

High-efficiency Transduction of Rhesus Hematopoietic Repopulating Cells by a Modified HIV1-based Lentiviral Vector

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Human immunodeficiency virus type 1 (HIV1) vectors poorly transduce rhesus hematopoietic cells due to species-specific restriction factors, including the tripartite motif-containing 5 isoform α (TRIM5 α) which targets the HIV1 capsid. We previously developed a chimeric HIV1 (χ HIV) vector system wherein the vector genome is packaged with the simian immunodeficiency virus (SIV) capsid for efficient transduction of both rhesus and human CD34⁺ cells. To evaluate whether χ HIV vectors could efficiently transduce rhesus hematopoietic repopulating cells, we performed a competitive repopulation assay in rhesus macaques, in which half of the CD34⁺ cells were transduced with standard SIV vectors and the other half with χ HIV vectors. As compared with SIV vectors, χ HIV vectors achieved higher vector integration, and the transgene expression rates were two- to threefold higher in granulocytes and red blood cells and equivalent in lymphocytes and platelets for 2 years. A recipient of χ HIV vector-only transduced cells reached up to 40% of transgene expression rates in granulocytes and lymphocytes and 20% in red blood cells. Similar to HIV1 and SIV vectors, χ HIV vector frequently integrated into gene regions, especially into introns. In summary, our χ HIV vector demonstrated efficient transduction for rhesus long-term repopulating cells, comparable with SIV vectors. This χ HIV vector should allow preclinical testing of HIV1-based therapeutic vectors in large animal models.

Received 15 February 2012; accepted 3 July 2012; advance online publication 7 August 2012. doi:10.1038/mt.2012.159

INTRODUCTION

Hematopoietic stem cell (HSC)-targeted gene therapy is potentially curative for a number of congenital and acquired disorders and indisputable efficacy has now been demonstrated in several clinical trials involving mostly subjects with immunodeficiencies using autologous HSC transplantation after *ex vivo* γ -retroviral

gene transfer.¹⁻⁵ However, four out of nine patients in an X-linked severe combined immunodeficiency gene therapy trial developed T cell type acute lymphoblastic leukemia, which was caused by insertional mutagenesis of the proto-oncogenes *LMO2* and *CCND2*.⁴ In another trial, correction of X-linked chronic granulomatous disease was associated with an *in vivo* clonal expansion of transduced cells (no leukemia development) with viral insertion into known γ -retroviral vector common integration sites, such as *MDS1-EV11*, *PRDM16*, and *SETBP1*.⁶ Evidence now confirms that γ -retroviral vectors tend to integrate near transcription start sites,⁷⁻⁹ which implies an inherent risk for integration into promoter regions that might induce insertional mutagenesis. In contrast, lentiviral vectors based on human immunodeficiency virus type 1 (HIV1) have a tendency toward equal integration into all gene regions.^{8,9} Therefore, HIV1 vectors are thought to possess a lower likelihood of insertional mutagenesis as compared with that of γ -retroviral vectors. In addition, current HIV1 vectors have been further modified to prevent activation of genes around viral integration sites through self-inactivation (SIN) of the long terminal repeats (LTR).¹⁰

HIV1 vectors have additional features which render them more suitable for gene therapy approaches to treat hemoglobin disorders. Gene transfer has long been envisioned as a tenable approach to correct hemoglobin disorders through the use of *ex vivo* transduction of autologous HSCs. The first demonstration of success in rodents was through the use of an HIV1-based lentiviral vector, TNS9, having erythroid specific β -globin expression sufficient to correct a murine model of thalassemia.¹¹ Confirmatory studies followed in both β -thalassemia and sickle cell disease models.¹²⁻¹⁷ As a preclinical step, we initiated studies to evaluate whether HIV1 vectors have an ability to drive therapeutically relevant levels of β -globin production in large animals. In rhesus macaques, HIV1 transduction is partially blocked by species-specific retroviral restriction.^{18,19} In order to circumvent this restriction, we modified the TNS9 vector by exchange of the cyclophilin A binding region in the HIV1 capsid with that of a macrophage-tropic HIV1 strain. This vector was previously shown to allow efficient

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transduction of simian (baboon) cells.²⁰ Shortly following transplantation of the TNS9 transduced rhesus CD34⁺ cells, human β -globin expression rates of 5% or higher were detectable by flow cytometry. However, long-term gene marking levels decreased to ~0.001% at 2 years, potentially due to additional species-specific HIV1 restriction factors such as the tripartite motif-containing 5 isoform α (TRIM5 α).^{18,19} The use of simian immunodeficiency virus (SIV)-based lentiviral vectors can circumvent this restriction.²¹ However, the SIV-based vector system is not able to use HIV1-based vector plasmids for preparing functional viral particles,²² a major limitation given that the majority of therapeutic vector constructs have been developed using HIV1-based vector systems.^{11–12,16,23–26}

To address this issue, we developed a chimeric HIV1-based lentiviral vector system (χ HIV vector), in which the HIV1 vector genome is packaged in the context of SIV capsid sequences, by passing the species-specific restriction. Using this χ HIV vector, we demonstrated efficient transduction of both human and rhesus CD34⁺ cells and superior short-term multi-lineage hematopoietic marking to a conventional HIV1 vector in the rhesus transduced CD34⁺ cell competitive repopulation transplantation model for 3–6 months.²² This χ HIV vector system has the advantage that it can use HIV1-based therapeutic vector constructs to prepare viral particles. In this study, we sought to evaluate whether χ HIV vectors efficiently transduce long-term repopulating rhesus hematopoietic cells, by comparison with standard SIV vectors in the rhesus HSC competitive repopulation model.

RESULTS

Rhesus hematopoietic stem cell transplantation with lentiviral transduction

We evaluated whether χ HIV vectors could transduce rhesus blood cells as efficiently as SIV vectors by performing a competitive repopulation assay in two rhesus macaques (RQ7307 and RQ7280) in which half of the CD34⁺ cells were transduced with the standard SIN-SIV vector and the other half with the SIN- χ HIV vector under otherwise identical conditions (Figure 1a). In a third animal (RQ7387), we used the SIN- χ HIV vector alone to evaluate both the transduction efficiency and integration sites of our χ HIV vector in rhesus hematopoietic repopulating cells.

After transplantation, bone marrow suppression followed by rapid reconstitution of peripheral blood cells was observed in all three animals (Table 1). The nadirs of peripheral blood counts were total white blood cells: 290 \pm 120/ μ l (day 6.3 \pm 0.3), granulocytes: 120 \pm 67/ μ l (day 6.3 \pm 0.3), lymphocytes: 56 \pm 17/ μ l (day 7.3 \pm 0.3), hemoglobin concentration 7.6 \pm 0.34g/dl (day 12.3 \pm 4.8), and platelets 30,000 \pm 8,200/ μ l (day 9.8 \pm 1.7). The durations of myelosuppression were 5.0 \pm 2.1 days for white blood cells <1,000/ μ l, and 4.7 \pm 1.9 days for granulocytes <500/ μ l. The recovery dates of each blood cell lineage were day 10.3 \pm 2.0 for white blood cells \geq 1,000/ μ l, day 10.0 \pm 1.7 for granulocytes \geq 500/ μ l and day 14.5 for platelets \geq 50,000/ μ l (the baseline platelets count for animal RQ7307 was never <50,000/ μ l) (Table 1). No animal required red blood cell (RBC) transfusion, and 60-ml platelet transfusion was needed for animal RQ7387 on day 13 after transplantation. No complications were observed in the three transplanted animals. After peripheral

blood reconstitution, similar blood counts continued for 2 years (Supplementary Figure S1 online).

Competitive repopulation assay between the χ HIV and SIV vectors in rhesus macaques

After an additional 2 days of liquid culture following transduction, lower transduction rates were found for the χ HIV vector than for the SIV vector (Figure 1b, $P < 0.01$) among bulk cultured CD34⁺ cells. A small aliquot of cells from the end of transduction was also cultured in methylcellulose media to evaluate their *in vitro* transduction. Transduction rates among colony forming units were, in contrast, not significantly different between the χ HIV and SIV vectors (Figure 1b, not significant).

