ARTICLE

doi:10.1016/j.ymthe.2005.01.005

Efficiency of Transduction of Highly Purified Murine Hematopoietic Stem Cells by Lentiviral and Oncoretroviral Vectors Under Conditions of Minimal *in Vitro* Manipulation

Gustavo Mostoslavsky, Darrell N. Kotton,* Attila J. Fabian, John T. Gray,† Jeng-Shin Lee, and Richard C. Mulligan‡

Department of Genetics, Harvard Medical School; Molecular Medicine, Children's Hospital; and Harvard Institute of Human Genetics, Boston, MA 02115, USA

**Present address: Department of Medicine, Division of Pulmonary and Critical Care Medicine, Boston University School of Medicine, Boston, MA, USA.*

†*Present address: Division of Experimental Hematology, Hematology–Oncology, St. Jude Children's Research Hospital, Memphis, TN, USA.*

‡*To whom correspondence and reprint request should be addressed at Molecular Medicine, Children's Hospital, 1 Blackfan Circle, Boston, MA 02115, USA. Fax: +1 617 730 0948. E-mail: mulligan@receptor.med.harvard.edu.*

Available online 12 February 2005

The development of leukemias in several children with severe combined immunodeficiency disease who were transplanted with retroviral vector-transduced bone marrow cells has renewed concerns about the risks associated with the random integration of proviral sequences into chromosomal DNA. One theoretical way to reduce the risks of insertional mutagenesis would be to employ transduction/transplantation protocols that minimize the total number of genetically modified cells and associated proviral integration "events" introduced into recipients. Toward this end, we have developed a transduction protocol that involves the short-term incubation of highly purified murine stem cells with high-titer recombinant lentivirus vectors in the presence of serum-free medium and the cytokines SCF and TPO. Competitive repopulation studies showed that stem cells transduced in this way possessed the same reconstitutive ability as fresh, unmanipulated cells. Animals transplanted with only 200–2000 transduced cells were efficiently reconstituted with the genetically modified cells, and most hematopoietic cells in the recipients expressed the transgene. In contrast, the use of high-titer oncoretroviral vectors in conjunction with the same transduction/transplantation protocol resulted in only low levels of gene marking *in vivo*. The use of a similar transduction/transplantation strategy in future clinical studies may offer distinct advantages over current protocols.

INTRODUCTION

Although simple oncoretroviral vectors have been extensively used to transduce hematopoietic stem cells (HSC) in preclinical models of gene therapy, recent clinical studies suggest that the standard transduction protocols used in conjunction with retroviral vectors generally do not lead to levels of gene transfer that are clinically relevant [1,2]. One feature of most transduction protocols involving oncoretroviral vectors is the use of cytokine "prestimulation," a procedure designed to induce stem cell populations to proliferate, an essential requirement for oncoretroviral integration. Although preclinical studies in mice have shown that prestimulation does indeed improve the transduction of reconstituting stem cells, presumably because the majority of HSCs are quiescent upon removal from the bone marrow, such *in vitro* manipulations clearly induce the differentiation of HSCs and thereby lead to the loss of reconstitution activity [3,4]. Effective gene transfer/reconstitution using protocols involving cytokine prestimulation would appear to necessitate finding a balance between achieving suitable levels of gene transfer and retaining acceptable levels of reconstituting activity. In the case of human cells, it may well be the inability to define effectively the experimental parameters that govern the relationship between these two important factors that accounts at least in part for the poor outcomes observed in the clinic.

Because lentiviruses are capable of infecting certain types of quiescent cells [5,6], there has been significant interest in the application of lentivirus-derived vectors to the transduction of HSCs, and indeed, a variety of investigators have defined protocols that lead to the

1525-0016/$30.00

efficient transduction of HSCs from different species, including human [7–13]. Interestingly, however, although proliferation per se does not appear to be necessary for efficient transduction of human HSCs, cytokine prestimulation does appear to improve gene transfer, likely because truly resting HSCs (in the G0 phase of the cell cycle) are relatively resistant even to lentiviral vector transduction, and cytokine prestimulation essentially serves to "activate" the cells to enter the G1 phase of the cell cycle, a stage at which transduction may be relatively more efficient [14]. Therefore, paradoxically, despite the success reported with the use of lentiviral vectors for transduction of HSCs, there are lingering questions regarding whether cytokine treatment of cells will prove to be essential for the achievement of high levels of gene transfer even with lentiviral vectors and therefore should be a component of lentiviral vector transduction protocols used in the clinic. Previous studies by Barrette and co-workers [15] have partially addressed this important issue by comparing the efficiency of transduction of murine HSCs afforded by standard oncoretroviral and lentiviral vectors under different conditions of transduction. Interestingly, they reported that under conditions of minimal *in vitro* manipulation (no added cytokines), neither lentiviral nor oncoretroviral vectors were capable of efficiently transducing murine HSCs, while under conditions of standard cytokine prestimulation, both vectors achieved a comparable level of transduction. Those studies have fueled the debate as to whether in fact lentiviral vectors would offer advantages over oncoretroviral vectors in the clinical gene therapy setting.

Here, we report studies aimed at further evaluating the relative abilities of oncoretroviral and lentiviral vectors to transduce highly purified murine hematopoietic stem cells. To enable the successful reconstitution of lethally irradiated recipients with small numbers of genetically modified cells, we sought to develop transduction conditions that do not compromise hematopoietic stem cell function yet permit efficient transduction. The results presented below indicate that lentiviral vectors are uniquely able to transduce efficiently highly purified reconstituting stem cell targets under conditions of minimal *in vitro* manipulation.

