPD cell lines. Transduction of hematopoietic cells with the PGK promoter vector resulted in substantially lower levels of dsGFP protein levels (three- to 10-fold) in all cell lines examined compared to the CMV vector. In contrast to the other promoter constructs, transduction with the EF1α promoter vector resulted in significantly higher levels of dsGFP protein in the T-cell lines (four- to seven-fold), myeloid cell lines (five- to 7.5-fold) and in the erythroid cell line (2.5-fold). Taken together, these data suggest that inclusion of the EF1α promoter in EIAV vectors is necessary to maximize both the levels of transgene expression and the number of transgene expressing cells.

Transduction of primary human hematopoietic cells with EIAV-derived vectors

FIV vectors are unable to transduce primary human hematopoietic cells.[14] To see if this was a characteristic of all non-primate lentiviral vectors, we investigated if EIAV vectors could transduce primary hematopoietic cells. EIAV WPRE vector was used to transduce 2-day stimulated peripheral or cord blood mononuclear cells. Flow cytometry was used to analyze GFP expression along with various CD markers 10 days post-transduction. GFP expression was observed in up to 11 and 24% of PBL $CD4^+$ and $CD8^+$ T-cells, respectively, and 21% of $CD19^+$ B cells (Figure 4). EGFP was also detected in $CD33^+$ and $CD34^+$ cord blood cells (Figure 4). These data demonstrate that EIAV vectors transduce both differentiated and immature hematopoietic cells, suggesting the suitability of EIAV vectors in the treatment of a variety of hematopoietic disorders.

Discussion

HIV-1 derived lentivirus vectors have been developed and optimized for gene transfer into human hematopoietic cells. However, since HIV-1 based vectors are

Table 3 Comparison of different promoters in E-SIN vectors

	Sup T1	*Jurkat*	*THP-1*	*MPD*	*K562*
Percentage of EGFP positive cells day 14 (average±s.d.)					
MPSV	9.4±0.9	1.3±0.2	4.4±2.8	1.8±0.8	17.1±4.1
MND	20.9±9.8	2.5±0.9	10.7±8.2	5.9±5.1	31.8±7.6
CMV	29.9±9.4	7.4±1.9	9.9±8.1	10.5±8.1	15.1±9.2
PGK	4.1±1.1	1.9±1.2	1.6±1.1	7.6±5.9	0.6±0.2
EF1α	**64.1±7.2**	**16.9±0.7**	**35.7±8.5**	**14.1±6.7**	**68.4±15.2**
Relative mean intensity day 14 (average±s.d.)					
MPSV	13.5±5.4	2.8±1.0	16±6.9	0.2±0.1	2.4±0.6
MND	3.5±1.5	0.8±0.3	3.1±1.0	0.4±0.1	3.6±2.1
CMV	2.6±1.6	2.2±1.4	2.9±1.3	0.9±0.4	2.0±0.9
PGK	0.7±0.6	0.3±0.2	0.8±0.6	0.5±0.2	0.2±0.08
EF1α	**19.8±12.1**	**8.2±3.6**	**15.3±8.9**	**6.1±0.8**	**4.7±2.1**

Bold indicates the most significant values.

