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ARTICLE

Molecular Evidence of Lentiviral Vector-Mediated Gene Transfer into Human Self-Renewing, Multi-potent, Long-Term NOD/SCID Repopulating Hematopoietic Cells

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A major challenge in gene therapy is to achieve efficient transduction of hematopoietic stem cells (HSC). It has previously been shown that lentiviral vectors (LV) transduce efficiently human cord blood-derived NOD/SCID mouse repopulating cells (SRC). Here we studied the effect of cytokines during the short *ex vivo* incubation with vector. Although SRC transduction was efficient without stimulation, the presence of cytokines significantly improved it. The treatment did not affect the engraftment level or the SRC frequency, but seemed to enhance SRC susceptibility to LV. SRC transduced in both conditions repopulated primary and secondary recipients, maintaining stable multi-lineage transgene expression. Using linear amplification-mediated PCR, we then analyzed vector integration in the bone marrow and CFC of the engrafted mice to monitor the clonal activity of the transduced SRC *in vivo*. We showed polyclonal engraftment, multi-lineage differentiation, and propagation to secondary recipients of individual SRC. We observed multiple integrations in most clones. These results provide the first formal demonstration that primitive human HSC with self-renewal and multi-lineage repopulation capacities were transduced by LV. Our findings are relevant for the design of clinical protocols that exploit this system to reach significant engraftment by genetically modified HSC in the absence of *in vivo* selection or strong conditioning regimens.

Key Words: gene transfer, lentiviral vectors, hematopoietic stem cells, bone marrow transplantation, clonal analysis, NOD/SCID mouse repopulation assay

INTRODUCTION

Hematopoietic stem cells (HSC) are attractive targets for the gene therapy of a variety of genetic and acquired diseases, including immunodeficiencies, hemoglobinopathies, metabolic disorders, and cancer. HSC are found in very small numbers in adult bone marrow, mobilized peripheral blood, umbilical cord blood (CB), and fetal liver. CB-derived HSC are the most extensively studied because of their ready availability, but their clinical use is limited by the amount that can be retrieved from a single source. A surrogate assay for *in vivo* repopulation has been developed to evaluate human HSC. Injection of human hematopoietic cells into sublethally irradiated nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice leads to the establishment of a human graft in the mouse bone marrow that contains both myeloid and lymphoid lineages [1,2]. Cells giving rise to multi-lineage repopulation of NOD/SCID mice are designated SCID-repopulating cells (SRC), and are assumed to be closely related to the HSC that repopulate human recipients. Early reports indicated poor SRC transduction by Moloney murine leukemia virus (MLV) vectors, in agreement with the unsatisfactory results obtained in clinical trials [3,4] using this type of vector. More recently, vector pseudotyping with different envelopes (for example, Gibbon ape leukemia virus [5,6] or feline endogenous retrovirus [7]) and improved transduction protocols allowed significant SRC marking [6–13], even though prolonged culture and cell proliferation (two conditions reported to decrease the frequency and long-term repopulating capacity of HSC [13–16]) remained constant requirements. Two recent clinical studies of HSC gene therapy of severe combined immunodeficiencies showed the improvement achieved

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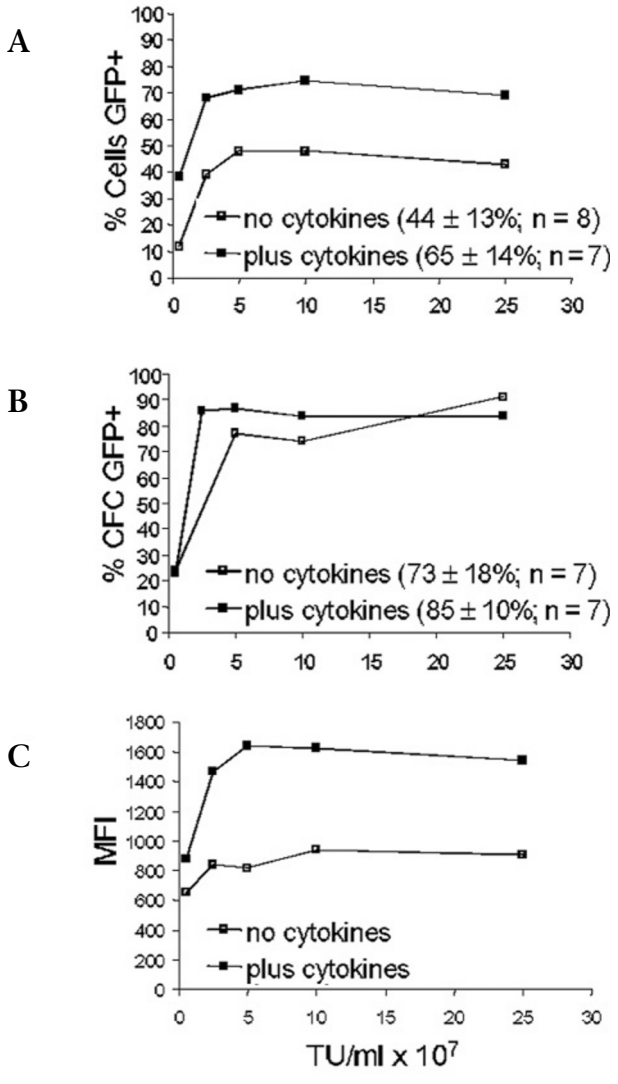