After reconstitution of peripheral blood cells, enhanced green fluorescent protein (GFP) and enhanced yellow fluorescent protein (YFP) expression was determined by fluorescence microscopy (Figure 2a) and by flow cytometry (Figure 2c). We detected the biconcave shape of RBCs by contrast of GFP (and YFP) fluorescence under UV light by a phase-contrast microscope (Figure 2a) and confocal microscope (Figure 2b), suggesting high expression of the transgene from viral constructs in erythroid cells. One month following transplantation, transgene expression rates had not yet reached plateau levels by flow cytometry, but higher GFP transgene expression rates with the χ HIV vector as compared with the SIV vector were seen in granulocytes (RQ7307: 12.4 versus 6.1% and RQ7280: 36.1 versus 27.2%) and equivalent transgene expression rates were seen in lymphocytes, RBCs, and platelets. Three to four months after transplantation, *in vivo* transgene expression rates plateaued, and two- to threefold higher transgene expression rates for the χ HIV vector as compared with the SIV vector were seen in granulocytes (RQ7307: 6.3 versus 2.5% and RQ7280: 30.4 versus 17.0%) and RBCs (RQ7307: 3.5 versus 1.0% and RQ7280: 13.8 versus 5.2%), and equivalent transgene expression rates in lymphocytes (RQ7307: 6.0 versus 5.1% and RQ7280: 25.9 versus 17.3%) and platelets (RQ7307: 2.8 versus 2.3% and RQ7280: 13.3 versus 15.4%) (Figure 2d). In peripheral blood cells of the two animals, similar transgene expression rates continued in all cell lineages for at least 2 years (Figure 2d).

Using cell type specific surface marker analysis in peripheral blood cells of the two animals, the χ HIV vector showed 1.3–7.5-fold higher transgene expression rates in CD33⁺ cells (granulocytes), CD71⁺ cells (reticulocytes), and RBC⁺ cells, and equivalent transgene expression rates in CD3⁺ cells (T cells), CD4⁺ cells (T cells), CD8⁺ cells (T cells), CD20⁺ cells (B cells), CD41a⁺ cells (platelets), and CD56⁺ cells (natural killer cells) ~1 year after transplantation (Figure 3). However, bone marrow samples demonstrated no distinct difference in transgene expression rates between the χ HIV and SIV vectors (Figure 3). When we followed transgene expression rates in CD3⁺ cells (T cells), CD20⁺ cells (B cells), CD33⁺ cells (granulocytes), RBC⁺ cells, and CD41a⁺ cells (platelets), similar transgene expression rates were detected in peripheral blood cells over 2 years (Figure 2e). These data demonstrated that after the transgene expression rates plateau, the χ HIV vector has equivalent transgene expression rates in rhesus macaques, comparable with the SIV vector. The χ HIV vector showed higher transgene expression rates in granulocytes and RBCs, as compared with the SIV vector.

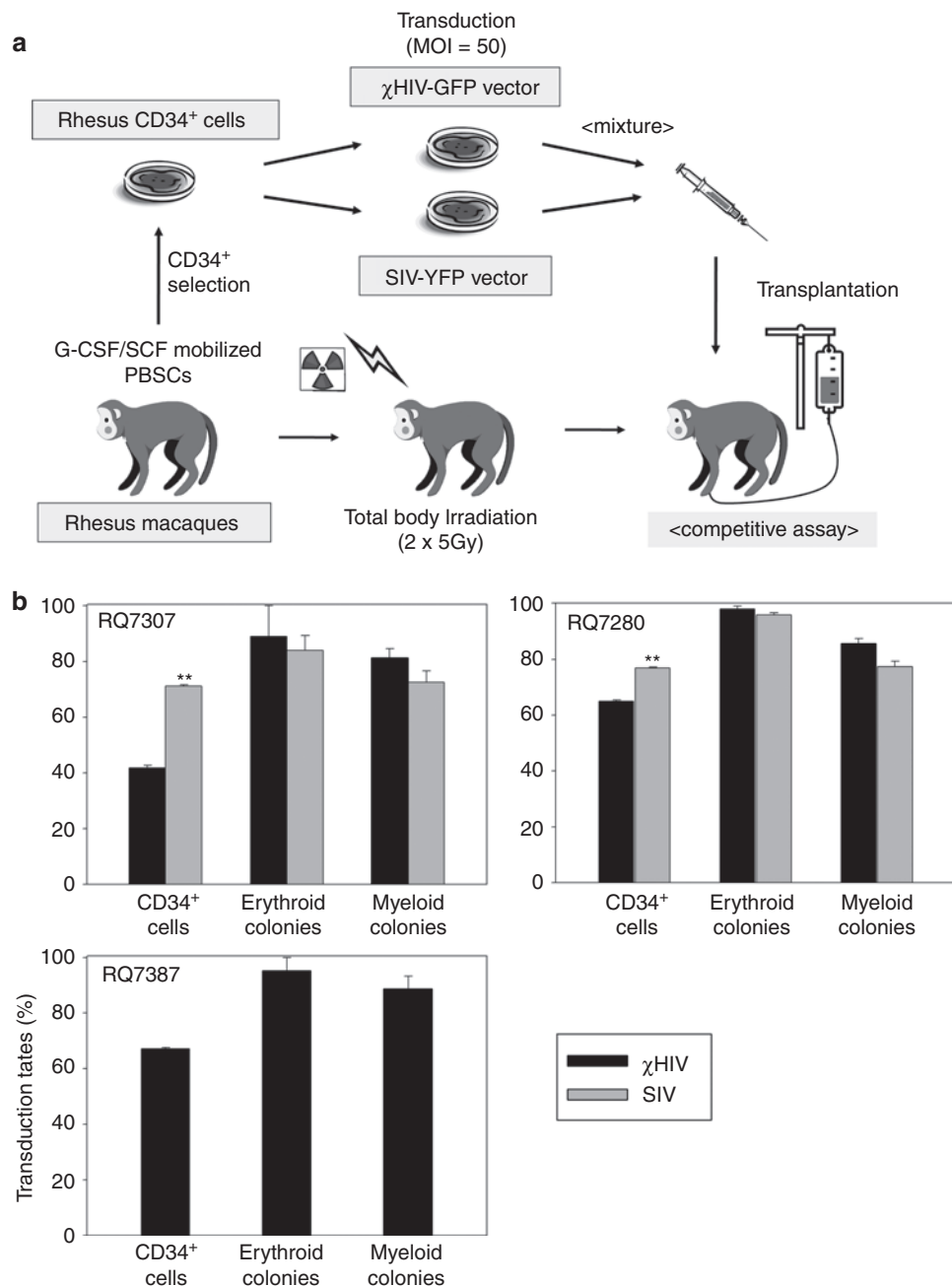


Figure 1 Competitive repopulation assay for transplantation of rhesus CD34⁺ cells transduced with the chimeric human immunodeficiency virus type 1 (χ HIV) vector and simian immunodeficiency virus (SIV) vector. **(a)** We evaluated whether the χ HIV vector can transduce rhesus hematopoietic cells as efficiently as SIV vectors by performing a competitive repopulation assay in two rhesus macaques (RQ7307 and RQ7280), in which half of the rhesus mobilized CD34⁺ cells were transduced with the χ HIV vector and the other half with the SIV vector under otherwise identical conditions. The third animal (RQ7387) received transplantation of CD34⁺ cells transduced with the χ HIV-green fluorescent protein vector alone to simply evaluate transduction efficiency and integration sites. The transduced CD34⁺ cells were infused into rhesus macaques following myeloablative total body irradiation (total 10 Gy). Transgene expression in rhesus peripheral blood was followed for over two years after the transduced hematopoietic stem cell transplantation. **(b)** A small aliquot of transduced CD34⁺ cells were cultured for an additional 2 days in liquid culture and for an additional 6 days in CFU assay, to evaluate *in vitro* transduction efficiency. Lower transduction rates in cultured bulk CD34⁺ cells were found for the χ HIV vector ($P < 0.01$), whereas no significant differences were noted for transduction rates in CFUs between the two vectors. G-CSF, granulocyte colony-stimulating factor; MOI, multiplicity of infection; PBSCs: peripheral blood stem cells; SCF, stem cell factor. * $P < 0.01$ (two-tailed *t*-test).

Efficient viral integration with the χ HIV vector in rhesus macaques

We also evaluated the integrated vector copy number of the χ HIV and SIV vectors in both transplanted animals (RQ7307 and RQ7280) by Southern blot analysis (Figure 4). Each vector

signal was separated by band size, and the band densities were compared with those of a control cell line that has a single copy of vector integration. We then calculated the average vector copy numbers in the peripheral blood mononuclear cells. The χ HIV vector showed several fold higher average vector copy numbers

as compared with the SIV vector (RQ7307: 0.37 versus 0.07 and RQ7280: 1.19 versus 0.37) (Figure 4b).

In addition, at various time points following transplantation, we evaluated the gene marking levels of both χ HIV and SIV vectors in granulocytes and lymphocytes using real time polymerase chain reaction (PCR) (Figure 5). In the all three animals, similar gene marking levels continued in the peripheral blood cells for 2 years. In the competitive assay animals (RQ7307 and RQ7280), the χ HIV vector demonstrated around fourfold

higher gene marking levels, as compared with the SIV vector. These integration data suggested higher transduction efficiency for rhesus hematopoietic repopulating cells for the χ HIV vector than for the SIV vector.

