

Efficient Marking of Murine Long-Term Repopulating Stem Cells Targeting Unseparated Marrow Cells at Low Lentiviral Vector Particle Concentration

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HIV-1-derived lentivirus vectors offer unique biological properties for gene delivery to hematopoietic stem cells and, when used at high multiplicities of infection (m.o.i.), permit efficient gene transfer after minimal target cell stimulation. However, such a strategy has been shown to promote multicopy proviral integration, potentially increasing the risk of insertional mutagenesis. To minimize cell manipulation, we targeted unseparated marrow and demonstrated that transduction at an m.o.i. of 1 resulted in up to 12% vector-modified peripheral blood leukocytes and successful repopulation of secondary recipients with vector-marked cells. Real-time PCR showed on average 1.8 proviral integrants per GFP-marked cell. By comparison, a cohort of animals transplanted with cells transduced at m.o.i. of 10 under otherwise unchanged conditions showed up to 45% marking with an average of 7 copies per GFP-expressing cell. Both m.o.i. groups demonstrated sustained proviral expression with stable GFP fluorescence intensity. In summary, we have identified conditions for lentiviral gene transfer involving minimal *ex vivo* target cell manipulation and have shown that the m.o.i. is a critical determinant of proviral copy number in lentivirus-transduced murine long-term repopulating cells. Thus, gene transfer efficiencies may be limited when single-copy integration is desired and additional strategies such as *in vivo* selection may be required to improve the frequency of gene-modified cells.

Key Words: gene transfer, lentivirus, proviral integration

INTRODUCTION

At present, oncoretrovirus vectors are the most widely used gene transfer vectors in animal models and clinical trials. However, a number of obstacles prevent their routine use. These include low integration frequency in stem cells, loss of stem cell properties during *in vitro* manipulation, silencing of transgene expression, and the potential for pathogenic genomic insertion and mutagenicity.

HIV-derived lentivirus vectors by contrast offer advantages for the genetic modification of hematopoietic stem cells due to their ability to transduce nondividing cells [1–3], a noted limitation of oncoretroviral vectors [4–6]. Gene transfer efficiency with lentivirus vectors can be substantially improved by the use of extended *ex vivo* culture and

cytokine stimulation [7–10]. However, this strategy also risks apoptosis and premature differentiation among target cells during prolonged periods of *in vitro* manipulation, including the enrichment of stem cells, cytokine activation, and long periods of exposure to vector [11–13].

Alternatively, improvement of gene delivery to repopulating cells can be accomplished by using high concentrations of vesicular stomatitis virus G-protein (VSV-G)-pseudotyped vector particles [10,14], especially in murine transplantation studies in which lentivirus gene transfer is relatively less efficient [15,16]. The resultant multicopy proviral integration, on the other hand, may increase the risk for activation of cellular genes, including oncogenes, and may thereby further contribute to the risk of insertional mutagenesis, as illustrated in recent reports [17–22].

Ideally, a protocol for the introduction of therapeutic genes should address all the following: (1) minimal manipulation of target cells, allowing maximum viability and retention of stem cell properties; (2) maximum number of target cells genetically modified; (3) single or minimal number of proviral insertions in each cell; and (4) sustained and uniform expression in cells of therapeutic interest. We envision that such a strategy of minimal manipulation and *ex vivo* stimulation would be particularly well suited to gene replacement therapy in Fanconi anemia (FA), in which progressive marrow aplasia and poor *ex vivo* viability are critical limitations to the number of stem cells available for genetic correction. In this study, we have therefore attempted to minimize *in vitro* time, cytokine exposure, and proviral insertion events per cell by using an abbreviated, low multiplicity of infection (m.o.i.) exposure of unfractionated marrow to lentiviral vectors.

RESULTS

Experimental design

We devised conditions of minimal hematopoietic target cell manipulation favoring single-copy proviral integration by transducing unseparated murine marrow cells at a low m.o.i. For the transduction of hematopoietic target cells with oncoretrovirus vectors, a dose–response relationship between vector particle to cell number ratio (m.o.i.) and gene transfer efficiency on the one hand and the resulting proviral copy number on the other hand has previously been demonstrated [23–25]. Hypothesizing that a similar relationship may exist using VSV-G-pseudotyped lentivirus vectors, our experimental design and cohort assignment, outlined in Table 1, focused on a comparison of m.o.i. of 1 and 10 and explored cytokine support and prestimulation before transduction as minor variables. Study endpoints were the repopulation of primary and secondary recipients with genetically marked cells, copy number determination in progeny of transduced cells, and, to a lesser extent, long-term transgene expression. These

experiments were not designed to provide an exhaustive evaluation of transduction parameters, but as a basis upon which to explore further strategies limiting target cell copy number while optimizing gene transfer efficiency.