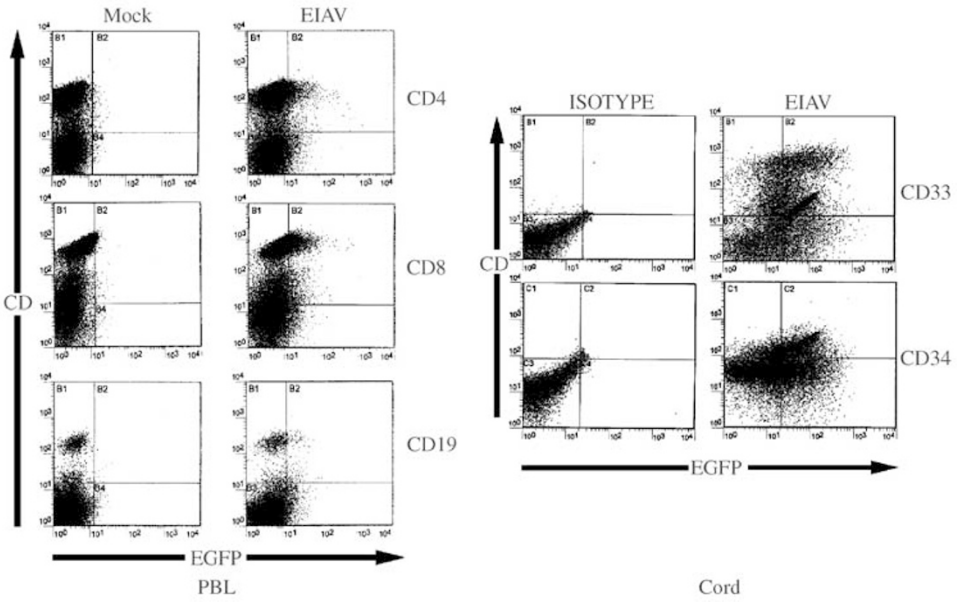

Figure 4 *Analysis of hematopoietic cell types transducted by EIAV vectors. Stimulated peripheral blood lymphocytes, and cord blood mononuclear cells transduced with WPRE-EIAV vector were analyzed 14 days post-transduction for expression of GFP in various cell types by two-color flow cytometry. (left) PBMC were analyzed for GFP expression in CD4 and CD8 positive T- and B cells (CD19). (right) Cord blood cells were analyzed for GFP expression in myeloid cells (CD33) and the progenitor/stem cell population (CD34).*

derived from a human pathogen, biosafety and ethical concerns, either real or perceived, exists for use of these vectors in human gene therapy protocols. Therefore, lentivirus phylogenetically distant from their primate counterparts such as FIV and EIAV may be more desirable for gene transfer into humans. FIV based vectors are proficient in transducing a variety of growing and growth arrested human cells,[13] but gene expression from FIV vectors is blocked in human hematopoietic cells via an unknown mechanism.[14] In contrast, we demonstrate EIAV vectors transduce up to 70% of human hematopoietic cell lines 2-weeks post-transduction (Figure 2). Importantly, we also demonstrate transduction of primary mature human T and B-lymphocytes, and cord blood derived myeloid and $CD34^+$ cells using EIAV derived vectors (Figure 4). We observed in preliminary Methocult colony forming assays using cord blood, bone marrow or purified $CD34^+$ cytokine mobilized peripheral blood cells, that first generation EIAV WPRE vectors transduce human hematopoietic progenitor cells (data not shown). Experiments are underway investigating the optimized EF1α E-SIN vector in transducing NOD/SCID repopulating cells.

We recently reported that lower gene expression levels of EIAV vectors compared to HIV-1 vectors was attributed, in part, to the relative instability of EIAV derived mRNA.[19] To overcome the low stability, we investigated a variety of post-transcriptional elements in the context of EIAV vectors. Incorporation of the constitutive transport elements CTE and DR-1 resulted in slightly lower gene expression than the parental EIAV vector, similar to our observation of these vectors in mouse skeletal muscle.[16] However, in T-cell lines and in the myeloid cell line MPD, addition of the CTE or DR-1 induced a significant increase in the number of EGFP positive cells. These data suggest that CTE and DR-1 increased the efficiency of transgene RNA processing/export leading to detectable gene expression in these cell types. This cell type difference may be due to cell type variation in the cellular cofactors necessary for effective RNA transport with the elements. It is of interest that similar enhancement and cell type specificity of transgene expressing cells were observed with both elements even though the CTE and DR-1 use different RNA export mechanisms.[24] Further experiments testing the effectiveness and cell type specificity of these transport elements in the contest of EIAV vectors in primary human hematopoietic cells, including tandem repeats of each element, which increases the effectiveness of the CTE, are needed.[25]