RESULTS

In Vivo Assessment of Stem Cell Transduction by Lentiviral Vectors under Conditions of Minimal Cytokine Stimulation and Optimization of Additional Experimental Parameters

We have previously reported the identification and isolation of a population of murine bone marrow cells highly enriched in hematopoietic reconstitution activity (termed side population or "SP" cells), based on the capacity of the cells to exclude actively the vital dye Hoechst 33342 [16,17]. Because it is possible to reconstitute lethally irradiated recipients fully with small numbers (500–200) of SP cells, we chose those cells as targets for our transduction studies. As a prelude to the evaluation of different transduction protocols, we first established the relative reconstitution activity of SP cells cultured in the presence of either SCF and TPO [12,18] or the combination of SCF, IL-3, and IL-6 [15], in the absence of exposure to virus, to study whether *in vitro* culture of SP cells under different conditions of cytokine prestimulation could compromise the reconstituting capacity of the cells. To quantify the engraftment potential of the cells manipulated in different ways, we employed a CD45.1/CD45.2 competitive repopulation assay involving the cotransplantation of genetically modified cells with a fixed number of unmanipulated congenic bone marrow cells [16] so that both the efficiency of transduction and the reconstitutive ability of the genetically modified cells could be independently determined. As shown in Fig. 1A, culture of SP cells overnight in SCF, IL-3, and IL-6 led to a significant reduction in their reconstitution ability relative to unmanipulated cells. In contrast, cells cultured solely in the presence of SCF and TPO maintained levels of reconstitution comparable to those observed with fresh, unmanipulated cells. Based on these results, we chose the culture of cells in the presence of SCF and TPO as an essential aspect of the transduction protocol.

As our expectation was that under conditions of minimal cytokine stimulation, lentiviral vector transduction would be more likely to result in efficient gene marking *in vivo* than retroviral vector transduction, we next examined a number of other potentially important experimental parameters of the transduction process using solely lentiviral vectors. In the first set of such experiments, we used a high lentiviral multiplicity of infection (m.o.i.) to transduce 100 CD45.1 SP cells that were transplanted together with 2×10^5 CD45.2 competitor cells (Fig. 1B). Transduction was performed in serum-free medium containing Polybrene, with or without minimal stimulation (SCF and TPO), for either 4 or 24 h at 37°C (Fig. 1C). We had previously observed that the use of Polybrene (5 μg/ml) per se does not compromise HSC potential, even after a 24-h incubation time (data not shown). We followed levels of engraftment and gene transfer for 8 months post-bone marrow transplantation (post-BMT). Efficient gene transfer was dependent on the presence of the cytokines (Fig. 1C, left) and was enhanced by extended incubation times (Fig. 1C, bottom). Under optimal conditions (Fig. 1C, bottom left), ~95% of donor-derived cells expressed GFP for the duration of the study.

To determine whether the absolute amount of virus used for transduction (e.g., viral m.o.i.) would affect HSC engraftment potential, we transduced purified SP cells with either 100 viral m.o.i. or 1000 viral m.o.i. in medium containing Polybrene and cytokines. The incuba-

doi:10.1016/j.ymthe.2005.01.005

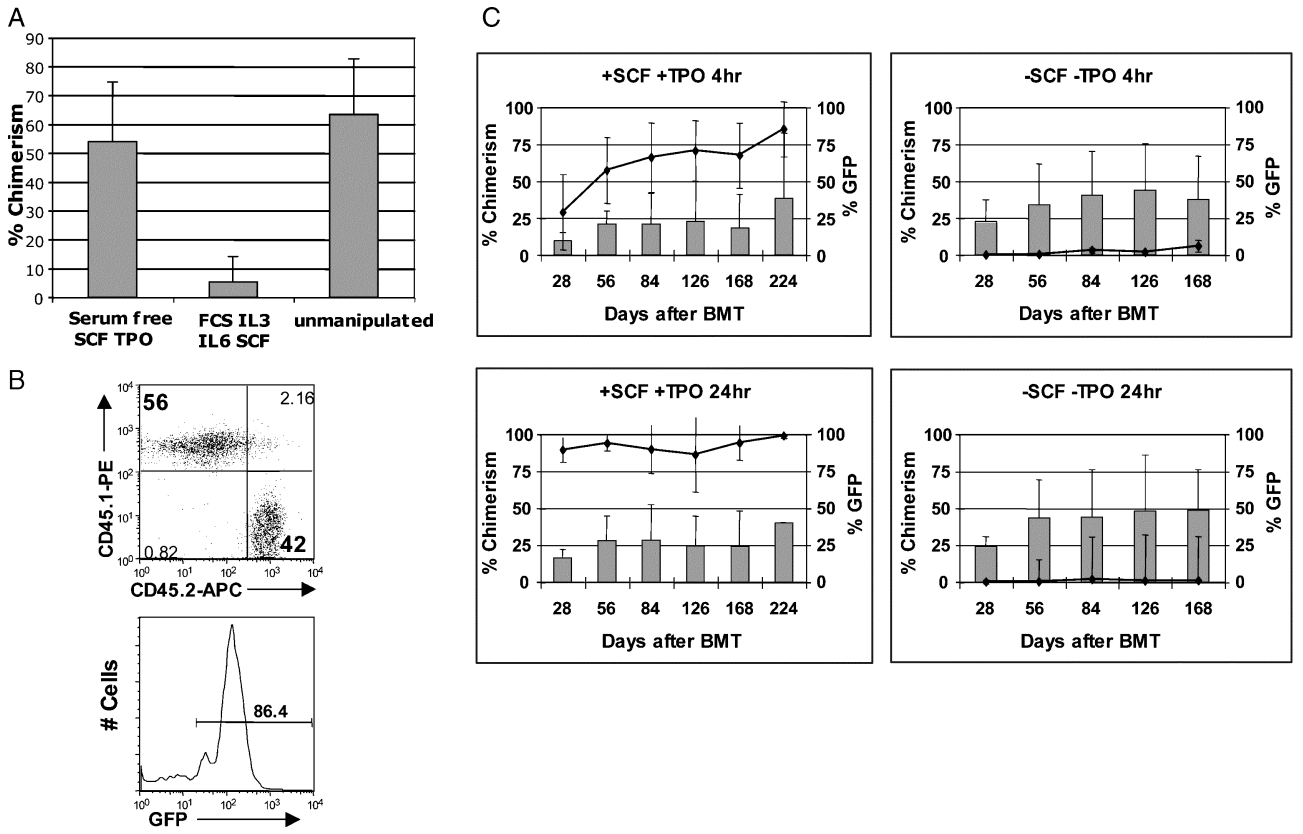

FIG. 1. Conditions for transduction of HSC. (A) Ly 5.1 SP cells were purified and used immediately (unmanipulated) or after 16 h incubation in either complete medium containing 15% FCS, IL-3, IL-6, and SCF or serum-free medium containing SCF and TPO, to compete against whole bone marrow from Ly 5.2 mice in their ability to reconstitute lethally irradiated mice. Each mouse received 200 SP cells together with 2×10^5 unfractionated whole marrow cells ($N = 4$ per group). Data are expressed as means ± SEM. (B) Representative plot and histogram analysis of one peripheral blood sample from a mouse transplanted with HSC transduced with a lentiviral vector. The top shows contribution from donor (CD45.1) and competitor population (CD45.2). The bottom shows percentage of GFP^+ cells within the donor population. (C) Purified SP cells were transduced with a lentiviral vector at 1000 m.o.i. Transduction was performed in serum-free medium containing 5 μg/ml Polybrene, with (left) or without (right) 10 ng/ml SCF and 100 ng/ml TPO, for 4 h (top) or 24 h (bottom) at 37°C. Bars show blood chimerism (%). Lines show percentage of GFP-expressing cells within the donor population. Each mouse received 100 SP cells together with 2×10^5 unfractionated whole marrow cells ($N = 8$ per group). Data are expressed as means ± SEM.