Cytokine exposure promotes gene transfer and *in vivo* persistence of murine marrow cells exposed to lentivirus vector at an m.o.i. of 1

Following transduction and injection of cells, animals rapidly repopulated except for one early death each in cohorts II and III and three animals in cohort IV (prestimulation and transduction in the absence of cytokines, Table 1). Animals were evaluated for gene transfer at serial time points after transplantation by flow-cytometric analysis of GFP expression in peripheral blood leukocytes (Fig. 1). As predicted, based on *in vitro* rates of gene transfer to bulk-transduced cells prior to infusion (Table 1), cohorts that received cells transduced in the absence of cytokines had substantially lower levels of gene transfer at all time points after transplantation, regardless of whether cells underwent prestimulation prior to transduction (Fig. 1A) or were transduced immediately upon harvest (Fig. 1B). Cytokine support with murine stem cell factor (mSCF) (S) or the combination of human interleukin-6 (IL-6), mSCF, megakaryocyte growth and development factor (MGDF), and Flt-3 ligand (Flt-3L) (6, S, M, F), on the other hand, led to improved short-term gene transfer rates (up to 23%). Overall transfer rates subsequently declined but remained higher at all time points in mice that received cells transduced in the presence of cytokines. Of note, long-term gene transfer rates in animals that received cells prestimulated and transduced in the presence of mSCF only were similar to those in animals in which the combination of all four cytokines had been used.

Lentiviral transduction of unseparated marrow results in hematopoietic repopulation with gene-modified myeloid and lymphoid progeny

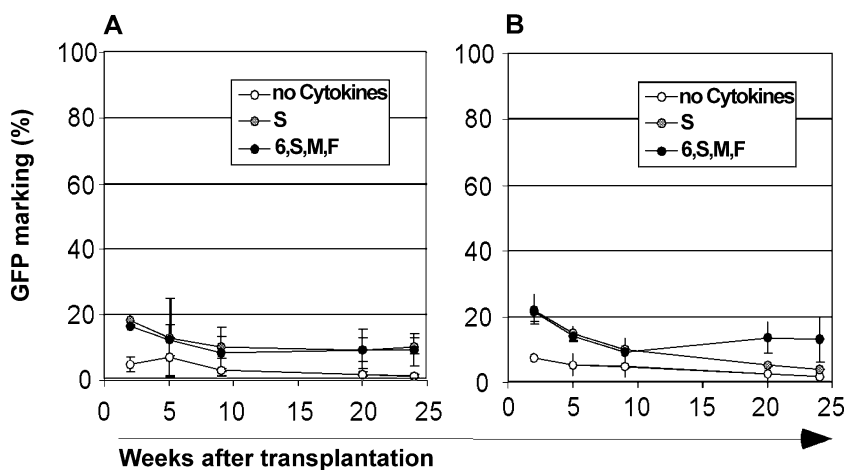
We next determined that the leukocyte subset composition in the peripheral blood at 16 weeks after

Table 1. Cohort assignment and *in vitro* marking

Cohort	m.o.i.	No. of animals at BMT/follow-up	Prestimulation 12 h	Cytokine support ^a	GFP marking <i>in vitro</i> (%)
I	1	5/5	—	—	6.6
II	1	4/3	—	S	14.5
III	1	4/3	—	6, S, M, F	12.2
IV	1	5/2	+	—	5.7
V	1	4/4	+	S	9.2
VI	1	4/4	+	6, S, M, F	7.1

Experimental design for transplant studies at m.o.i. 1. Mice were transplanted as outlined under Materials and Methods. Prestimulation/transduction per cohort were carried out with cytokine support as noted. Gene transfer rates *in vitro* were assessed flow cytometrically on day 5 after transduction in small aliquots of bulk-transduced cells injected into recipient animals. ^aS, murine stem cell factor; 6, interleukin-6; F, Flt-3 ligand; M, megakaryocyte growth and development factor.

FIG. 1. Repopulation of recipients with GFP lentivirus-transduced cells. All animals received grafts of 2×10^6 cells after exposure of cells to concentrated vector supernatant for 12 h under conditions outlined in Table 1. Animals were subjected to retroorbital blood sampling after transplant at the time points indicated, and gene transfer was analyzed. Cohorts of animals received cells (A) without or (B) with prestimulation. *Ex vivo* culture and transduction were performed in the absence of cytokines (open symbols), the presence of mSCF (S; shaded symbols), or the presence of IL-6, mSCF, MGDF, and Flt-3 ligand (6, S, M, F; solid symbols).



transplantation was indistinguishable from that of a steady-state control group (data not shown). To ascertain gene transfer to pluripotent cells, we analyzed GFP marking in myeloid and lymphoid progeny in a group of four animals. Using fluorochrome-labeled, lineage-specific antibodies, flow-cytometric analysis of GFP expression demonstrated genetic marking in B and T lymphocytes (B220, Thy-1) as well as in granulocytes (Gr-1) from all four animals, suggesting that multipotent progenitor or stem cells were transduced. A representative example from one animal with high marking is shown (Fig. 2).