Integration site analysis of the χ HIV vector in hematopoietic repopulating cells

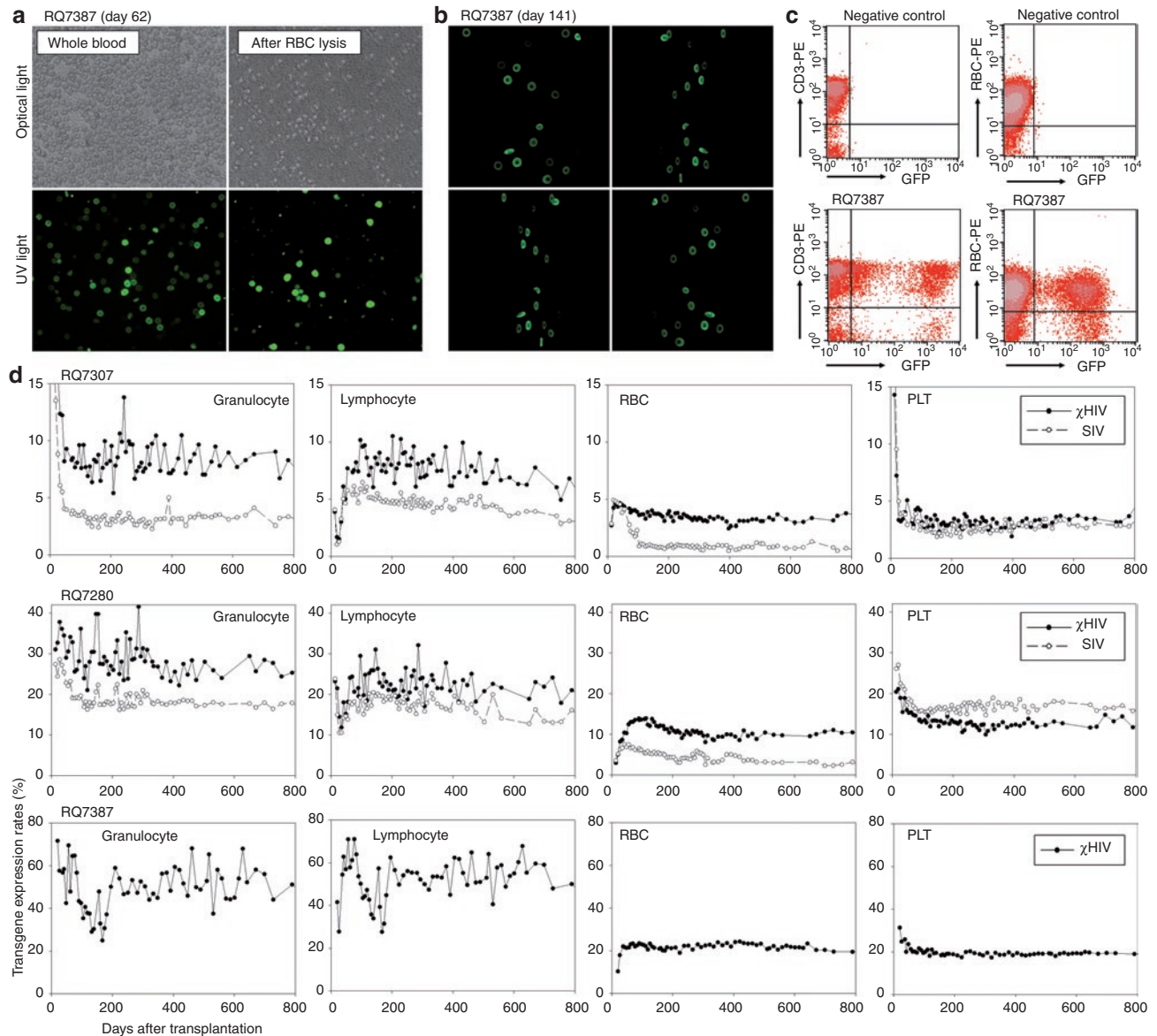
We performed transplantation using cytokine mobilized, immunoselected rhesus CD34⁺ cells transduced with the χ HIV vector

Table 1 Number of days to recovery of peripheral blood counts after transplantation

Animals	Cell #	WBC ≥ 1000	Gr ≥ 500	Lym ≥ 500	Lym $\geq 1,000$	Hb ≥ 10.0	PLT $\geq 50,000$
RQ7307	2.26e7	10	10	17	18	32	N/A ^a
RQ7280	4.63e7	7	7	20	30	19	9
RQ7387	4.94e7	14	13	20	30	30	20

Cell #, the number of mobilized CD34⁺ cells; Gr, granulocytes (μ l); Hb, hemoglobin concentration (g/dl); Lym, lymphocytes(μ l); N/A, not applicable; PLT, platelets (μ l); WBC, white blood cells (μ l).

^aThe platelet count never decreased below 50,000/ μ l in this animal.



alone to evaluate integration sites. The transgene expression rates among peripheral blood cells in this animal (RQ7387) plateaued 1–3 months after transplantation, with transgene expression rates of 30–40% in granulocytes and lymphocytes and ~20% in RBCs and platelets (Figure 2d). Multi-lineage marking was observed for 2 years after transplantation by cell surface marker analysis (Figures 2e and 3).

We evaluated integration sites for the χ HIV vector in rhesus hematopoietic repopulating cells by linear amplification-mediated PCR, using peripheral blood cells of animal RQ7387 0.5–1.5 years after transplantation. The integration site data were compared with computer-generated random controls (Table 2 and Figure 6).^{27–29} We amplified a total of 344 integration sites for the χ HIV vector, and these data demonstrated that the χ HIV vector integrated into gene regions, especially introns, as compared with the integration of random controls (Table 2, $P < 0.01$). On the other hand, the data revealed fewer integrations of the χ HIV vector into the regions 30 kb upstream of genes than would be expected at random (Table 2, $P < 0.01$ and Figure 6). Most of the integration sites were in gene density regions of 0–10 genes within 1 Mb upstream or downstream of integration sites ($P < 0.01$), as compared with that of random controls (Table 2). No specific trend was noted for the number of integration sites around CpG islands and the common integration sites with the χ HIV vector (Table 2). These data suggest that the χ HIV vector has integration patterns comparable with HIV1 and SIV vectors.

DISCUSSION

Previously, we demonstrated our χ HIV vector allows escape from species-specific restriction in rhesus macaque cells, as judged by the efficient transduction of both rhesus and human CD34⁺ cells for 3–6 months.²² In the present study, the χ HIV vector demonstrated long-term multi-lineage transgene expression comparable with that achieved with the SIV vector, which had previously shown high marking levels in the rhesus HSC transplantation model.^{21,27} Erythroid cells, in particular, showed better transgene expression rates with the χ HIV vector in two transplanted rhesus macaques than with the SIV vector. The high level of GFP marking allowed us to recognize the biconcave shape of RBCs using only fluorescence microscopy, and was confirmed by three dimension images using confocal microscopy. Unlike SIV vectors, the χ HIV vector system can use HIV1-based therapeutic vector plasmids, which have been extensively tested in mouse disease models. Thus the χ HIV vector may be more suitable for preclinical evaluation of transgene expression in rhesus RBCs *in vivo*.