tion was performed for 4 or 24 h at 37°C. As shown in Fig. 2, the use of high viral m.o.i. clearly reduced the engraftment potential of transplanted SP cells (compare Figs. 2C to 2B and 2F to 2E). This effect was more dramatic when the time of incubation of virus and cells was extended to 24 h (compare Figs. 2F to 2E). Importantly, at the lower m.o.i. (2B and 2E), the engraftment potential of transduced cells was equal to that of mock-infected cells and high levels of gene transfer were observed, thus establishing conditions for achieving efficient gene transfer that do not compromise stem cell function. The length of time of exposure of cells to virus did significantly influence gene transfer efficiencies ($P = 0.0004$), as incubating cells and virus for 24 h instead of 4 h improved the levels of gene transfer from an average of ~35 to ~65%, with individual mice showing up to 90% of donor-derived cells expressing GFP (compare Figs. 2E to 2B).

Since the above experiments involved the cotransplantation of transduced cells and unmanipulated "competitor" cells, to enable the quantitative assessment of engraftment potential of the vector-transduced cells, it was important to confirm the ability of transduced cells to reconstitute lethally irradiated mice in the absence of such competitor cells. Toward this end, we transplanted CD45.2 animals with 2000 infected CD45.1 SP cells and subsequently analyzed them for hematopoietic reconstitution and gene transfer. HSC transplantation without competition allowed us to obtain almost complete donor-derived reconstitution of recipients, with all mice showing more than 90% of their peripheral blood content as derived from the donor cells, and the vast majority of those cells expressed GFP for the duration of the study (data not shown).

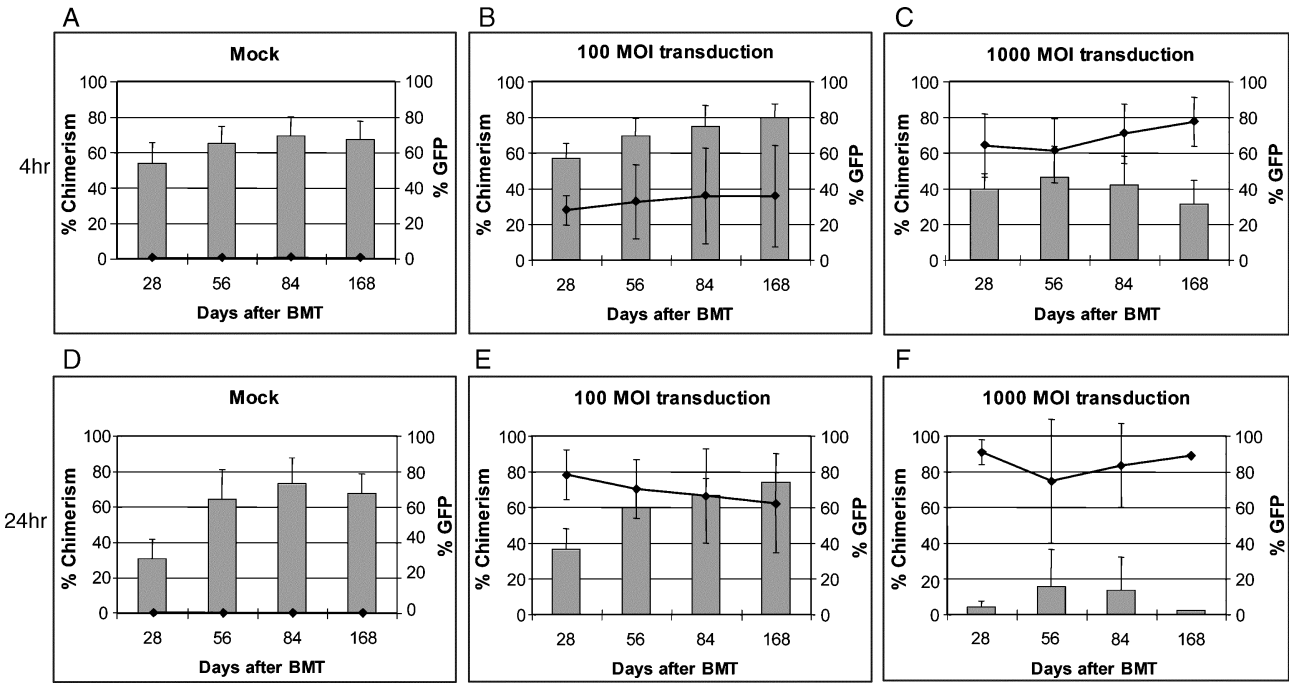

FIG. 2. Effect of viral m.o.i. on repopulating capacity of HSC. Purified SP cells were incubated in TPO and SCF for 4 h (A–C) or 24 h (D–F) and exposed to no treatment (mock) (A, D) or lentivirus at 100 m.o.i. (B, D) or 1000 m.o.i. (C, F). Bars show blood chimerism (%). Lines show percentage of GFP-expressing cells within the donor population. Each mouse received 200 SP cells together with 2×10^5 unfractionated whole marrow cells ($N = 4$ per group). Data are expressed as means ± SEM.