Repopulation of secondary recipients with genetically marked cells

To confirm the transduction of long-term repopulating cells, we sacrificed seven primary recipients at 6 ($n = 2$) and 9 ($n = 5$) months after primary stem cell transplantation. Animals were selected on the basis of higher gene transfer from cohorts III, V, and VI, all of which received cells transduced in the presence of cytokines. By design, and since gene transfer and copy number are known covariates [23], this should have maximized the probability of finding multicopy integrants (see below). We transplanted a total of 17 myeloablated secondary recipients with 2×10^6 donor cells each. Recipient animals ($n = 2-4$ per donor) showed rapid hematopoietic recovery with variable peripheral blood GFP marking and, in some recipients, at a much lower level than the respective donor animals (Fig. 3A). The variability and lower overall frequency of GFP-marked cells in secondary recipients may in part reflect the relatively limited number of cells taken from donor animals and are consistent with oligoclonal marking (below). Taken together with the experiments above indicating gene transfer to myeloid and lymphoid subsets, these studies strongly suggest that a single 12-h vector exposure of unseparated marrow at an m.o.i. of 1 resulted in

genetic marking and long-term persistence of murine hematopoietic stem cells.

Cells transduced with lentivirus vector at an m.o.i. of 1 demonstrate low average copy number in target cell progeny

Average proviral copy numbers can be readily assessed by real-time PCR technology [15,16,26–28]. We there-

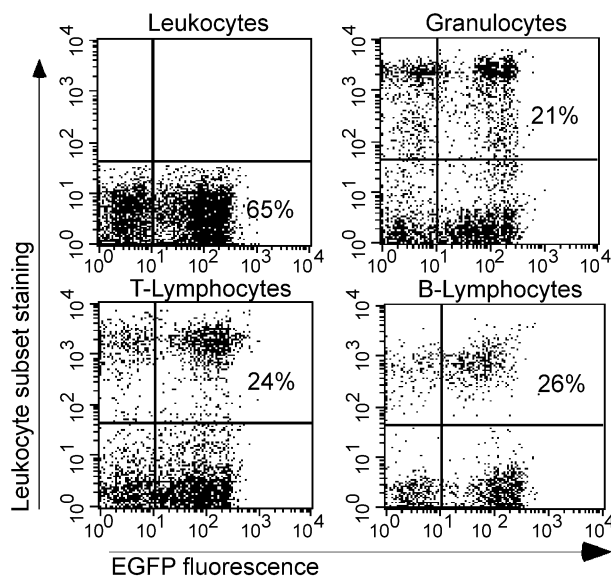


FIG. 2. Gene transfer to murine repopulating/stem cells occurs with marking in lymphoid and myeloid subsets. Analysis of GFP expression 16 weeks after transplantation in an animal with a high gene transfer rate. Phycoerythrin-labeled monoclonal antibodies (expression analyzed in fluorescence channel 2 (FL-2)) were directed against murine B and T cells and granulocytes. GFP expression was analyzed in FL-1. This analysis was performed in three additional animals with similar results.

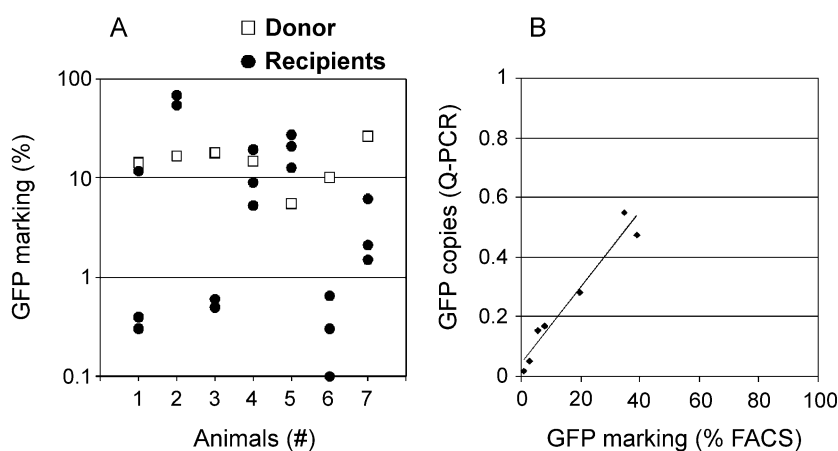


FIG. 3. Transduction at m.o.i. of 1. Engraftment and proviral copy number of genetically marked marrow cells used to transplant secondary recipients. Animals from cohorts III (animals 3 and 4), V (1, 2, 5, and 7), and VI (6) were sacrificed to serve as donors for secondary transplants at 6 ($n = 2$) or 9 ($n = 5$) months. (A) GFP marking in marrow leukocytes in the primary animals at sacrifice (open squares) and the gene transfer rates in individual secondary recipients (solid circles) at 9 weeks after transplantation. Donors were taken from cohorts V, III, and VI. (B) Flow-cytometric analysis for GFP expression and persistence of proviral DNA by real-time PCR. Integrating PCR and FACS data for a given animal, the graph illustrates the integration of 1.8 (SD 0.5) copies on average among GFP-expressing cells, with a strong correlation among the seven animals ($R^2 = 0.95$). Note the y axis range up to 1 copy per cell.

fore used a real-time PCR assay to determine the average proviral copy number in DNA extracted from marrow cells of animals sacrificed at 6 ($n = 2$) or 9 ($n = 5$) months after transplantation. Further correlation with gene transfer rates determined by flow cytometric expression of GFP can provide additional information to calculate the average copy number among genetically marked cells. Results of our PCR and FACS analyses (determined on aliquots from the same bone marrow samples) illustrate the strong correlation between the two modalities and demonstrate an average of 1.8 (standard deviation (SD) 0.5) proviral copies per cell in the seven animals analyzed (Fig. 3B).