The use of WPRE increases gene expression from a variety of viral vectors. In contrast to the transport elements, addition of the WPRE into EIAV vectors also resulted in a significant increase in the levels of EGFP as well as the number of EGFP expressing cells in all hematopoietic cell lines. The only exception is the MPD where WPRE containing vectors resulted in markedly lower levels of EGFP expressing cells. Additionally, the incorporation of the WPRE element in EIAV vectors had no effect in primary human hematopoietic cells and was slightly inhibitory in mouse muscle *in vivo* (data not shown).[16] Mangeot *et al*[21] reported that WPRE significantly reduced gene transfer to primary human dendritic cells in the context of SIV vectors. These data suggest the use of the WPRE elements should be investigated in a cell type and vector specific manner.

We also investigated a variety of viral and cellular promoters in the context of EIAV vectors driving a destabilized form of EGFP as a reporter. Many studies using HIV-1 vectors demonstrated little differences in transgene levels using different promoters. However, many of these studies employed the highly stable EGFP as a reporter gene, which, due to accumulation over time, may mask differences in promoter strength and may not

be reflective of gene expression with therapeutic genes. Promoter analysis is even more critical in the context of EIAV vectors due to the relatively labile nature of EIAV derived RNA. Inclusion of the intron containing EF1α promoter into EIAV vectors resulted in a significant increase in reporter gene protein levels and number of transgene expressing cells (up to seven- and four-fold respectively over CMV) in all hematopoietic cell lines investigated, whereas the often utilized PGK promoter performed significantly poorer than CMV. The enhanced gene expression found with the EIAV EF1α promoter construct may be specific to hematopoietic cells as higher levels of transgene was observed with the CMV promoter in 293 fibroblast and D17 osteosarcoma cell lines (data not shown). We did not perform nuclear run on experiments and, therefore, cannot rule out that the improved gene expression seen with the EF1α promoter is due to the presence of the EF1α intron and corresponding improved processing and transport of viral RNA and not increased promoter strength. Addition of a synthetic intron into HIV-1 vectors increased gene expression.[26] Further, long-term experiments with EF1α promoter vectors in primary human hematopoietic cells are necessary to fully access the effectiveness of this promoter in the context of EIAV vectors.

To improve the performance and biosafety of EIAV vectors, second-generation vectors containing a 3′ SIN LTR, the EIAV rev responsive element (RRE), the EIAV central polypurine tract (cppt) and WPRE was developed. Viral titers from the second-generation vector improved five- to 10-fold over first-generation EIAV vectors (data not shown) to levels comparable to HIV-1 vectors. Surprisingly, transduction of hematopoietic cell lines using the second-generation vector lead only to slight increases in the number of EGFP positive cells and levels of EGFP when compared to first-generation vectors WPRE containing vectors. This is in contrast to our studies of EIAV vectors in mouse skeletal muscle where use of the second-generation vector lead to markedly improved levels of transduction *in vitro* and *vivo* compared to the first-generation EIAV WPRE vector.[16] In HIV-1 vectors, modified SIN LTRs lead to increased gene expression possibly due to a decrease in promoter interference between LTR and the internal promoter[27] and Yamada *et al*[28] suggested that low expression from EIAV vectors compared to HIV-1 in EBV transformed lymphoblasts was due, in part, to promoter interference. Our data show little enhancement of transgene protein levels using EIAV vectors containing the SIN LTR, suggesting that promoter interference may play only a minor role in gene expression levels with EIAV vectors in hematopoietic cells.