We previously reported three rhesus macaques that received transplantation of CD34⁺ cells transduced with the SIV vector alone.^{21,27} In these animals, transgene expression rates plateaued around 6 months after transplantation, and transgene expression in peripheral blood cells (4–12% in granulocytes and lymphocytes) continued at the same levels for more than 4 years. Our current study also demonstrated stable transgene expression (up to 40% in

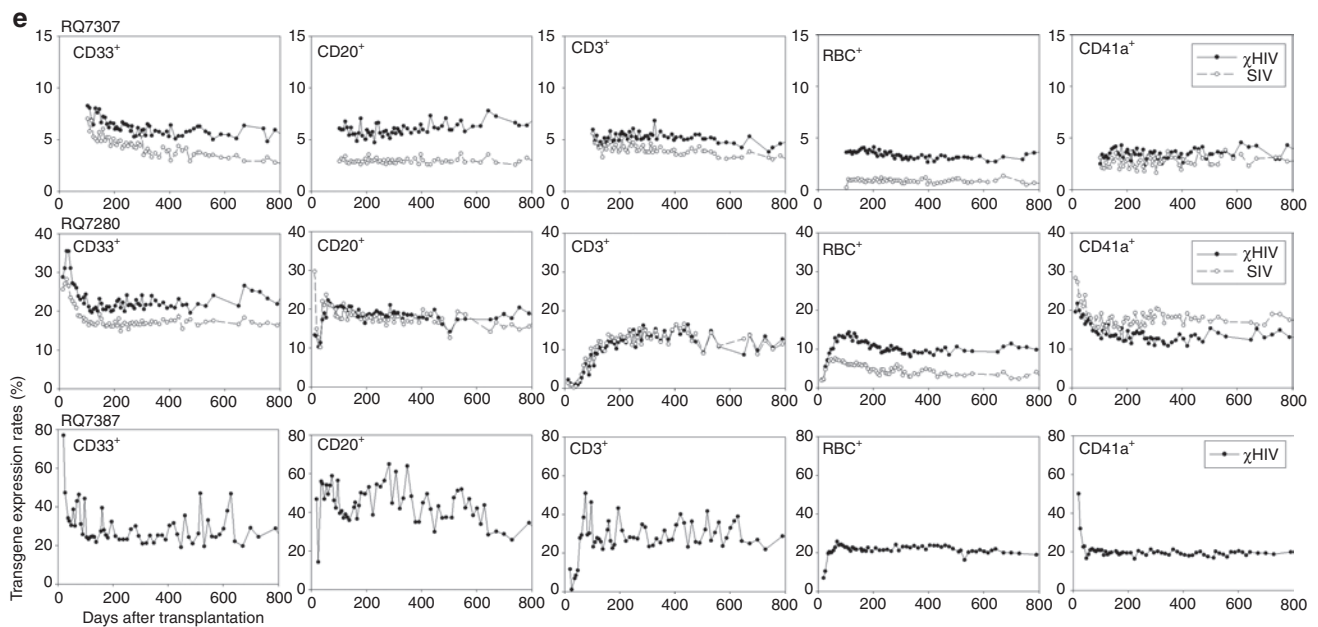


Figure 2 High transgene expression rates with the chimeric human immunodeficiency virus type 1 (χ HIV) vector in rhesus peripheral blood cells. (a, b, and c) Following engraftment in the transplanted animals (RQ7387), transgene expression in peripheral blood cells was evaluated by fluorescence microscopy (a) and flow cytometry (c). The brightness of green fluorescent protein (GFP) allowed detection of the biconcave shape of red blood cells by a phase-contrast microscope (a) and confocal microscopy (b). (d) Three to four months after transplantation, *in vivo* transgene expression rates reached plateau levels in all animals, and 1.5–2 fold higher marking levels, in granulocytes, two- to threefold higher marking levels in red blood cells, and equivalent marking levels in lymphocytes and platelets, were found for the χ HIV vector as compared with the SIV vector, over 2 years (RQ7307 and RQ7280). In the third animal (RQ7387) who received cells transduced with the χ HIV-GFP vector alone, the transgene expression rates of peripheral blood cells demonstrated high transgene expression rates of 30–40% in granulocytes and lymphocytes and ~20% in red blood cells (RBCs) and platelets. (e) Transgene expression was followed by cell surface analysis of CD33⁺ granulocytes, CD20⁺ B lymphocytes, CD3⁺ T lymphocytes, rhesus RBC-antibody⁺ red blood cells, and CD41a⁺ platelets over 2 years. The CD33⁺ and RBC⁺ cells showed higher transgene expression rates with the χ HIV vector, whereas CD20⁺, CD3⁺, and CD41a⁺ cells had similar transgene expression rates with both vectors. PE, phycoerythrin; PLT, platelets; RBC, red blood cells; UV, ultraviolet.

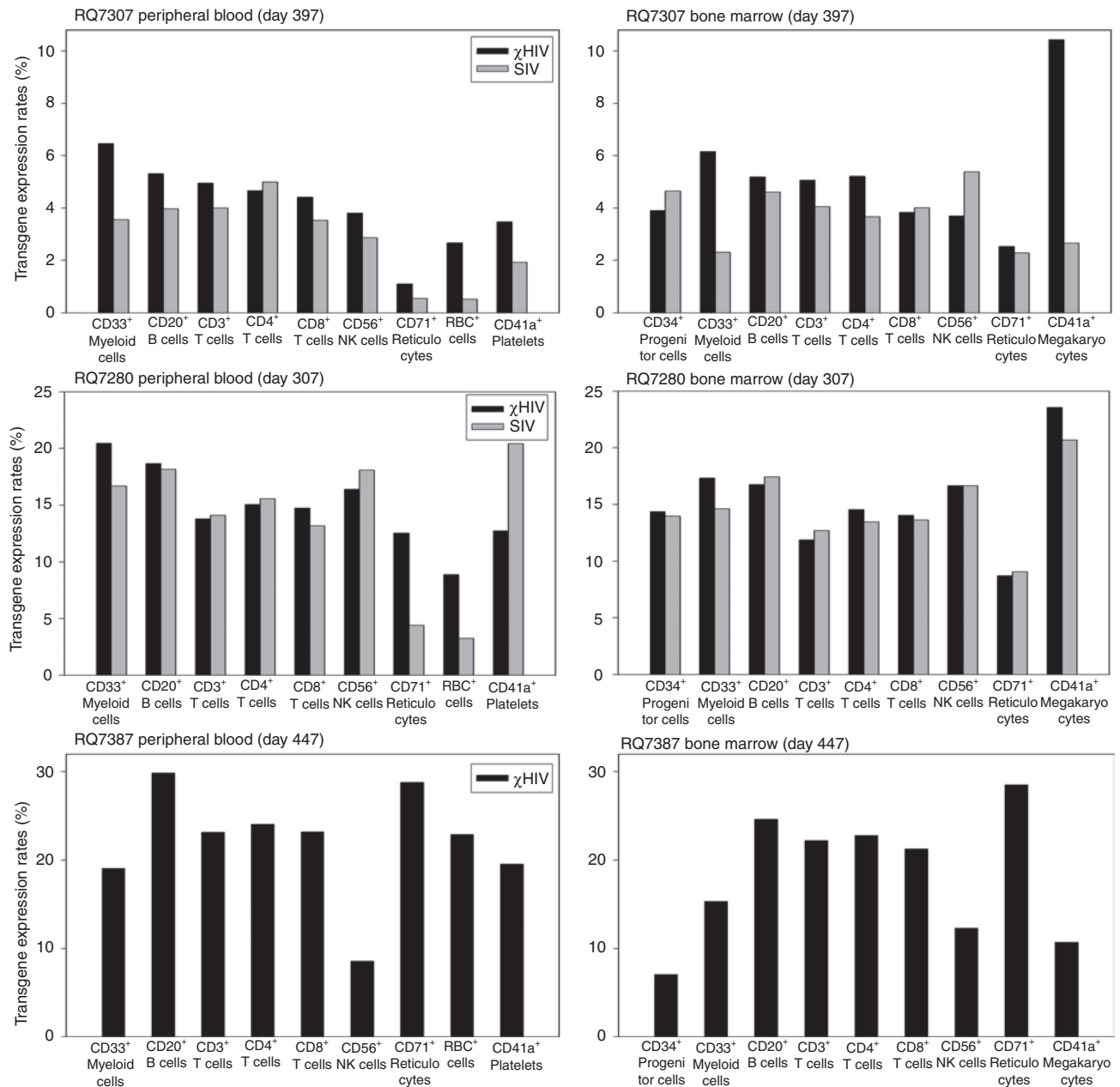


Figure 3 Multi-lineage transgene expression in peripheral blood cells and bone marrow cells. The chimeric human immunodeficiency virus type 1 (χ HIV) vector showed multi-lineage marking in both peripheral blood cells and bone marrow cells that was comparable to that of simian immunodeficiency virus (SIV), with superior transduction rates observed in red blood cells (RBCs).

granulocytes and lymphocytes) from both χ HIV and SIV vectors in transplanted rhesus macaques for at least 2 years after arriving at the plateau levels. These data suggest that the transplanted animals acquired immunological tolerance to GFP (and YFP) proteins which were transferred by the χ HIV and SIV vectors at the stem cell level. In addition, in the best marking animal in the previously reported recipients of SIV transduced cells, the transgene expression rates were 3% in RBCs and <1% in platelets. In the present study, significantly higher levels (20% in RBCs and 20% in platelets) were observed in the best marking animal (RQ7387).

In the competitive assay animals, stable transgene expression with the GFP-expressing χ HIV vector (χ HIV-GFP) was observed at

5–10% (RQ7307) and 20–30% (RQ7280) among granulocytes and lymphocytes. These GFP-positive blood cells derived from only half of transplanted HSCs, since one half of transplanted CD34⁺ cells were transduced with the χ HIV vector. Therefore, if we had transduced all rhesus CD34⁺ cells with the χ HIV vector alone for transplantation in these two animals, theoretically the transgene expression rates might have been twice as high at ~40% in granulocytes and lymphocytes and ~20% in RBCs and platelets in animal RQ7280, which were indeed the rates of transgene expression observed in animal RQ7387 (who received cells transduced with the χ HIV vector alone).