Single-copy integration at the clonal level in genetically marked progenitor cells from a primary recipient

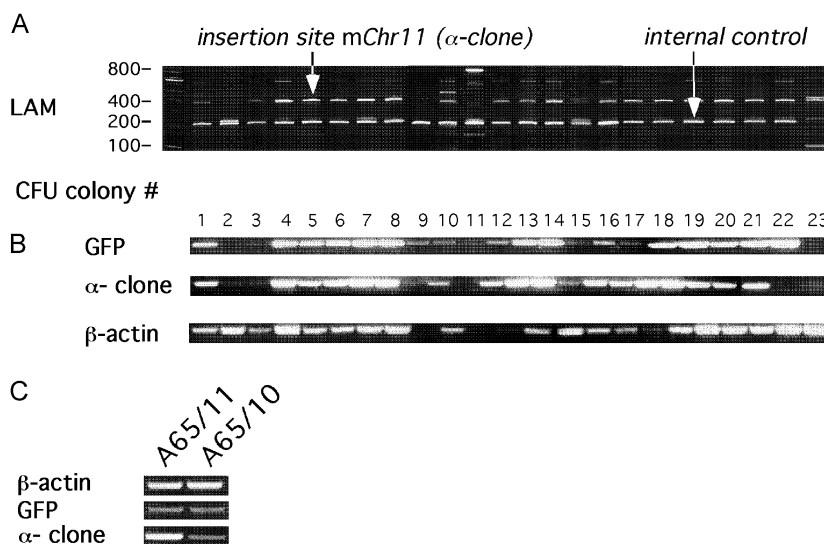
In an effort to analyze proviral copy numbers at the clonal level, we studied hematopoietic progenitor colonies from a single primary recipient of cohort V (prestimulation and transduction in the presence of mSCF) in detail. This was performed not in an attempt to study the number of clones contributing, but to confirm and illustrate the number of integrants per cell. Given the complexity of the analysis, we focused on an animal with high *in vivo* marking, predicting a greater number of provirus-containing progenitor colonies and an increased overall likelihood of finding multicopy integrants, if present. Linear amplification (LAM)-PCR (Fig. 4A) was performed and independently confirmed by direct amplification of GFP and β -actin control PCR from progenitor cell DNA extracts (Fig. 4B). Results indicate repopulation in this animal with a predominant ($=\alpha$) clone indicated by the recurrent pattern of internal control band and a unique recurrent band at 400 bp found in 21 of 23 colonies. Additional distinct clones were identified only in colonies 2 (β -clone) and 23 (γ -

clone). Based on direct sequencing and comparison with the mouse genome draft (April 2003 freeze), the predominant proviral integration site in this animal mapped to chromosome 11 (mChr 11; 16,388,630). To investigate the identity of additional bands on the polyacrylamide gel image (Fig. 4C) and exclude the presence of multicopy insertions in the progeny of CFU-forming cells, we sequenced additional individual bands ($n = 28$) in lanes 2, 3, 10, 11, 13, 15, and 23. Direct sequencing revealed these bands to be PCR recombination artifacts of the known proviral α -clone sequence. This illustrative analysis in a single animal revealed oligoclonal marking with predominance of a single hematopoietic clone. No evidence was found for a second distinct integration site in any of the colonies studied.

A 10-fold increase in lentiviral vector concentration leads to substantial improvements in gene transfer and promotes multicopy integration

While gene transfer levels under the above conditions may be adequate for some therapeutic applications and can be the basis for *in vivo* selection, we were encouraged by the low copy number to explore how an increase in m.o.i. might improve overall marking and test its impact on proviral copy number. We therefore transplanted an additional group of animals with cells transduced under identical conditions (in the presence of cytokines 6, S, M, F), except for a 10-fold increase in vector concentration at transduction. As predicted, these animals showed high-level GFP marking in up to 70% of peripheral blood leukocytes short term and 40% long term after transplantation (Fig. 5A). Again, animals showed normal leukocyte subset composition and substantial marking in all leukocyte subsets (data not shown). Similar to animals that received cells transduced at an m.o.i. of 1, animals from this group served as donors for the successful reconstitution of

FIG. 4. Transduction at m.o.i. of 1 in the presence of mSCF only results in oligoclonal repopulation and single-copy integration. (A) A single animal from cohort V (prestimulation and transduction in the presence of mSCF) was sacrificed 6 months after transplantation, and DNA from marrow progenitor colonies was extracted and subjected for analysis of proviral integration sites. The gel image shows the constant internal control band and additional bands (= presumed proviral inserts) that were directly sequenced. Unique band sequences were compared to the mouse genome database (April 2003 freeze). The majority of bands of minor intensity were PCR recombinant artifacts (see Results). A predominant unique integrant in mChr 11 was identified in 21 of 24 colonies picked (α -clone). (B) Sequence-specific primers amplifying a provirus genomic DNA junction from all colonies directly for the presence of the α -clone, GFP, and β -actin control. (C) DNA was extracted from peripheral blood obtained from two secondary recipients repopulated with marrow cells from this animal. Direct PCR for α -clone, GFP, and β -actin demonstrated the presence of α -clone and GFP proviral progeny in both secondary recipients.