To document that HSCs transduced under the above conditions retained their full differentiation potential after BMT, we analyzed peripheral blood from transplanted mice using specific markers to identify macrophages, granulocytes, B lymphocytes, T lymphocytes, and erythroblasts. As shown in Fig. 3A, transduced HSC

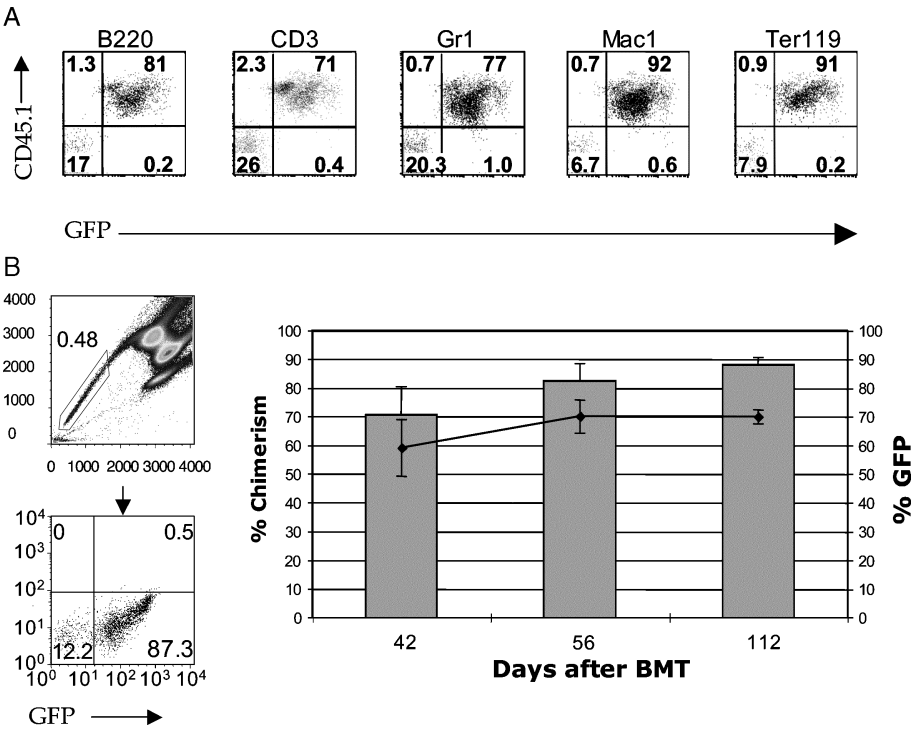

FIG. 3. Multilineage contribution and secondary BMT using transduced HSC. (A) 18 weeks post-BMT, peripheral blood was stained using antibodies to detect B lymphocytes (B220), T lymphocytes (CD3), granulocytes (Gr1), macrophages (Mac1), and erythroblasts (Ter119). After gating on each marker, cells were analyzed for donor contribution and GFP expression. Similar results were found in all transplanted animals. (B) Bone marrow cells from a primary recipient mouse that was transplanted with lentivirally transduced HSC were purified 6 months after transplantation and stained with Hoechst 33342 (upper left). Gated SP cells were sorted and used to transplant secondary irradiated recipients. Greater than 80% of sorted SP cells expressed GFP (bottom left). Bars show blood chimerism (%). Lines show percentage of GFP-expressing cells within the donor population. Each mouse received 2000 SP cells ($N = 4$). Data are expressed as means ± SEM.

doi:10.1016/j.ymthe.2005.01.005

robustly reconstituted all blood cell lineages and high level GFP expression was detected in all cell lineages. In addition, to demonstrate that transduced SP cells retain their full reconstitution ability, we performed secondary BMT using SP cells purified from BM of mice that had been transplanted 6 months earlier. As shown in Fig. 3B, secondary transplanted recipients were efficiently reconstituted by donor-derived cells, showing levels of GFP expression that were as high as in the primary transplanted donor mouse and did not decline with time.

Direct Comparison of *in Vivo* Gene Marking Efficiencies by Oncoretroviral and Lentiviral Vectors Under Conditions of Minimal Cytokine Stimulation

Having established conditions for efficient gene transfer into HSC using lentiviral vectors that do not compromise stem cell function, we compared directly the abilities of oncoretroviral and lentiviral vectors to transduce HSCs under those conditions. In a first experiment, we infected SP cells with either an oncoretroviral or a lentiviral vector encoding the reporter GFP for 24 h, in the presence of SCF and TPO (see above); subsequently we cultured the transduced cells for 48 h in the same medium and finally analyzed them by FACS for expression of GFP. As shown in Fig. 4A, we observed efficient gene transfer (up to 90% transduction) using either type of vector. These results suggested that both vectors were capable of efficiently transducing SP cells under these conditions.

For a more direct assessment of the efficiency of HSC transduction by each vector type, we performed *in vivo* repopulation studies in which 2000 SP cells transduced by the different vectors were used to transplant lethally