It is possible that the higher *in vivo* transgene expression rates seen in our current study were improved not only by the χ HIV

Table 2 Distribution of integration sites for the χ HIV vector in peripheral blood cells

	χ HIV vector (n = 344)	Random control (n = 344 × 10 ⁴)	P value
Relative to genes			
Within exon	1.74%	1.05%	0.16
Within intron	43.31%	31.98%	<0.0001
Within gene (exon or intron)	45.06%	33.03%	<0.0001
Within 30 kb upstream of ≥1 genes	6.10%	12.22%	<0.0001
Within 30 kb downstream of ≥1 genes	4.94%	7.16%	0.07
Within 30 kb window of ≥1 genes	56.10%	52.41%	0.10
Relative to CpG islands			
Within CpG island	0.00%	0.10%	0.72
Within 1 kb window of CpG island	0.39%	0.97%	0.36
Within 5 kb window of CpG island	3.20%	4.88%	0.09
Gene density			
0-10 genes within 1 Mb window	91.57%	79.61%	<0.0001
11-20 genes within 1 Mb window	7.27%	15.36%	<0.0001
≥21 genes within 1 Mb window	1.16%	5.02%	<0.0001
Number of CISs			
≥2 vectors in 30 kb window	4	1.42	0.06
≥3 vectors in 50 kb window	1	0.01	<0.01
≥4 vectors in 100 kb window	0	0.00	1.00

CIS, common integration site; χ HIV, chimeric human immunodeficiency virus type 1.

vector system, but also other factors in the present protocol of rhesus HSC transduction and transplantation. In this study, we used a culture condition of 24-hour prestimulation (with cytokines and without vectors) and 24-hour transduction (with cytokines and vectors, single transduction) with the vesicular stomatitis virus G (VSV-G)-pseudotyped χ HIV or SIV vectors at a multiplicity of infection 50, whereas the previous study (with SIV vector only) used a culture condition of 36-hour prestimulation and 36-hour transduction (twice transduction of 24 hours and 12 hours) using the SIV vector with an amphotropic envelope at a multiplicity of infection of 1. Perhaps the longer *ex vivo* culture previously used reduced the repopulating ability of rhesus HSCs, resulting in less efficient engraftment and lower marking levels. This explanation is supported by our recent data which demonstrated that 24-hour *ex vivo* culture showed higher repopulating ability of human CD34⁺ cells in humanized xenograft mice, as compared with that of 48-hour culture.³⁰ The other possibility is that the amphotropic envelope might have lower transduction efficiency for long-term hematopoietic progenitor cells as compared with VSV-G envelope, which has been previously suggested in a humanized mouse model using HIV1 vectors.³¹

CD34 expression is widely used clinically as a HSC marker. However, the CD34⁺ cell population contains not only HSCs but also early progenitor cells.^{32,33} Previously, we demonstrated a

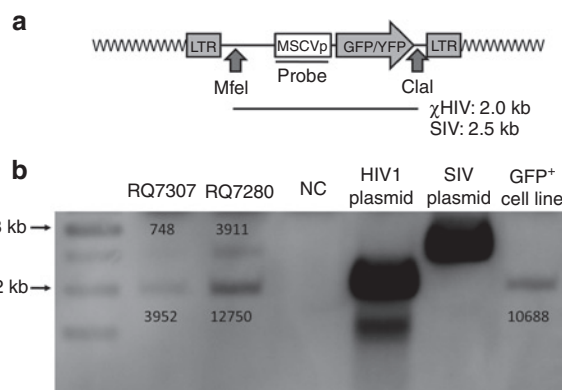


Figure 4 Integrated copy numbers of chimeric human immunodeficiency virus type 1 (χ HIV) and simian immunodeficiency virus (SIV) vectors *in vivo*. **(a)** We evaluated the integrated vector copy number of the χ HIV vector and SIV vector in transplanted animals (RQ7307 in 11 months after transplantation and RQ7280 in 8 months) by Southern blot analysis. As control, we used a monoclonal GFP⁺ cell line in which one copy of HIV1 vector was integrated into single cells. The vector sequences were cut out by MfeI and ClaI restriction enzymes, and these signals were detected by the MSCV promoter probe as 2.0 k bands for the χ HIV vector and 2.5 k bands for the SIV vector. **(b)** The densities of vector signals are described next to the appropriate bands. The vector signals were divided by the control signal to calculate the average vector copy numbers. The χ HIV vector showed three- to fivefold higher average vector copy numbers as compared with the SIV vector (RQ7307: 0.37 versus 0.07 and RQ7280: 1.19 versus 0.37). LTR, long terminal repeat; MSCV, murine stem cell virus; NC, negative control.

significant correlation between *in vitro* %GFP of cultured CD34⁺ cells before transplantation and *in vivo* %GFP of rhesus peripheral blood cells 6 months after transplantation.³⁴ In this study, we cultured a small amount of the transduced CD34⁺ cells to evaluate transduction rates of bulk CD34⁺ cells (cultured for an additional 2 days) and colony forming units (cultured for an additional 6 days). The cultured CD34⁺ cells showed higher transduction rates with SIV vectors than with the χ HIV vector ($P < 0.01$), whereas there was no significant difference in transduction rates in colony forming units between the χ HIV and the SIV vectors. In rhesus peripheral blood cells, the χ HIV vector showed equivalent or higher transgene expression rates as compared with those of the SIV vector. This suggests that the SIV vector transduced early progenitor cells more efficiently than the χ HIV vector.

We evaluated integrated vector copy numbers of both χ HIV vector and SIV vector in rhesus peripheral blood cells using Southern blot analysis and real time PCR. The χ HIV vector showed an estimated three- to fivefold higher vector integration *in vivo*, as compared with the SIV vector, whereas the χ HIV vector had estimated one- to threefold higher transgene expression rates. The χ HIV vector resulted in a more efficient transduction of hematopoietic repopulating cells, over the long term. These results suggest that higher transgene expression rates in the χ HIV vector might result from increased viral integration. In addition, our real time PCR data revealed a relative overestimation of the average vector copy numbers as compared with Southern blot analysis, especially in the animal RQ7280 who has high transgene expression rates (20–30% in the χ HIV vector). We have empirically used Southern blot analysis to calculate average vector copy numbers because PCR techniques are suitable for sensitive detection with