secondary recipients (Fig. 5B). Importantly, the analysis for proviral copy number in marrow cells harvested from primary recipient animals at the time of sacrifice for secondary stem cell transplantation showed a substantial increase in average proviral copy number to 7 (SD 1.9) in GFP-expressing cells (Fig. 5C). These results demonstrate a consistent increase in proviral copy number as a function of vector particle concentration (= dose) at transduction, consistent with similar *in vitro*

observations by others using oncoretrovirus vectors [23].

Proviral GFP expression remains stable over time in primary and secondary recipients regardless of proviral copy number

Given evidence of proviral silencing and downregulation of expression from oncoretroviral integrants [29–31], we investigated the stability of proviral expression by serial

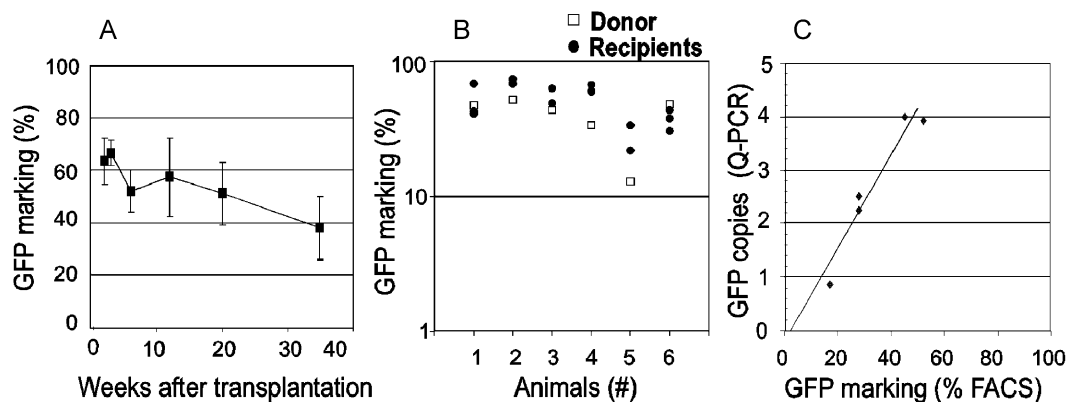


FIG. 5. Transduction at m.o.i. of 10. Gene transfer in primary and secondary recipients and proviral copy number in marrow cells used to transplant secondary recipients. (A) A comparison cohort of six animals received grafts of 2×10^6 cells after a single 12-h vector exposure at an m.o.i. of 10 in the presence of cytokines IL-6, mSCF, MGDF, and Flt-3L under otherwise unchanged conditions. Animals were subjected to retroorbital blood sampling after transplant at the time points indicated, and GFP expression in peripheral blood leukocytes was analyzed. (B) GFP marking in marrow leukocytes in the primary animals at sacrifice (open squares) and the gene transfer rates in individual secondary recipients (solid circles) at 9 weeks after transplantation. (C) Analysis of GFP expression by flow cytometry and persistence of proviral DNA by real-time PCR in five of six animals (no amplification in DNA from one animal was accomplished despite repeated attempts). The graph illustrates the presence of seven (SD 1.9) proviral copies per GFP-expressing cell after transduction at m.o.i. of 10, with a strong correlation among animals ($R^2 = 0.93$). Note the y axis range up to five copies per cell.

posttransplantation analyses of GFP mean fluorescence intensity (MFI; in random units) among peripheral blood leukocytes. To examine the fluorescence intensity in primary and their secondary recipients, we have restricted our analysis to the seven animals who subsequently served as donors for secondary transplants. While this was not a primary endpoint in our study, our observations from serial MFI determinations illustrate a remarkable stability of proviral expression (Fig. 6A) that is maintained in peripheral blood leukocytes of secondary recipients of marrow grafts from these animals (Fig. 6B). These results illustrate sustained and undiminished expression from what are likely predominantly single lentiviral inserts. Predictably, proviral GFP expression from peripheral blood leukocytes in animals that received cells transduced at an m.o.i. of 10, and their secondary recipients, were stable and similarly persistent (Figs. 6C and 6D).

Taken together, the above results establish a correlation between lentiviral vector particle concentration, gene transfer, and target cell proviral copy number.

DISCUSSION

The current study demonstrates that lentiviral gene transfer to murine long-term hematopoietic repopulating cells can be accomplished after a brief *ex vivo* exposure of unseparated marrow cells at a low m.o.i. of 1 and after single cytokine stimulation with mSCF. Using minimal target cell manipulation, we have demonstrated sustained transgene expression and gene transfer to murine stem cells, the efficiency of which is closely linked to target cell copy numbers.