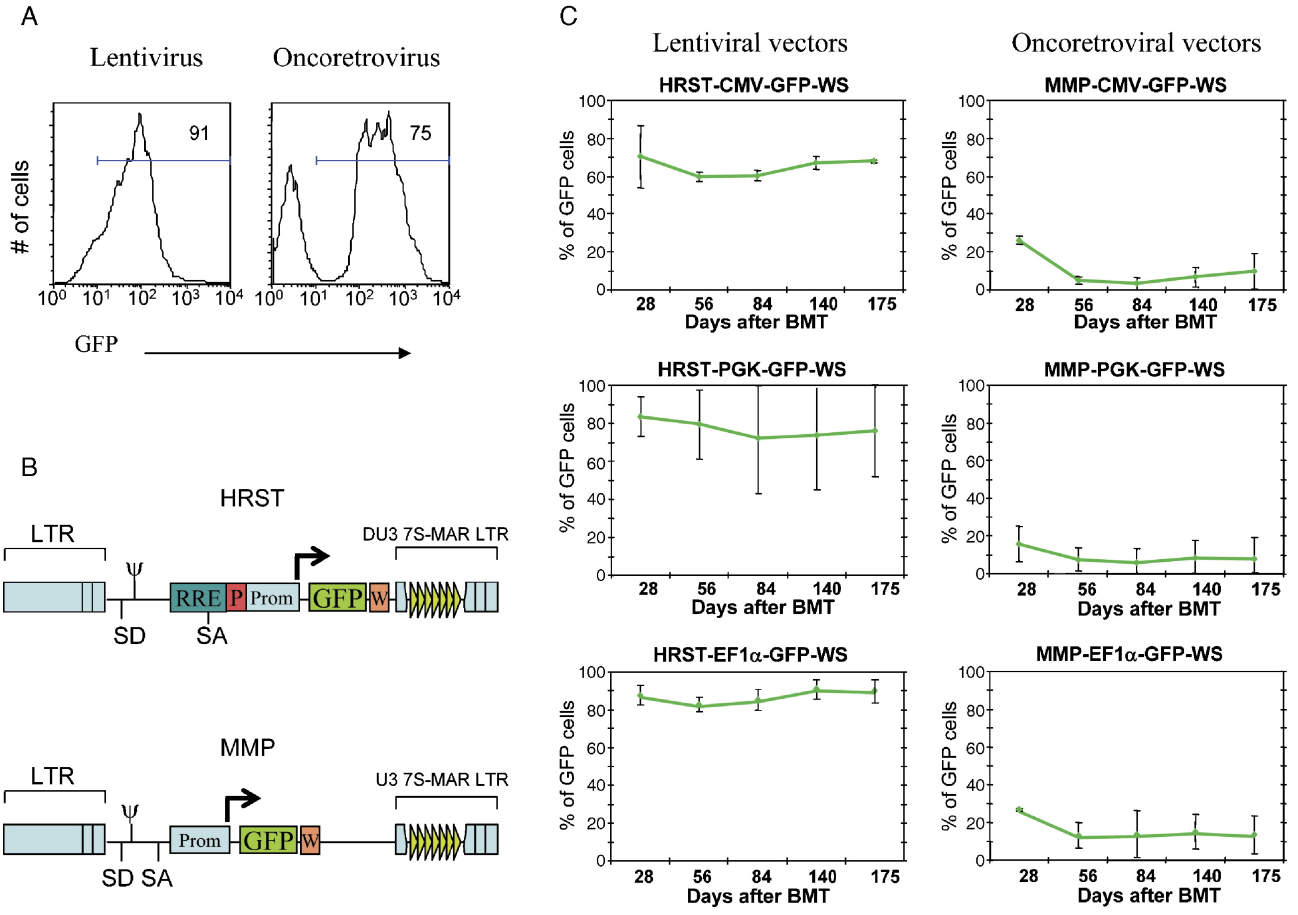

FIG. 4. Comparison of lentiviral and oncoretroviral transduction of HSC. (A) Purified SP cells were transduced with lentiviruses or simple retroviruses, using serum-free medium with minimal conditioning (SCF + TPO), and GFP levels were analyzed 72 h after transduction. Results are representative of four independent experiments. (B) Schematic representations of the lentivirus (HRST) and oncoretrovirus (MMP) used in this study. LTR, long terminal repeat. Ψ, Psi packaging signal. SD, splice donor. SA, splice acceptor. RRE, Rev-responsive element. P, central polypurine tract. Prom, internal promoter. W, woodchuck hepatitis virus posttranscriptional regulatory element. DU3, deleted U3. S-MAR, synthetic matrix attachment region. (C) Lethally irradiated Ly5.2 recipient mice ($N = 4$ per group) were transplanted with 2000 Ly5.1 SP cells that had been transduced for 24 h at 37°C using 200 m.o.i. of either a lentiviral (i.e., HRST) or an oncoretroviral (i.e., MMP) vector in which GFP expression was driven by three different internal promoters, CMV, PGK, or EF1α. Lines show percentage of GFP-expressing cells within the donor population. Data are expressed as means ± SEM.

irradiated mice. In an effort to reduce the possibility that analysis of reporter gene expression might not properly reflect gene transfer efficiencies, we compared the performances of a series of both vector types, which made use of different internal promoters (CMV, PGK, and EF1α) to drive expression of the reporter GFP (Fig. 4B) (see Materials and Methods for details). As shown in Fig. 4C, while transduction with oncoretroviral vectors led, in some cases, to detectable levels of reporter gene expression, the proportion of cells expressing the reporter was dramatically lower than that achieved using lentiviral vectors ($P = 0.0001$). These *in vivo* results contrast with the *in vitro* analysis of gene transduction presented in Fig. 4A. Interestingly, although all internal promoters used in the study presented in Fig. 4C were able to induce GFP expression efficiently, the CMV promoter showed the highest levels of mean fluorescence (data not shown).

To rule out completely that difficulties with oncoretroviral vector-mediated gene expression *in vivo* could have in part accounted for the poor apparent "gene transfer" observed, we sacrificed all transplanted mice and analyzed their marrow cells for chimerism, for the efficiency of transduction both in whole bone marrow and within the stem cell compartment (SP cells), and for proviral copy number. As shown in Fig. 5A, bone marrow from all mice showed levels of chimerism that correlated well with that seen in peripheral blood. Moreover, all marrow samples expressed GFP at the same level as peripheral blood. Analysis of the level of GFP expression specifically in SP cells isolated from mice transplanted with lentiviral vector-transduced cells demonstrated efficient gene vector-mediated gene expression in those cells as well (Fig. 5B). In contrast, mice receiving SP cells transduced with oncoretrovirus showed low to undetectable levels of GFP expression within the HSC fraction of marrow.

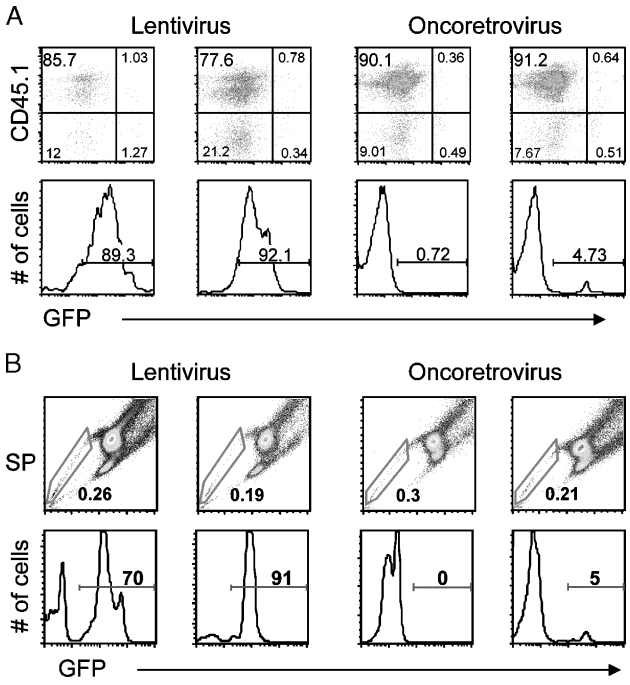

FIG. 5. Analysis of engraftment and gene transduction efficiency in bone marrow of transplanted mice. (A) Whole marrow cells were purified from transplanted mice and analyzed for donor contribution and GFP expression. The top shows levels of chimerism and the bottom shows percentage of GFP cells within the donor population. (B) Bone marrow cells were stained with Hoechst 33342 and SP cells (representing the stem cell compartment) were analyzed for levels of GFP expression. The top shows SP plots and the bottom shows percentage of GFP cells within the SP gate. Results from two representative mice in each group are shown. Each graph represents the analysis of at least 1000 events.