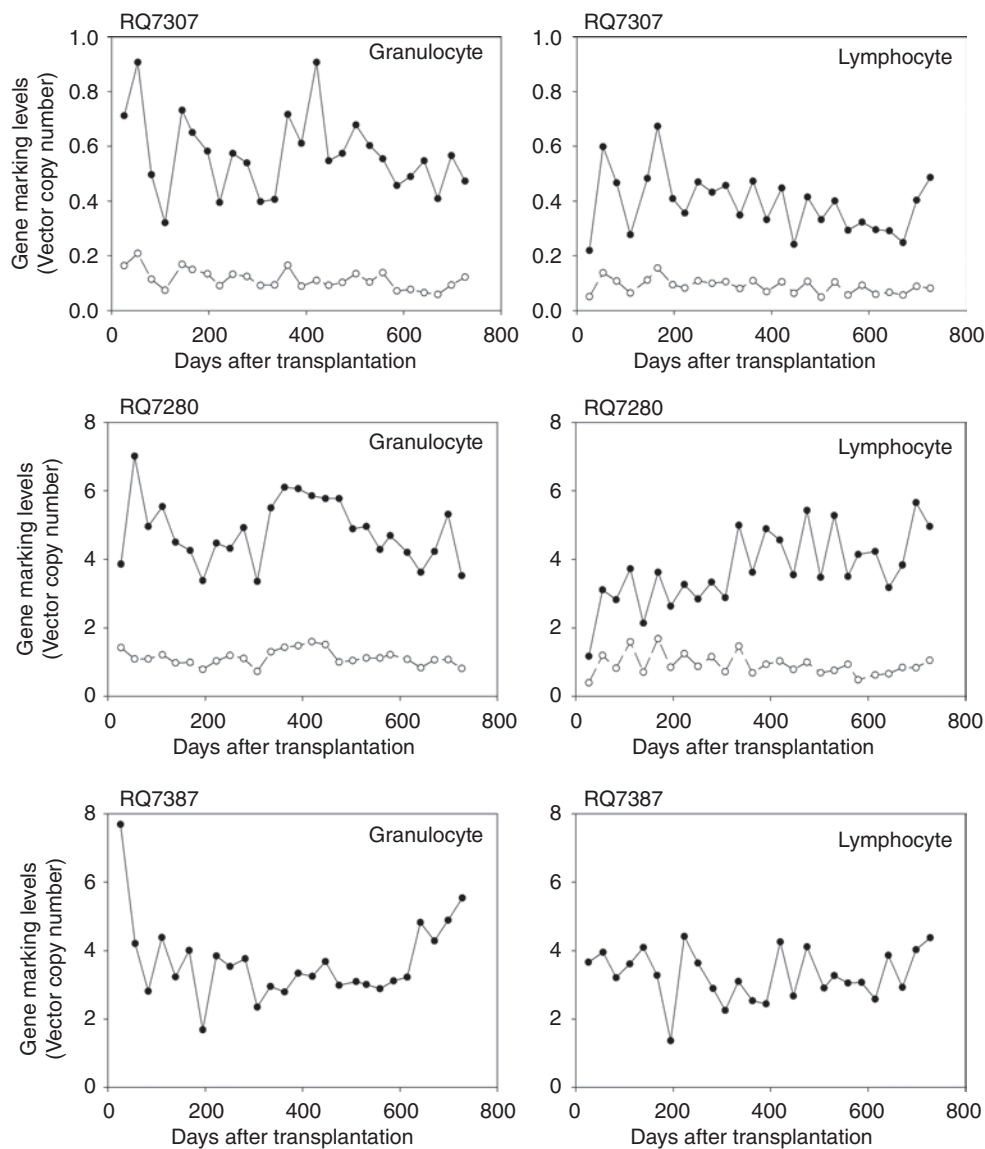


Figure 5 Long-term gene marking in peripheral blood cells of transplanted rhesus macaques. We evaluated gene marking levels of the chimeric human immunodeficiency virus type 1 (χ HIV) vector and simian immunodeficiency virus (SIV) vector in granulocytes and lymphocytes by real time polymerase chain reaction at various time points following transplantation. In all three animals, similar gene marking levels continued in peripheral blood cells for 2 years. The χ HIV vector had around fourfold higher gene marking levels, as compared with the SIV vector, in the competitive assay animals (RQ7307 and RQ7280).

a potential risk of overestimation. In our high marking animals, real time PCR might be too sensitive to accurately estimate vector copy numbers.

We then evaluated integration sites of the χ HIV vector in rhesus peripheral blood cells using linear amplification-mediated PCR techniques. We found a total of 344 unique integration sites, which suggested polyclonal reconstitution of rhesus peripheral blood cells. Previously, we demonstrated no monoclonal expansion in peripheral blood cells with stable transgene expression rates in χ HIV transplanted rhesus macaques.²² In the three rhesus macaques in this study, no clonal expansion of transduced blood cells was also suggested by stable blood counts for 2 years, stable transgene expression rates and marking levels, and no overexpression of *LMO2* and *HMG2* genes in peripheral blood mononuclear

cells (**Supplementary Figure S2** online), which were reported as genes responsible for insertional mutagenesis in previous gene therapy trials.^{4,35}

In the present study, integration analysis showed a propensity of integration of the χ HIV vector into gene regions, especially introns, and less integration upstream of genes. No specific relationship was noted for the integration sites in CpG islands (suggesting promoter regions) and the common integration sites with the χ HIV vector. When compared with previous data, the integration sites of the χ HIV vector were similar to those of both HIV1 and SIV vectors, but they differed from those of γ -retroviral vectors in keeping with previous reports.^{7-9,27-29,36} These data are consistent with theoretically predictable results because the χ HIV vector system was constructed similarly to the conventional HIV1

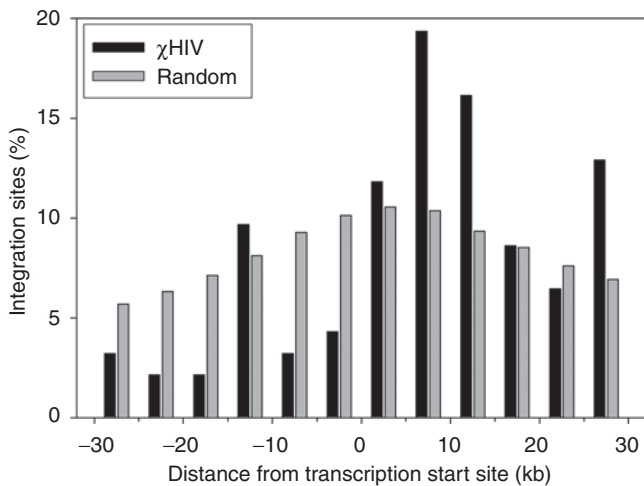


Figure 6 Distribution of chimeric human immunodeficiency virus type 1 (χ HIV) vector integration sites in a 30 kb window around transcription start sites (TSSs). We evaluated the distribution of χ HIV vector integration sites from TSSs in peripheral blood cells of the animal RQ7387, and compared these to sites from a random control. The χ HIV vector demonstrated a tendency towards more integrations inside of genes and fewer integration upstream of the TSSs.

vector system in combination with the SIV capsid and the VSV-G envelope.

Interestingly, the present study showed that the vast majority of integration sites occur in regions of average gene density (0–10 genes per Mb), whereas other studies of SIV and γ -retroviral vectors showed integration into regions of higher gene density.^{27,28} This difference may be explained by our shorter *ex vivo* transduction protocol.³⁰ These data also suggest that our transduction protocol can potentially induce less gene activation in long-term progenitor cells, resulting in lower gene density around integration sites.

Lentiviral vectors thus appear well suited for the hemoglobin disorders, and clinical trials have already been initiated. An HIV1 vector system was used recently in a β -thalassemia gene therapy trial in which two patients received autologous bone marrow CD34⁺ cells that were transduced with a SIN-HIV1 vector containing the β -globin gene under the control of the β -globin locus control region, the β -globin promoter, and the 3'-untranslated region.³⁵ The first patient experienced engraftment failure and received HSC rescue by back-up CD34⁺ cells, whereas the second patient became transfusion independent at 1 year after transplantation. This transfusion independence was, however, due in equal parts to a rise in endogenous hemoglobin F, endogenous hemoglobin E, and vector derived β -globin. In addition, the rise of vector derived β -globin was associated with an increase of a dominant clone that had an activation of the *HMG2* gene. These results, although encouraging for the field, also demonstrate that significant progress will be necessary before this approach can be expected to benefit those with other genotypes in which there is no production of the β -chain or especially, production of a pathologic β -chain such as that characterizing sickle cell disease.

In summary, this study demonstrates that the χ HIV vector can efficiently transduce rhesus long-term hematopoietic progenitor cells at levels comparable with SIV-based vectors. This χ HIV

vector system should allow preclinical testing of a broad range of HIV1-based therapeutic vectors in large animal models, especially for RBC diseases.

METHODS

Lentiviral vector preparation. We previously developed the χ HIV vector system to allow for efficient transduction of both human and rhesus hematopoietic cells.^{22,37} The χ HIV vector and SIV vector were prepared by four plasmid cotransfections of 293T cells, as previously described.^{21,30,38–40} The GFP-expressing χ HIV vector (χ HIV-GFP) was prepared by χ HIV Gag/Pol plasmid, HIV1 Rev/Tat plasmid, VSV-G envelope plasmid, and GFP-expressing SIN-HIV1 vector plasmid (pCL20cMpGFP).²² The YFP-expressing SIV vector (SIV-YFP) was prepared by SIV Gap/Pol plasmid, SIV Rev/Tat plasmid, and VSV-G envelope plasmid, YFP-expressing SIN-SIV vector plasmid (pCL20c SLFR MSCV-YFP) which was constructed by exchanging GFP to YFP in pCL20c SLFR MSCV-GFP.²¹ In both vectors, the transgenes were driven by a murine stem cell virus promoter.