Several lines of evidence from our studies suggest that the strategy of targeting unseparated marrow cells rather than purified stem cells resulted in significant transduction of pluripotent long-term repopulating cells even at limiting vector concentrations. First, flow cytometric analysis demonstrates long-term gene transfer to myeloid and lymphoid subsets, consistent with studies by others, albeit under different transduction and culture conditions [10,16]. Second, we demonstrate hematopoietic recovery of secondary recipients with

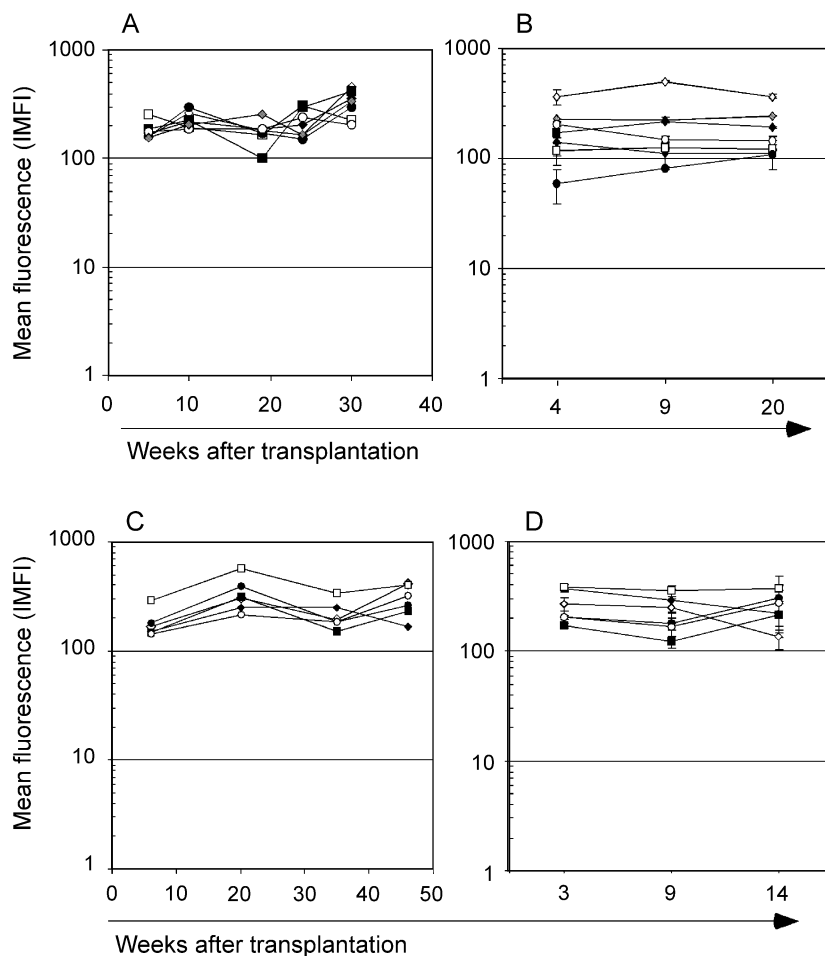


FIG. 6. Persistent transgene expression in primary and secondary recipients of genetically marked cells. Flow-cytometric analysis of GFP mean fluorescence intensity (MFI) was determined using CellQuest software in peripheral blood leukocytes at serial time points after primary and secondary transplant. Analysis of fluorescence intensity in mice that received cells transduced at an m.o.i. of 1 in (A) primary and (B) their secondary recipients is shown. Analysis of fluorescence intensity in mice that received cells transduced at an m.o.i. of 10 in (C) primary and (D) their secondary recipients is also shown. Error bars reflect MFI standard deviations from three secondary recipients per donor animal.

GFP-marked cells from primary recipients, and third, we demonstrate amplification of specific proviral inserts in progenitor colonies from a single primary recipient in the peripheral blood cells of the respective secondary recipients. In targeting unseparated marrow cells, this strategy significantly reduced the manipulation and *in vitro* time required for gene transfer, thereby limiting the loss of stem cells due to apoptosis and differentiation. However, this strategy also appears to substantially limit clonal diversity of genetically marked cells.

In our murine model, a log-fold increase in lentiviral vector particle concentration at the time of transduction led to substantial increases in long-term gene transfer (12% vs 40%) with a concomitant increase in proviral copy number (1.8 vs 7), suggesting a vector dosage effect for both cell marking efficiency and individual cell integration events. These results are relevant with regard to the potential risk of insertional mutagenesis after hematopoietic stem cell gene therapy, in general, and its emerging relationship with oncoretroviral vector dose and multicopy integration in particular [18–21,32]. Assuming that lentiviral vectors carry similar mutagenic properties, and extrapolating recent data showing a correlation with oncoretrovirus vector copy number [32], our protocol provides a potential strategy to reduce that risk after lentiviral transduction by promoting single/low-copy proviral integration. However, such efforts to control for copy numbers in stem cells targeted by lentiviral vectors will limit gene transfer efficiency and may necessitate additional *in vivo* selection strategies to accomplish higher levels of gene transfer for clinical applications [33,34]. Therefore, the clinical relevance may initially be restricted to diseases in which stem cell gene transfer imparts a selective survival advantage on phenotypically corrected cells.