We performed Southern blot analysis of bone marrow DNA derived from recipients transplanted with either lentiviral or retroviral vector-transduced cells, both using DNA cleaved either with restriction enzymes that recognized sequences within the viral LTRs (see Fig. 6) or with enzymes that recognized a unique sequence in the interior of the proviral genomes (data not shown). In all cases we detected limited numbers of integrants (data not shown). In the case of DNA derived from animals transplanted with lentiviral-transduced cells, the proviral copy number averaged 3 copies per genome. As observed through analysis of reporter gene expression, the efficiency of gene marking with oncoretroviral vectors was poor, yet in some cases detectable (see Fig. 6).

DISCUSSION

The studies reported here were motivated in large part by recent reports by Fischer and co-workers, which described the development of leukemia-like syndromes in severe combined immunodeficiency disease (SCID) patients transplanted with cells transduced by oncoretroviral vectors [19–23]. Those studies have appropriately led to dramatically increased concerns about the safety of retroviral (and lentiviral) vector-mediated gene therapy approaches for diseases affecting hematopoietic cells and established a firm rationale for the development of new safer approaches to their genetic modification. The development of leukemia in the specific SCID-γ C patient population has also provoked keen interest in understanding whether the nature of the target cells used for transduction and/or the specific manipulations of them *in vitro* used to achieve gene transfer may have contributed to the oncogenic process. Unfortunately, to date, few of these issues have been adequately addressed.

Our studies were based on the notion that even in the absence of mechanistic information explaining the serious adverse events associated with the SCID-γ C trial, it may be possible to develop "safer" protocols for transferring genes into hematopoietic cells. Specifically, we focused on the development of transduction protocols that would result in a reduction in the total number of

doi:10.1016/j.ymthe.2005.01.005

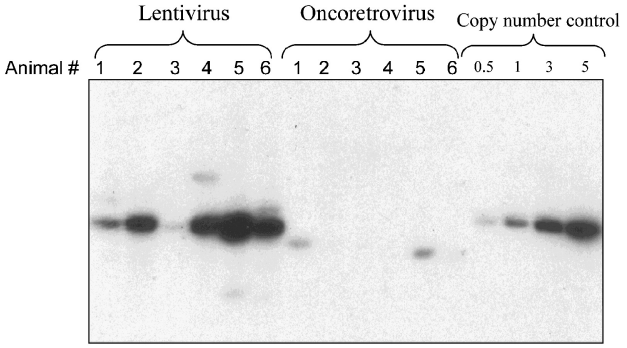

FIG. 6. Analysis of proviral copy number in BM cells of transplanted mice. Genomic DNA from whole marrow cells was purified and digested with *Afl*II to obtain the average number of viral integrants per genome. Samples from mice transplanted with cells transduced with lentiviruses or oncoretroviruses are shown. Increasing amounts of known plasmid vector DNA was used for copy number control.

proviral insertions "introduced" into a recipient after bone marrow transplantation, relative to standard transduction protocols currently in widespread use, and that would limit the range of biologically distinct cell types introduced *in vivo* via transplantation. While several elements of the protocol we have developed have been employed previously in different experimental contexts by others [12], our studies addressed directly a very specific issue of practical import that had not been adequately addressed in the past: the relative utility of lentiviral and oncoretroviral vectors for transduction of hematopoietic stem cells under conditions under which stem cell function is not compromised. As indicated earlier, Barrette *et al.* [15] concluded from their studies that lentiviral vectors offer no significant advantages over oncoretroviral vectors for the transduction of hematopoietic stem cells, based on studies that compared transduction protocols that made use of either a standard cytokine cocktail or no cytokines at all. Notably, those studies did not evaluate the conditions of minimal cytokine stimulation that we have shown here to both preserve stem cell function and provide for the effective "activation" of stem cells that renders them susceptible to efficient transduction. Our results clearly show that there is a distinct advantage to the use of lentiviral vectors for the transduction of purified stem cells under conditions that preserve their biological properties. It is interesting and somewhat surprising that the *in vitro* analysis of transduction presented in Fig. 4A did not reveal the superiority of lentiviral vectors for the transduction of SP cells. It is possible that either the subpopulation of SP cells most capable of expansion *in vitro* is compromised in their capacity for *in vivo* repopulation or that the levels of reporter expression observed could in part reflect expression from unintegrated proviral copies.

While our studies suggest that "some" addition of cytokines is indeed required for efficient transduction of purified stem cells, other recent reports have described lentiviral vector-based protocols for the transduction of enriched or purified stem cells that do not involve the addition of any cytokines [24,25]. Our studies, in fact, do not really conflict with those reports other than in the demonstration under our specific transduction conditions (m.o.i., serum-free medium, etc.) that transduction can be "improved" by minimal cytokine stimulation. Notably, in neither of the studies cited above were the effects of the transduction conditions on the reconstitution activity of the cells used for bone marrow transplantation assessed, an important issue for our studies.

As shown in Fig. 6, the conditions we have utilized for transduction of purified cells led to highly efficient gene transfer, as reflected by the presence of multiple proviral copies per cell in some cases. While such a result would appear to be contrary to the goal of "limiting" the total number of integrations, there will likely be a need to "weigh" the possible disadvantages of multiple proviral insertions per cell (e.g., safety issues) with the possible therapeutic importance of obtaining robust transgene expression through the introduction of multiple proviral copies. In fact, we have demonstrated that it is possible to reduce the number of proviral copies per cell when making use of the protocol we have developed by simply reducing the m.o.i. (data not shown). However, in the clinical setting, it may be practically difficult to achieve a "low" average proviral copy number via variation of m.o.i., without compromising the "efficacy" of the therapy. For this reason, we believe that it may be most advantageous (and practical) to make use of high m.o.i.'s but to limit dramatically the total number of provirally "marked" cells that are introduced into the recipient, as the protocol we have described here achieves.