The cppt/central flap region of HIV-1 is thought to be critical in wild-type HIV-1 nuclear import and inclusion of a 178 bp HIV-1 cppt into HIV vectors increase transduction levels of cell lines *in vitro*, primary hematopoietic cells and noncycling hepatocytes *in vivo*.[22,29,30] Furthermore, cppt also increases levels of transgene via a poorly understood mechanism. Sakuma *et al*,[31] however, demonstrated that addition of the 282 bp fragment of the HIV-1 cppt resulted in impaired integration and a significant reduction in transduction. Our results with the EIAV cppt in our vector suggest that it does not effect transduction into human hematopoietic cell lines (compare EIAV-WPRE and E-SIN: Table 2). However, we have not investigated other sizes or orientation of EIAV cppt, so we cannot discount that the EIAV cppt would increase/decrease transduction in human cell types. Since EIAV cannot replicate in human cells, it would be of interest to test the cppt vector in equine cells and compare it to human cell to see if any species specific effects are observed.

In conclusion, we demonstrate transduction of human hematopoietic cells including primary cells, using EIAV derived vectors. We began optimization of EIAV vectors for gene transfer into hematopoietic cells, which is of critical importance to achieve effective transduction levels with a minimal number of viral particles, and thereby reducing the possibility of insertional mutagenesis. Further optimization of EIAV vectors including investigating scaffolding regions, chromatin insulators, and alternative envelopes may further increase the effectiveness of EIAV for use in hematopoietic cells. Taken together, these data suggest that optimized EIAV based vectors may be a suitable alternative to HIV-1 vectors for hematopoietic gene transfer.

Materials and methods

Cell lines and cell culture

The human hematopoietic cell lines SupT1, Jurkat, THP-1, and K562, cells were all purchased from ATCC (ATCC # 1942-CRL, CRL-10915, TIB-202, and CCL-243 respectively). The human myeloid cell line MPD was a generous gift from Dr Michael A Baumann (Wright State University, Dayton, OH, USA). The SupT1, Jurkat, and THP-1 cells were grown in RPMI 1640 Glutamax media supplemented with 10% fetal bovine serum (FBS) and 1 × antibiotic/antimytotic solution. The MPD and K562 cells were maintained in MEM media supplemented with 10% FBS and 1 × antibiotic/antimytotic solution. All cell culture products were purchased from Gibco (Carlsbad, CA, USA). The 293 and 293T fibroblast cell lines and the D17 canine osteosarcoma cell line were purchased from ATCC (ATCC # CRL-1513, CRL-11268, and CRL-6248 respectively). The 293, 293T, and D17 cells were maintained in DMEM Glutamax media supplemented with 10% FBS and 1 × X antibiotic/antimytotic solution. Human peripheral blood and cord blood were obtained from healthy donors. Mononuclear cells were then isolated by density gradient centrifugation with Ficoll (Sigma, St Louis, MO, USA), and cultured on plastic dishes for 24 h to remove monocytes. The nonadherent lymphocytes were maintained in X-Vivo 15 media (Stemcell Technologies, Vancouver, Canada) supplemented with 10% FBS, and 10 U/ml IL-2 (Stemcell Technologies, Vancouver, Canada). Lymphocytes were either used immediately for transduction assays or cryopreserved for later use. No difference in transduction levels was observed between the fresh and cryopreserved cells.

Plasmids

The EIAV transfer plasmids PEC-EIAV, PEC-WPRE, PEC-CTE, and PEC-DR are described.[19] Briefly, the woodchuck hepatitis post-transcriptional element (WPRE), the constitutive transport element (CTE) from the Mason-Pfizer monkey virus, or the direct repeat element 1 (DR) from the Rous Sarcoma virus was ligated

into the Cla I site of first-generation PEC EIAV vector directly downstream of the enhanced green fluorescent protein (EGFP). The second-generation EIAV self-inactivating vector E-SIN is described (O'Rourke *et al*[16] and Olsen *et al* manuscript in preparation.). Briefly, the E-SIN vector consists of a U3 deleted 3′LTR, and contains the EIAV RRE, the EIAV central polypurine tract and the WPRE element. To create the various E-SIN promoter vectors, the EGFP gene was replaced with the 2 h destabilized GFP gene (dsGFP, Clontech, Stanford, CA, USA). Promoters from the elongation factor 1 gene (ef1α; 2.3 kb) the phosphoglycerol kinase gene (PGK; 800 kb), the myloproliferative sarcoma virus LTR (MPSV), the MND LTR, and the immediate early CMV promoter were cloned into the dsGFP E-SIN vectors.