In keeping with our efforts to devise a protocol minimizing cell activation and maximizing hematopoietic stem cell yields, we avoided *in vivo* mobilization with 5-fluorouracil and explored conditions of minimal cytokine support during *ex vivo* lentivirus transduction. Our results show that gene transfer rates at an m.o.i. of 1 drastically declined in the absence of cytokine support and thereby confirm the improved transduction efficiency of hematopoietic stem cells with lentiviral vectors after target cell activation [1,9,35,36]. Considering that prolonged *ex vivo* stimulation with multiple cytokines compromises the engraftment potential of hematopoietic stem cells and can contribute to multicopy integration [8,11,13,37], the finding that gene transfer rates can be maintained in the presence of a single cytokine (mSCF) after a brief vector exposure of unseparated marrow leukocytes is particularly noteworthy.

Lentiviral vectors appear to be less prone to silencing and positional effects on transgene expression, thereby

providing an additional advantage over oncoretrovirus vectors [27,30,38]. While not a primary endpoint in these experiments, we provide substantial additional indirect evidence to expand on that aspect with a long-term analysis of GFP expression from progeny of lentivirally transduced hematopoietic stem cells followed through serial transplantation. We show that proviral expression is remarkably stable in primary and their secondary recipients and is maintained through hematopoietic differentiation in lymphoid and myeloid lineages (data not shown). This stability of expression was similar in recipients of cells transduced at low and high m.o.i. and thus appeared less affected by the difference in average proviral copy number. Overall, we show that proviral expression appeared remarkably stable over time and from primary to secondary recipients. Future studies will have to clarify whether this is due to the integration properties of lentiviral vectors, the use of a phosphoglycerate kinase (PGK) promoter and woodchuck hepatitis virus posttranscriptional regulatory element (wPRE) driving expression [39], or a combination of both. The promoter choice may certainly explain the discrepancy between our own and the study by Chen and colleagues, who describe significant silencing of CMV promoter-directed GFP expression in lentivirus-transduced PCR-positive murine progenitor cells [40].

In conclusion, we herein provide evidence that minimally manipulated hematopoietic stem cells present in unseparated marrow and genetically modified using lentiviral vectors at low m.o.i. contribute to long-term repopulation in murine recipients. While we cannot comment on stem cell recovery (CRU) under these circumstances or compare directly our own to CRU and clonality data from other investigators, we believe such a strategy of minimal target cell manipulation and short *ex vivo* duration offers a number of advantages, as outlined above and recently reviewed by Richter and Karlsson [41]. Furthermore, confined to the two vector concentrations tested, our study suggests a correlation between (VSV-G-pseudotyped) lentiviral vector particle concentration and proviral copy number in murine stem cells and provides a strategy to minimize multicopy integration. However, this is associated with relatively lower gene transfer rates and oligoclonality among marked cells and may require additional *in vivo* selection strategies. We believe that these conditions are uniquely suited to the gene replacement therapy of hematopoietic disorders with small residual stem cell pools such as FA, in which extensive *ex vivo* manipulation should be avoided and of which the inherent genetic instability mandates strategies that minimize additional genotoxicity from multiple proviral integrants. Our studies may be instructive in designing similar experiments to identify conditions optimizing transduction of human hematopoietic stem cells with lentivirus vectors.

MATERIALS AND METHODS

Mice, marrow harvest, and transplantation. Eight- to 12-week-old mice (C57BL/6J) were kept according to institutional guidelines and provided chow and water *ad libitum*. All experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee at the Fred Hutchinson Cancer Research Center. Marrow cells were harvested from donor animals following CO₂ euthanasia, sterile dissection, and flushing of femurs and tibias. Cells were processed by ACK hemolysis, passed through a 70- μ m cell strainer, and enumerated by trypan blue exclusion. Cells were pooled and washed after transduction, and equal numbers (2×10^6) per animal were subsequently injected via tail vein into lethally irradiated (1050 cGy) recipients.

Vector design and packaging. We used a central polypurine tract containing a self-inactivating lentiviral backbone containing an internal PGK promoter, enhanced green fluorescent protein (EGFP) expression cassette, and a wPRE (RRLSin.cppt.hPGKGFp.wPRE, kindly provided by L. Naldini, Turin, Italy) [42]. This vector was produced by transient cotransfection of 293T cells with transfer vector, gag-pol construct, and VSV-G envelope expression construct according to established protocols [43,44]. Vector was harvested, filtered (22- μ m pore size), and 100-fold concentrated by ultracentrifugation. Titer estimates were calculated after flow-cytometric determination of GFP expression in HT 1080 target cells, indicating a titer between 1.5 and 2×10^8 transducing units (TU)/ml concentrated vector supernatant.