Overall, our studies offer promise for the implementation of clinical protocols for gene therapies involving human hematopoietic cells that may have an improved safety profile and that may lead to improved gene marking *in vivo*. In addition to limiting the absolute number of cells carrying proviral integrations introduced into patients, the use of small numbers of highly purified cells may have additional advantages from the standpoint of safety, if, for example, the target cell for oncogenic conversion sometimes represents a progenitor cell (abnormal or normal) rather than a normal reconstituting stem cell or a cell that uniquely arises through specific *in vitro* manipulations associated with cytokine prestimulation and significant *in vitro* culture. Such targets could be "biologically" more susceptible to oncogenic events than relatively "unmanipulated" cells, due to their proliferative status and/or other unique biological properties. Furthermore, standard cytokine activation protocols may lead to the activation of a large number of chromosomal genes involved in the control of stem cell proliferation and thereby increase the accessibility of chromosomal sequences associated with those genes to

proviral insertion. In addition to the potential "safety" advantages of the type of transduction protocol we have described here, we speculate that the ability to preserve levels of reconstitution activity comparable to fresh, unmanipulated cells, as we have documented, may prove to be an extremely critical determinant of successful gene marking *in vivo*. Clearly, the evaluation of transduction protocols similar to those we have described herein in a clinical setting is warranted.

Materials and Methods

Viral production. Generation of lentiviral vectors was accomplished by a five-plasmid transfection procedure. Briefly, 293T cells were transfected using TransIT 293 (Mirus, Madison, WI, USA) according to the manufacturer's instructions with the backbone HRST vector together with four expression vectors encoding the packaging proteins Gag-Pol, Rev, Tat, and the G protein of the vesicular stomatitis virus (VSV). HRST is a third-generation lentiviral SIN nonreplicative vector derived from the original pHR′ CMV-lacZ vector previously described [5]. The expression of GFP is driven by one of three different internal promoters, CMV, PGK, and EF1α. The *gagpol* helper plasmid has been codon optimized for efficient mammalian expression and modified to reduce severely the homology with the *gag* sequences present in the vector packaging signal. In addition, it makes the *gagpol* expression Rev independent. All of the expression helper plasmids contain only the coding sequences, with minimal 5′ or 3′ untranslated sequences and no introns. In addition, the backbone contains the woodchuck hepatitis virus posttranscriptional regulatory element [26], seven synthetic scaffold–matrix attachment regions [27], and the central polypurine tract to enhance levels of transcription and gene expression. Viral supernatants were collected starting 24 h after transfection, for four consecutive times every 12 h, pooled, and filtered through a 0.45-μm filter. Viral supernatants were then concentrated ~100-fold by ultracentrifugation in a Beckman centrifuge, for 3 h at 15,000 rpm. Using these protocols titers of ~5×10^8 to 1×10^9/ml were achieved. For oncoretroviral vector production the same transfection protocol detailed above was used, except that three plasmids were cotransfected into 293 T cells, including an MPSV-derived viral vector and its corresponding *gagpol* and VSV-G envelope.

HSC purification and viral transduction. Purified HSC were obtained by isolating bone marrow SP cells using fluorescence-activated cell sorting after Hoechst staining as previously described [16], with some modifications. Briefly, femurs and tibias from mice were homogenized and bone marrow cells were filtered through a 70-μm filter and washed in PBS containing 2% FCS and 0.5% sodium azide. Cells were then resuspended in HBSS containing 2% FCS, 10 mM Hepes buffer, and antibiotics (all from Gibco, Grand Island, NY, USA) and stained with 8.8 μg/ml Hoechst 33342 (Molecular Probes, Eugene, OR, USA) at a cell concentration of 5×10^6/ml. After the cells were incubated for 90 min at 37°C, they were washed once and further purified by using a gradient of Ficoll–Paque Plus (Amersham Biosciences AB, Uppsala, Sweden) to remove red blood cells. Purified marrow cells were then sorted using a MoFlo high-speed cell sorter (DakoCytomation, Fort Collins, CO, USA). Except when specified, cells were kept on ice during the entire procedure. Viral transduction of sorted HSC was performed using StemPro SFM-34 medium (Gibco). When indicated, 20 ng/ml IL-3, 50 ng/ml IL-6, and 50 or 10 ng/ml SCF and 100 ng/ml TPO were added to the medium containing 5 μg/ml Polybrene. All cytokines were purchased from R&D Systems (Minneapolis, MN, USA). Transduction was performed in round bottom 96-well plates, using 20 μl reaction volume, for either 4 or 24 h at 37°C. Cells were then resuspended in 100 μl for transplantation or incubated for 3 days for FACS analysis.

Bone marrow transplantation. All mice were purchased from The Jackson Laboratory and maintained in a specific-pathogen-free animal facility at Harvard Medical School. All experiments included at least four mice per group. CD45.2 recipient mice were lethally irradiated with two doses of 7 Gy, 3 h apart, 1 day before BMT and maintained with antibiotic-supplemented water for 15 days. Transduced SP cells from CD45.1 donors alone or, when indicated, mixed with 2×10^5 CD45.2 unfractionated marrow cells were injected retro-orbitally into recipient mice under isoflurane anesthesia. Peripheral blood was obtained from the retro-orbital plexus every 4 weeks and stained using fluorescence-conjugated anti CD45.1-PE and CD45.2-biotin/streptavidin–APC antibodies (BD Biosciences Pharmingen, San Diego, CA, USA), to calculate levels of chimerism. Levels of gene transfer were measured directly by detection of GFP expression. Analysis of multilineage gene transfer was done by staining peripheral blood with biotin-conjugated antibodies against B220, CD3, Mac1, Gr1, and TER119 surface antigens (BD Biosciences Pharmingen). Cells were incubated with antibodies for 30 min on ice, washed once, and resuspended in PBS/1% BSA for analysis. Dead cells were excluded using propidium iodide stain. Samples were analyzed in a FACSCalibur machine (Becton–Dickinson) and data processed by FlowJo software (Tree Star, Ashland, OR, USA). For secondary bone marrow transplantations, marrow samples were collected from primary recipient mice 6 months after primary BMT and used to reconstitute lethally irradiated recipients as described above. Statistical analyses were performed using Student's *t* test. All animal procedures were approved by the Standing Committee on Animals of Harvard Medical School.