Vector production and transduction

Recombinant EIAV viruses were produced by transient triple transfection of packaging plasmid, transfer plasmid and VSV-G envelope plasmid at a ratio of 1:1:0.2 into 293T cells using standard calcium phosphate methods. Viral supernatants were collected at 24-h intervals and concentrated by centrifugation for 16 h at 7000 *g*. Viral pellets were resuspended with a volume of PBS between 1–0.1% of the starting volume. Parental, first-generation PEC-EIAV was concentrated $\sim$500-fold with 5–20% loss of infectious titer. To determine viral titer, 1×10^5 D17 cells were plated in a poly-L-lysine six-well plate. After 24 h, serial dilutions of concentrated viral stock was placed on cells and centrifuged at 2600 rpm for 1 h at 32°C. The percentages of EGFP positive cells were determined 2 days later by flow cytometry (Coulter Epics Elite EPS, Beckman-Coulter, Fullerton, CA, USA). The viral dilutions giving between 10–50% positive cells were used to determine titers. Titers were between 5×10^7 and 4×10^8 infectious particles (ip) per ml. The overall yield of recombinant first-generation EIAV vector alone or with the CTE or the DR-1 elements were about 10-fold less than comparable HIV vector preps. Addition of WPRE increased viral yield approximately two-fold and the amount of EIAV-SIN vectors was comparable to HIV-1 vector preps. Transduction of hematopoietic cells were similar as above with the following changes: 10^5 cells were plated in 12 well dishes in 1 ml growth media and the various EIAV vectors at a multiplicity of infection (moi) of 5 were added. After spin inoculation, percentage of EGFP positive cells and mean channel fluorescence of EGFP were determined 14 days post-transduction.