Ex vivo culture and transduction. Following marrow harvest and processing, cells were resuspended in Iscove's medium supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA; 1% penicillin/streptomycin) and combinations of human IL-6, mSCF, Flt-3L, and MGDF as noted. All cytokines were used at a final concentration of 100 ng/ml. All transductions were performed as 12-h vector exposures by adding 100-fold concentrated vector supernatant to 1×10^6 cells plated in individual CH296 (Takara Shuzo, Japan) fibronectin fragment-coated wells in non-tissue culture-treated six-well plates in the presence of protamine sulfate at 4 μ g/ml plate. Concentrated vector was added to a final volume of 1 ml per well at an m.o.i. of 1 (or 10) HT1080 TU/cell. This translates into a vector concentration (VC) of 1 (or 10×10^6) HT1080 TU/ml. Under the current conditions and given unchanged transduction culture volumes, 10-fold increases in VC resemble 10-fold increases in m.o.i. We have therefore used the two terms interchangeably throughout the current study.

Hematology and flow cytometry. Blood from animals was obtained via retroorbital eye bleed at scheduled intervals, collected in EDTA-coated microtainers, and submitted for complete blood count analysis to a clinical hematology laboratory for determination of hematocrit, white blood cell, and platelet counts. A smaller aliquot treated with ACK hemolysis buffer was resuspended in buffered saline containing propidium iodide and analyzed for expression of GFP on a FACSCalibur instrument. Leukocyte immunophenotyping was performed using anti-mouse monoclonal antibodies B-220 (B lymphocytes), Thy-1.2 (T lymphocytes), and Gr-1 (granulocytes) (all purchased from PharMingen, San Diego, CA, USA).

Progenitor CFU assays. Marrow cells were harvested following CO₂ euthanasia via bilateral aspiration of cells from tibia and femur. Cells were resuspended in PBS wash solution, and white blood cells were isolated after red cell hemolysis and subsequently resuspended in Iscove's medium supplemented with 10% FBS (Hyclone). Progenitor colony-forming assays were performed in triplicate, plating 2×10^4 cells/35-mm dish according to the manufacturer's instructions in cytokine-supplemented (rhIL-6, rmlL-3, rmSCF, and rh erythropoietin) methylcellulose medium (Methocult GF M3434; Stem Cell Technologies, Vancouver, Canada). Colonies from a donor animal for secondary stem cell transplantation were plated at 6 months after primary transplantation, picked for DNA extraction as described [45], and analyzed by LAM-PCR for proviral integrants, as described below.

Modified LAM-PCR. Determination of proviral copy numbers from hematopoietic progenitors grown in methylcellulose was modified from the method performed by Schmidt and colleagues [46]. Briefly, 5 μ l colony DNA extract, representing 25–250 cells, was subjected to 100 cycles of linear amplification with a biotinylated lentiviral LTR primer P711b (biotin-5'AAGCCTCAATAAAGCTTGCC). The single-stranded LTR–host junction product was purified on streptavidin-coated magnetic beads, made double-stranded in a random-primed Klenow reaction, and digested with *Tsp509I*, and the extended end was ligated to a double-stranded synthetic anchor primer (AP662, GACGGAGATCTGAATTCAGTGGCAGCAGTAGG, and AP716, AATTCCTAACTGCTGCCACTGAATTCAGATC). The LTR–host junction region was then amplified by two rounds of nested PCR (first primer set LTR712, GCTTCAAGTAGTGTGTGCC, and Anchor 664, GACCCGGGAGATCTGAATTC; second primer set LTR713, ACTCTGGTAACTAGAGATCC, and Anchor 674, GATCTGAATTCAGTGGCAGCAG). PCRs used Advantage II enzyme (Clontech) under the following cycling conditions: hold at 95°C for 5 min; 30 cycles at 95°C for 1 min, 60°C for 0.75 min, and 68°C for 1.5 min; followed by elongation at 68°C for 10 min. Products were analyzed by electrophoresis on Novex 4–20% polyacrylamide gels in Tris borate buffer (Invitrogen), followed by ethidium bromide staining. For sequence analysis, products were electrophoresed on 3% agarose gels, and bands were excised, purified on QIAquick gel extraction columns (Qiagen), and sequenced using the “Big Dye” reagent system and the ABI Prism 7700 sequence detection system (Applied Biosystems, Branchburg, NJ, USA).

Real-time PCR assay. PCR amplification of the lentiviral vector sequence from genomic DNA extracted from bone marrow leukocytes was performed by using a quantitative real-time PCR assay. DNA (300 ng) was amplified in duplicate with EGFP-specific primers (5'-CTGACCACCGGCAA-3' and 5'-GTAGCGGCTGAAGCACTG-3') and a fluorescence-tagged probe (5'-FAM-CCACCCTGACCTACGGCGTG-TAMRA-3'; SynGene, Houston, TX, USA). The DNA amount in duplicate wells was normalized by mouse IL-3 amplification with mIL-3-specific primers (5'-CCAGCATCCACACCAGTCT-3' and 5'-CCGGCCACTGATTGAAGCT-3') and a fluorescence-tagged probe (5'-FAM-CTCCTGATGCTTCCACCTGGGACTC-TAMRA-3'). The primers and probes were designed using Primer Express' software v.1.5 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Standards consisted of diluting DNA extracted from baboon B cells transduced with a single copy of pMND.EGFP.SN. Reactions were run using the ABI universal master mix (Applied Biosystems) on the ABI Prism 7700 sequence detection system (Applied Biosystems) using the following thermal cycling conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The spectrum was then analyzed using Sequence Detector v.1.6.3.

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