Rhesus hematopoietic stem cell transplantation. We previously developed the autologous peripheral blood stem cells transplantation model in rhesus macaques by granulocyte colony-stimulating factor (kindly provided by Amgen, Thousand Oaks, CA) and stem cell factor (kindly provided by Swedish Orphan Biovitrum AB, Stockholm, Sweden) mobilization (Figure 1a).⁴¹ After immunoselection of rhesus CD34⁺ cells, as previously described,²² the CD34⁺ cells were cultured in fibronectin CH-296-coated (RetroNectin; TaKaRa, Otsu, Shiga, Japan) flasks using serum-free X-VIVO10 media (Lonza, Allendale, NJ) containing stem cell factor, FMS-related tyrosine kinase 3 ligand, and thrombopoietin (all 100 ng/ml; R&D Systems, Minneapolis, MN). After 24-hour prestimulation, half of the cells were transduced at multiplicity of infection 50 with the χ HIV-GFP vector in the fresh media with the same concentration of cytokines and the other half of the cells were transduced with SIV-YFP vector, using the same conditions, for both animals (RQ7307 and RQ7280). In an additional animal (RQ7387), the CD34⁺ cells were transduced with the χ HIV-GFP vector alone at multiplicity of infection 50. After 24-hour transduction, the transduced cells were infused into rhesus macaques, which had undergone myeloablative conditioning therapy of split dose total body irradiation (5Gyx2, total 10Gy). The *in vitro* transduction efficiency was evaluated by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ) using a small number of transduced rhesus CD34⁺ cells following culture in liquid media for an additional 2 days. In addition, the transduced rhesus CD34⁺ cells were cultured in methylcellulose media (1,000 cells in 1.5 ml) for an additional 5–6 days. The transduction efficiency in colony forming units was calculated by GFP (or YFP) positive and negative colony counts, which were evaluated by fluorescence microscope.²² After engraftment, we evaluated the complete blood counts, transgene expression by fluorescence microscope and flow cytometry (Figure 2c), and integration site analysis. In addition, we evaluated GFP expression in RBCs by confocal microscope (Carl Zeiss, Thornwood, NY) under ultraviolet light. Three dimension images were produced by LSM Image Browser Rel. 4.2 (Carl Zeiss).

Southern blot analysis. Viral integration into genomic DNA was evaluated by digesting 10 μ g total DNA extracted from peripheral blood mononuclear cells with MfeI and ClaI restriction enzymes (New England Biolabs, Ipswich, MA) (Figure 4a). The signals were detected by the murine stem cell virus-promoter probe (the 457b MluI-Tth111I fragment from pCL20c MpGFP) using chemifluorescent reactions. The two vector signals were separated by size (2.0 k bands for the χ HIV vector and 2.5 k bands for the SIV vector). The density of vector signals was evaluated by ImageJ (National Institutes of Health, Bethesda, MD) and these were compared with that of a monoclonal cell line in which a single copy of HIV1 vector was integrated. The vector signals were divided by the control signal to calculate average integration vector copy numbers.

Real time PCR. Viral gene marking levels in peripheral blood cells were evaluated by real time PCR. Genomic DNA was extracted from granulocytes and lymphocytes in transplanted animals, which were separated by mononuclear cell separation. The χ HIV-GFP and SIV-YFP vector signals were detected by GFP specific probe/primers and YFP specific probe/primers, as previously described.²² Average vector copy numbers were calculated by as compared with those of a monoclonal cell line in which a single copy of HIV1 vector was integrated.

Gene expression levels of *LMO2* and *HMG2* in peripheral blood cells were evaluated by real time reverse transcription PCR. Total RNA was extracted from peripheral mononuclear cells in transplanted animals, and cDNA was synthesized by a reverse transcriptase (SuperScript III Reverse Transcriptase; Life Technologies, Carlsbad, CA). The gene signals were detected by specific probe/primers targeting rhesus *LMO2* exons 4 and 5 and *HMG2* exons 2 and 3 (TaqMan Gene Expression Assay; Life Technologies) and standardized by 18S ribosomal RNA (TaqMan Ribosomal RNA Control Reagents; Life Technologies).

Integration site analysis. We evaluated the integration sites of the χ HIV vector in rhesus long-term repopulating cells by performing linear amplification-mediated PCR using peripheral blood samples from animal RQ7387 at 0.5–1.5 years after transplantation, as previously described.^{7,21,38,42} Briefly, the HIV3-I primer (5'-biotin-TTTTGCCTGTACTGGGTCTCTCTG-3') targets the LTR sequence and it can be used for linear amplification of the 3'-end junction. The amplified DNA was digested by Tsp509I restriction enzyme, and it was ligated to asymmetric DNA-linker (LC-Tsp-a: 5'-ACTGACAGCGGAT AATCGGTGCAGTAGCATACTAGA G-3', LC-Tsp-b: 5'-AAT TCTCTA GTA TGC TAC TCG CAC CGA TTA TCT CCG CTG TCA GT-3'). The ligated DNA of 3'LTR-genomic DNA-linker was amplified by nested PCR using HIV3-II (5'-TCTCTGCTAAC TAGGGAAC-3) and LC-1 (5'-ACTGACAGCGGAT AATCG-3') primers for first PCR, and HIV3-III (5'-GCC TTG AGT GCT TCA AGT AGT G-3') and LC-2 (5'-GTG CGA GTA GCA TAC TAG AG-3') primers for second PCR. The HIV3-II and HIV3-III primers target the LTR sequence, and the LC-1 and LC-2 primers target the linker sequence. Integration site sequences were validated with the intact LTR-genomic junction. We aligned the 344 unique integration sites to the rhesus macaque genome assembly (Mmul 1.0, January 2006) and determined their genomic context by comparing their locations to those of the 21,905 protein coding genes in Ensembl 61 (February 2011). To evaluate the statistical significance of our findings, we created 10,000 random datasets, each with 344 mock integration sites, as previously described.^{27–29} We evaluated common integration sites with the χ HIV vector using three criteria: second order: two or more integration sites within a 30 kb window, third order: three or more integration sites within a 50 kb window, and fourth order: four or more integration sites within a 100 kb window.²⁸

Statistical analysis. Statistical analyses were performed using JMP 9 software (SAS Institute, Cary, NC). Transduction rates of two vectors were evaluated by the student's *t*-test. A *P* value of <0.01 was deemed significant. Standard errors of the mean are shown as error bars.

SUPPLEMENTARY MATERIAL

Figure S1. Peripheral blood counts after transplantation.

Figure S2. *LMO2* and *HMG2* gene expression in peripheral blood cells of transplanted rhesus macaques.

ACKNOWLEDGMENTS

This work is supported by the intramural research programs of the National Institute of Diabetes, Digestive, and Kidney Diseases, the National Heart, Lung, and Blood Institute, and the National Human Genome Research Institute at the National Institutes of Health. We thank the animal care staff and technicians at 5 Research Court for their excellent care and handling of the animals. We would also like to thank William DeGraff and Dr Jim Mitchell of the Radiation Biology Branch, National Cancer Institute,

for the use of the Eldorado Cobalt-60 irradiator. We would like to thank Heidi Dorward, Medical Genetics Branch, National Human Genome Research Institute, for the use of the confocal microscope. The authors have declared that no conflict of interest exists.