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References

1 Bordignon C, Roncarolo MG. Therapeutic applications for hematopoietic stem cell gene transfer. *Nat Immunol* 2002; **3**: 318–321.
2 Vollweiler JL *et al*. Hematopoietic stem cell gene therapy: progress toward therapeutic targets. *Bone Marrow Transplant* 2003; **32**: 1–7.
3 Brenner S, Malech HL. Current developments in the design of onco-retrovirus and lentivirus vector systems for hematopoietic cell gene therapy. *Biochim Biophys Acta* 2003; **1640**: 1–24.
4 Moore MA. Cytokine and chemokine networks influencing stem cell proliferation, differentiation, and marrow homing. *J Cell Biochem Suppl* 2002; **38**: 29–38.
5 Chinnasamy D *et al*. Lentiviral-mediated gene transfer into human lymphocytes: role of HIV-1 accessory proteins. *Blood* 2000; **96**: 1309–1316.
6 Naldini L *et al*. *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 1996; **272**: 263–267.
7 Case SS *et al*. Stable transduction of quiescent CD34(+)CD38(−) human hematopoietic cells by HIV-1-based lentiviral vectors. *Proc Natl Acad Sci USA* 1999; **96**: 2988–2993.
8 Miyoshi H *et al*. Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science* 1999; **283**: 682–686.
9 Hawley RG. Progress toward vector design for hematopoietic stem cell gene therapy. *Curr Gene Ther* 2001; **1**: 1–17.
10 Curran MA, Nolan GP. Nonprimate lentiviral vectors. *Curr Top Microbiol Immunol* 2002; **261**: 75–105.
11 Sauter SL, Gasmi M. FIV vector systems. *Somat Cell Mol Genet* 2001; **26**: 99–129.
12 Olsen JC. Gene transfer vectors derived from equine infectious anemia virus. *Gene Therapy* 1998; **5**: 1481–1487.
13 Poeschla EM, Wong-Staal F, Looney DJ. Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors. *Nat Med* 1998; **4**: 354–357.
14 Price MA *et al*. Expression from second-generation feline immunodeficiency virus vectors is impaired in human hematopoietic cells. *Mol Ther* 2002; **6**: 645–652.
15 Maury W. Regulation of equine infectious anemia virus expression. *J Biomed Sci* 1998; **5**: 11–23.
16 O'Rourke JP *et al*. Analysis of gene transfer and expression in skeletal muscle using enhanced EIAV lentivirus vectors. *Mol Ther* 2003; **7**: 632–639.
17 Mitrophanous K *et al*. Stable gene transfer to the nervous system using a non-primate lentiviral vector. *Gene Therapy* 1999; **6**: 1808–1818.
18 Waddington SN *et al*. Long-term transgene expression by administration of a lentivirus-based vector to the fetal circulation of immuno-competent mice. *Gene Therapy* 2003; **10**: 1234–1240.
19 O'Rourke JP *et al*. Comparison of gene transfer efficiencies and gene expression levels achieved with equine infectious anemia virus- and human immunodeficiency virus type 1-derived lentivirus vectors. *J Virol* 2002; **76**: 1510–1515.
20 Haas DL, Case SS, Crooks GM, Kohn DB. Critical factors influencing stable transduction of human CD34(+) cells with HIV-1-derived lentiviral vectors. *Mol Ther* 2000; **2**: 71–80.
21 Mangeot PE *et al*. High levels of transduction of human dendritic cells with optimized SIV vectors. *Mol Ther* 2002; **5**: 283–290.

22 Sirven A *et al*. The human immunodeficiency virus type-1 central DNA flap is a crucial determinant for lentiviral vector nuclear import and gene transduction of human hematopoietic stem cells. *Blood* 2000; **96**: 4103–4110.
23 Wahlers A *et al*. Influence of multiplicity of infection and protein stability on retroviral vector-mediated gene expression in hematopoietic cells. *Gene Therapy* 2001; **8**: 477–486.
24 Paca RE *et al*. Rous sarcoma virus DR posttranscriptional elements use a novel RNA export pathway. *J Virol* 2000; **74**: 9507–9514.
25 Schambach A *et al*. Context dependence of different modules for posttranscriptional enhancement of gene expression from retroviral vectors. *Mol Ther* 2000; **2**: 435–445.
26 Royal RE *et al*. Increased functional expression of transgene in primary human lymphocytes using retroviral vectors modified with IRES and splicing motifs. *Gene Therapy* 2002; **9**: 1085–1092.
27 Ginn SL, Fleming J, Rowe PB, Alexander IE. Promoter interference mediated by the U3 region in early-generation HIV-1-derived lentivirus vectors can influence detection of transgene expression in a cell-type and species-specific manner. *Hum Gene Ther* 2003; **14**: 1127–1137.
28 Yamada K *et al*. Functional correction of fanconi anemia group C hematopoietic cells by the use of a novel lentiviral vector. *Mol Ther* 2001; **3**: 485–490.
29 Zennou V *et al*. The HIV-1 DNA flap stimulates HIV vector-mediated cell transduction in the brain. *Nat Biotechnol* 2001; **19**: 446–450.
30 VandenDriessche T *et al*. Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells *in vivo*. *Blood* 2002; **100**: 813–822.
31 Sakuma R, Kobayashi N, Ae K, Kitamura Y. Inhibitory and enhancing effects of insertion of central polypurine tract sequence on gene expression with vectors derived from human immunodeficiency virus type 1. *Biochem Biophys Res Commun* 2003; **302**: 489–495.