Southern blot analysis. Genomic DNA was extracted 6 months after BMT from tibias, femurs, hips, and sternum bones of transplanted animals. Southern blot analysis using standard methods was performed on DNA digested with *Afl*II (New England Biolabs, Beverly, MA, USA), which cuts once in each of the two viral LTRs, to estimate the proviral copy number per genome, or with *Bam*HI (New England Biolabs), which cuts only once within the provirus, to analyze different viral integrants.

Acknowledgments

We thank Peggy Russell for technical assistance and Alejandro Balazs, George Murphy, and Raul Mostoslavsky for their comments on the manuscript. This work was supported by NIH 5PO-HL54785, a Lady TATA Memorial Fund Postdoctoral Award (G.M.), and NIH 1 KO8 HL071640-01 (D.N.K.).

RECEIVED FOR PUBLICATION DECEMBER 9, 2004; ACCEPTED JANUARY 6, 2005.

References

1. Somia, N., and Verma, I. M. (2000). Gene therapy: trials and tribulations. *Nat. Rev. Genet.* **1:** 91–99.
2. Kohn, D. B., *et al.* (2003). American Society of Gene Therapy (ASGT) Ad Hoc Subcommittee on Retroviral-Mediated Gene Transfer to Hematopoietic Stem Cells. *Mol. Ther.* **8:** 180–187.
3. Yonemura, Y., Ku, H., Hirayama, F., Souza, L. M., and Ogawa, M. (1996). Interleukin 3 or interleukin 1 abrogates the reconstituting ability of hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* **93:** 4040–4044.
4. Peters, S. O., Kittler, E. L., Ramshaw, H. S., and Quesenberry, P. J. (1996). Ex vivo expansion of murine marrow cells with interleukin-3 (IL-3), IL-6, IL-11, and stem cell factor leads to impaired engraftment in irradiated hosts. *Blood* **87:** 30–37.
5. Naldini, L., *et al.* (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272:** 263–267.
6. Kafri, T., Blomer, U., Peterson, D. A., Gage, F. H., and Verma, I. M. (1997). Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat. Genet.* **17:** 314–317.
7. Uchida, N., *et al.* (1998). HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* **95:** 11939–11944.
8. Miyoshi, H., Smith, K. A., Mosier, D. E., Verma, I. M., and Torbett, B. E. (1999). Transduction of human $CD34^+$ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science* **283:** 682–686.
9. Mikkola, H., *et al.* (2000). Lentivirus gene transfer in murine hematopoietic progenitor cells is compromised by a delay in proviral integration and results in

doi:10.1016/j.ymthe.2005.01.005

transduction mosaicism and heterogeneous gene expression in progeny cells. *J. Virol.* **74:** 11191–11198.
10. Piacibello, W., *et al.* (2002). Lentiviral gene transfer and ex vivo expansion of human primitive stem cells capable of primary, secondary, and tertiary multilineage repopulation in NOD/SCID mice: nonobese diabetic/severe combined immunodeficient. *Blood* **100:** 4391–4400.
11. Schmidt, M., *et al.* (2002). Polyclonal long-term repopulating stem cell clones in a primate model. *Blood* **100:** 2737–2743.
12. Tahara-Hanaoka, S., Sudo, K., Ema, H., Miyoshi, H., and Nakauchi, H. (2002). Lentiviral vector-mediated transduction of murine CD34(−) hematopoietic stem cells. *Exp. Hematol.* **30:** 11–17.
13. Horn, P. A., *et al.* (2002). Lentivirus-mediated gene transfer into hematopoietic repopulating cells in baboons. *Gene Ther.* **9:** 1464–1471.
14. Sutton, R. E., Reitsma, M. J., Uchida, N., and Brown, P. O. (1999). Transduction of human progenitor hematopoietic stem cells by human immunodeficiency virus type 1-based vectors is cell cycle dependent. *J. Virol.* **73:** 3649–3660.
15. Barrette, S., Douglas, J. L., Seidel, N. E., and Bodine, D. M. (2000). Lentivirus-based vectors transduce mouse hematopoietic stem cells with similar efficiency to Moloney murine leukemia virus-based vectors. *Blood* **96:** 3385–3391.
16. Goodell, M. A., Brose, K., Paradis, G., Conner, A. S., and Mulligan, R. C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J. Exp. Med.* **183:** 1797–1806.
17. Goodell, M. A., *et al.* (1997). Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat. Med.* **3:** 1337–1345.
18. Ema, H., Takano, H., Sudo, K., and Nakauchi, H. (2000). In vitro self-renewal division of hematopoietic stem cells. *J. Exp. Med.* **192:** 1281–1288.
19. Hacein-Bey-Abina, S., *et al.* (2003). A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.* **348:** 255–256.
20. Marshall, E. (2003). Gene therapy: second child in French trial is found to have leukemia. *Science* **299:** 320.
21. Kohn, D. B., Sadelain, M., and Glorioso, J. C. (2003). Occurrence of leukaemia following gene therapy of X-linked SCID. *Nat. Rev. Cancer* **3:** 477–488.
22. Baum, C., *et al.* (2003). Side effects of retroviral gene transfer into hematopoietic stem cells. *Blood* **101:** 2099–2114.
23. McCormack, M. P., and Rabbitts, T. H. (2004). Activation of the T-cell oncogene LMO2 after gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.* **350:** 913–922.
24. Yamada, K., *et al.* (2003). Phenotype correction of Fanconi anemia group A hematopoietic stem cells using lentiviral vector. *Mol. Ther.* **8:** 600–610.
25. Biffi, A., *et al.* (2004). Correction of metachromatic leukodystrophy in the mouse model by transplantation of genetically modified hematopoietic stem cells. *J. Clin. Invest.* **113:** 1118–1129.
26. Zufferey, R., Donello, J. E., Trono, D., and Hope, T. J. (1999). Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.* **73:** 2886–2892.
27. Murray, L., *et al.* (2000). Addition of the human interferon beta scaffold attachment region to retroviral vector backbones increases the level of in vivo transgene expression among progeny of engrafted human hematopoietic stem cells. *Hum. Gene Ther.* **11:** 2039–2050.