REFERENCES

- Schwarzwaelder, K, Howe, SJ, Schmidt, M, Brugman, MH, Deichmann, A, Glimm, H *et al.* (2007). Gammaretrovirus-mediated correction of SCID-X1 is associated with skewed vector integration site distribution in vivo. *J Clin Invest* **117**: 2241–2249.
- Aiuti, A, Slavin, S, Aker, M, Ficara, F, Deola, S, Mortellaro, A *et al.* (2002). Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* **296**: 2410–2413.
- Aiuti, A, Cattaneo, F, Galimberti, S, Benninghoff, U, Cassani, B, Callegaro, L *et al.* (2009). Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med* **360**: 447–458.
- Hacein-Bey-Abina, S, Garrigue, A, Wang, GP, Soulier, J, Lim, A, Morillon, E *et al.* (2008). Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest* **118**: 3132–3142.
- Hacein-Bey-Abina, S, von Kalle, C, Schmidt, M, Le Deist, F, Wulffraat, N, McIntyre, E *et al.* (2003). A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* **348**: 255–256.
- Ott, MG, Schmidt, M, Schwarzwaelder, K, Stein, S, Siler, U, Koehl, U *et al.* (2006). Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EV11, PRDM16 or SETBP1. *Nat Med* **12**: 401–409.
- Hematti, P, Hong, BK, Ferguson, C, Adler, R, Hanawa, H, Sellers, S *et al.* (2004). Distinct genomic integration of MLV and SIV vectors in primary hematopoietic stem and progenitor cells. *PLoS Biol* **2**: e423.
- Mitchell, RS, Beitzel, BF, Schroder, AR, Shinn, P, Chen, H, Berry, CC *et al.* (2004). Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol* **2**: E234.
- Wu, X, Li, Y, Crise, B and Burgess, SM (2003). Transcription start regions in the human genome are favored targets for MLV integration. *Science* **300**: 1749–1751.
- Ryu, BY, Evans-Galea, MV, Gray, JT, Bodine, DM, Persons, DA and Nienhuis, AW (2008). An experimental system for the evaluation of retroviral vector design to diminish the risk for proto-oncogene activation. *Blood* **111**: 1866–1875.
- May, C, Rivella, S, Callegari, J, Heller, G, Gaensler, KM, Luzzatto, L *et al.* (2000). Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin. *Nature* **406**: 82–86.
- Pestina, TI, Hargrove, PW, Jay, D, Gray, JT, Boyd, KM and Persons, DA (2009). Correction of murine sickle cell disease using gamma-globin lentiviral vectors to mediate high-level expression of fetal hemoglobin. *Mol Ther* **17**: 245–252.
- Rivella, S, May, C, Chadburn, A, Riviere, I and Sadelain, M (2003). A novel murine model of Cooley anemia and its rescue by lentiviral-mediated human beta-globin gene transfer. *Blood* **101**: 2932–2939.
- Persons, DA, Allay, ER, Sabatino, DE, Kelly, P, Bodine, DM and Nienhuis, AW (2001). Functional requirements for phenotypic correction of murine beta-thalassemia: implications for human gene therapy. *Blood* **97**: 3275–3282.
- Imren, S, Payen, E, Westerman, KA, Pawliuk, R, Fabry, ME, Eaves, CJ *et al.* (2002). Permanent and panerythroid correction of murine beta thalassemia by multiple lentiviral integration in hematopoietic stem cells. *Proc Natl Acad Sci USA* **99**: 14380–14385.
- Puthenveetil, G, Scholes, J, Carbonell, D, Qureshi, N, Xia, P, Zeng, L *et al.* (2004). Successful correction of the human beta-thalassemia major phenotype using a lentiviral vector. *Blood* **104**: 3445–3453.
- Malik, P, Arumugam, Pl, Yee, JK and Puthenveetil, G (2005). Successful correction of the human Cooley's anemia beta-thalassemia major phenotype using a lentiviral vector flanked by the chicken hypersensitive site 4 chromatin insulator. *Ann N Y Acad Sci* **1054**: 238–249.
- Stremlau, M, Owens, CM, Perron, MJ, Kiessling, M, Autissier, P and Sodroski, J (2004). The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* **427**: 848–853.
- Stremlau, M, Perron, M, Lee, M, Li, Y, Song, B, Javanbakht, H *et al.* (2006). Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor. *Proc Natl Acad Sci USA* **103**: 5514–5519.
- Kootstra, NA, Munk, C, Tonn, N, Landau, NR and Verma, IM (2003). Abrogation of postentry restriction of HIV-1-based lentiviral vector transduction in simian cells. *Proc Natl Acad Sci USA* **100**: 1298–1303.
- Hanawa, H, Hematti, P, Keyvanfar, K, Metzger, ME, Krouse, A, Donahue, RE *et al.* (2004). Efficient gene transfer into rhesus repopulating hematopoietic stem cells using a simian immunodeficiency virus-based lentiviral vector system. *Blood* **103**: 4062–4069.
- Uchida, N, Washington, KN, Hayakawa, J, Hsieh, MM, Bonifacino, AC, Krouse, AE *et al.* (2009). Development of a human immunodeficiency virus type 1-based lentiviral vector that allows efficient transduction of both human and rhesus blood cells. *J Virol* **83**: 9854–9862.
- Hanawa, H, Hargrove, PW, Kepes, S, Srivastava, DK, Nienhuis, AW and Persons, DA (2004). Extended beta-globin locus control region elements promote consistent therapeutic expression of a gamma-globin lentiviral vector in murine beta-thalassemia. *Blood* **104**: 2281–2290.
- Persons, DA, Hargrove, PW, Allay, ER, Hanawa, H and Nienhuis, AW (2003). The degree of phenotypic correction of murine beta-thalassemia intermedia following lentiviral-mediated transfer of a human gamma-globin gene is influenced by chromosomal position effects and vector copy number. *Blood* **101**: 2175–2183.
- Samakoglu, S, Lisowski, L, Budak-Alpdogan, T, Usachenko, Y, Acuto, S, Di Marzo, R *et al.* (2006). A genetic strategy to treat sickle cell anemia by coregulating globin transgene expression and RNA interference. *Nat Biotechnol* **24**: 89–94.
- Arumugam, Pl, Scholes, J, Perelman, N, Xia, P, Yee, JK and Malik, P (2007). Improved human beta-globin expression from self-inactivating lentiviral vectors carrying the chicken hypersensitive site-4 (CHS4) insulator element. *Mol Ther* **15**: 1863–1871.

27. Kim, YJ, Kim, YS, Larochelle, A, Renaud, G, Wolfsberg, TG, Adler, R *et al.* (2009). Sustained high-level polyclonal hematopoietic marking and transgene expression 4 years after autologous transplantation of rhesus macaques with SIV lentiviral vector-transduced CD34+ cells. *Blood* **113**: 5434–5443.
28. Métais, JY, Topp, S, Doty, RT, Borate, B, Nguyen, AD, Wolfsberg, TG *et al.* (2010). Feline leukemia virus integrase and capsid packaging functions do not change the insertion profile of standard Moloney retroviral vectors. *Gene Ther* **17**: 799–804.
29. Winkler, T, Cantilena, A, Métais, JY, Xu, X, Nguyen, AD, Borate, B *et al.* (2010). No evidence for clonal selection due to lentiviral integration sites in human induced pluripotent stem cells. *Stem Cells* **28**: 687–694.
30. Uchida, N, Hsieh, MM, Hayakawa, J, Madison, C, Washington, KN and Tisdale, JF (2011). Optimal conditions for lentiviral transduction of engrafting human CD34+ cells. *Gene Ther* **18**: 1078–1086.
31. Kim, YS, Wielgosz, MM, Hargrove, P, Kepes, S, Gray, J, Persons, DA *et al.* (2010). Transduction of human primitive repopulating hematopoietic cells with lentiviral vectors pseudotyped with various envelope proteins. *Mol Ther* **18**: 1310–1317.
32. Bonnet, D (2002). Haematopoietic stem cells. *J Pathol* **197**: 430–440.
33. Guo, Y, Lübbert, M and Engelhardt, M (2003). CD34- hematopoietic stem cells: current concepts and controversies. *Stem Cells* **21**: 15–20.
34. Uchida, N, Bonifacino, A, Krouse, AE, Metzger, ME, Csako, G, Lee-Stroka, A *et al.* (2011). Accelerated lymphocyte reconstitution and long-term recovery after transplantation of lentiviral-transduced rhesus CD34+ cells mobilized by G-CSF and plerixafor. *Exp Hematol* **39**: 795–805.
35. Cavazzana-Calvo, M, Payen, E, Negre, O, Wang, G, Hehir, K, Fusil, F *et al.* (2010). Transfusion independence and HMGA2 activation after gene therapy of human β -thalassaemia. *Nature* **467**: 318–322.
36. Hayakawa, J, Washington, K, Uchida, N, Phang, O, Kang, EM, Hsieh, MM *et al.* (2009). Long-term vector integration site analysis following retroviral mediated gene transfer to hematopoietic stem cells for the treatment of HIV infection. *PLoS ONE* **4**: e4211.
37. Hatzioannou, T, Princiotta, M, Piatak, M Jr, Yuan, F, Zhang, F, Lifson, JD *et al.* (2006). Generation of simian-tropic HIV-1 by restriction factor evasion. *Science* **314**: 95.
38. Uchida, N, Hanawa, H, Dan, K, Inokuchi, K and Shimada, T (2009). Leukemogenesis of b2a2-type p210 BCR/ABL in a bone marrow transplantation mouse model using a lentiviral vector. *J Nihon Med Sch* **76**: 134–147.
39. Uchida, N, Washington, KN, Lap, CJ, Hsieh, MM and Tisdale, JF (2011). Chicken HS4 insulators have minimal barrier function among progeny of human hematopoietic cells transduced with an HIV1-based lentiviral vector. *Mol Ther* **19**: 133–139.
40. Hanawa, H, Kelly, PF, Nathwani, AC, Persons, DA, Vandergriff, JA, Hargrove, P *et al.* (2002). Comparison of various envelope proteins for their ability to pseudotype lentiviral vectors and transduce primitive hematopoietic cells from human blood. *Mol Ther* **5**: 242–251.
41. Donahue, RE, Kirby, MR, Metzger, ME, Agricola, BA, Sellers, SE and Cullis, HM (1996). Peripheral blood CD34+ cells differ from bone marrow CD34+ cells in Thy-1 expression and cell cycle status in nonhuman primates mobilized or not mobilized with granulocyte colony-stimulating factor and/or stem cell factor. *Blood* **87**: 1644–1653.
42. Schmidt, M, Zickler, P, Hoffmann, G, Haas, S, Wissler, M, Muessig, A *et al.* (2002). Polyclonal long-term repopulating stem cell clones in a primate model. *Blood* **100**: 2737–2743.