FIG. 1. Transduction efficiency of CD34+ cells and CFC. (A) CD34+ cells were exposed to increasing concentrations of vector, as indicated in transducing unit$_{HeLa}$ (T.U.)/ml, in the absence or presence of a cytokine cocktail (20 ng/ml IL6, 20 ng/ml TPO, 100 ng/ml SCF, and 100 ng/ml FLT3LG) for 20 hours, washed, and assessed for *GFP* expression by FACS 5 days later. In addition, the mean transduction efficiency of CD34+ cells from multiple experiments performed at vector concentrations of ϑ 5 $\times$ 10^7 T.U./ml are indicated in parentheses in the graph legend. A significant difference in transduction efficiencies was observed in the absence or presence of cytokines ($P < 0.001$ by paired Student's *t*-test). (B) CD34+ cells transduced at the indicated vector concentrations were plated in colony assays and the proportion of GFP+ CFC was determined by fluorescence microscopy. Again, the mean transduction efficiencies of CFC from multiple experiments performed at vector concentrations of ϑ 5 $\times$ 10^7 T.U./ml are indicated in parentheses. A lesser but still significant difference in transduction efficiencies of CFC was observed in the absence or presence of cytokines ($P < 0.05$ by paired Student's *t*-test). (C) Mean fluorescence intensity (MFI) of GFP+ CD34+ cells at increasing vector doses. This is a representative experiment out of five performed.

by the new protocols [17,18]. However, a major factor in the successful outcomes of these studies was the selective growth advantage conferred *in vivo* to transduced cells that could enable amplification of even a small input of transduced HSC. To broaden significantly the scope of HSC-based gene therapy, transduction of the majority of HSC in a transplant would be required, allowing for significant levels of engraftment by transduced cells even in the absence of *in vivo* selection or strong conditioning regimens.

Lentiviral vectors, such as those derived from HIV-1, may provide a means to achieve these ambitious goals [19–21]. The genomes of both HIV-1 and the vectors derived from it are actively imported to the nucleus and integrate in the absence of cell division [19,22–25], thus potentially bypassing the requirement for *ex vivo* cell cycle induction. Moreover, mitosis-independence allows a high frequency of transduction in a given target cell population even upon a short vector incubation. Others and we demonstrated that vesicular stomatitis virus (VSV)-pseudotyped HIV vectors transduce efficiently CB and adult bone marrow-derived SRC by a short *ex vivo* incubation in the absence or presence of cytokines [25–31]. In the course of these studies, improved vector design allowed higher transduction efficiencies, while the cytokine requirement for HSC transduction has remained controversial [26,27,29]. It was proposed that the minimal *ex vivo* manipulation allowed by lentiviral vectors may better preserve the long-term repopulation and self-renewal capacity of transduced SRC [13,27]. However, SRC quiescence may limit transduction even by lentiviral vectors [32], as was shown for T lymphocytes, which require activation out of G_0 for HIV-1 infection to be productive [33,34]. More importantly, no previous study provided direct evidence that the transduced SRC were capable of self-renewal, a distinguishing feature of stem cells.

In this study, we used improved lentiviral vectors containing the central polypurine tract-central termination sequence (cPPT-CTS) sequence to optimize transduction of CB-derived HSC and to characterize the effect of cytokines. By a highly sensitive PCR-based technique that identifies vector integration sites in the target cell genome and allows monitoring of clonal activity of transduced cells, we demonstrate highly efficient multi-copy gene transfer into SRC leading to polyclonal engraftment and long-term repopulation of primary and secondary mice. The transduced cells were capable of self-renewal and multilineage differentiation *in vivo*, while maintaining stable transgene expression.

RESULTS

Vector Dose Response and Effect of Cytokines on the Transduction Efficiency of CD34+ Cells and CFC

To determine the optimal vector concentration for the transduction of human CB-derived CD34+ cells, we carried out a dose response assay. We incubated 10^5 purified

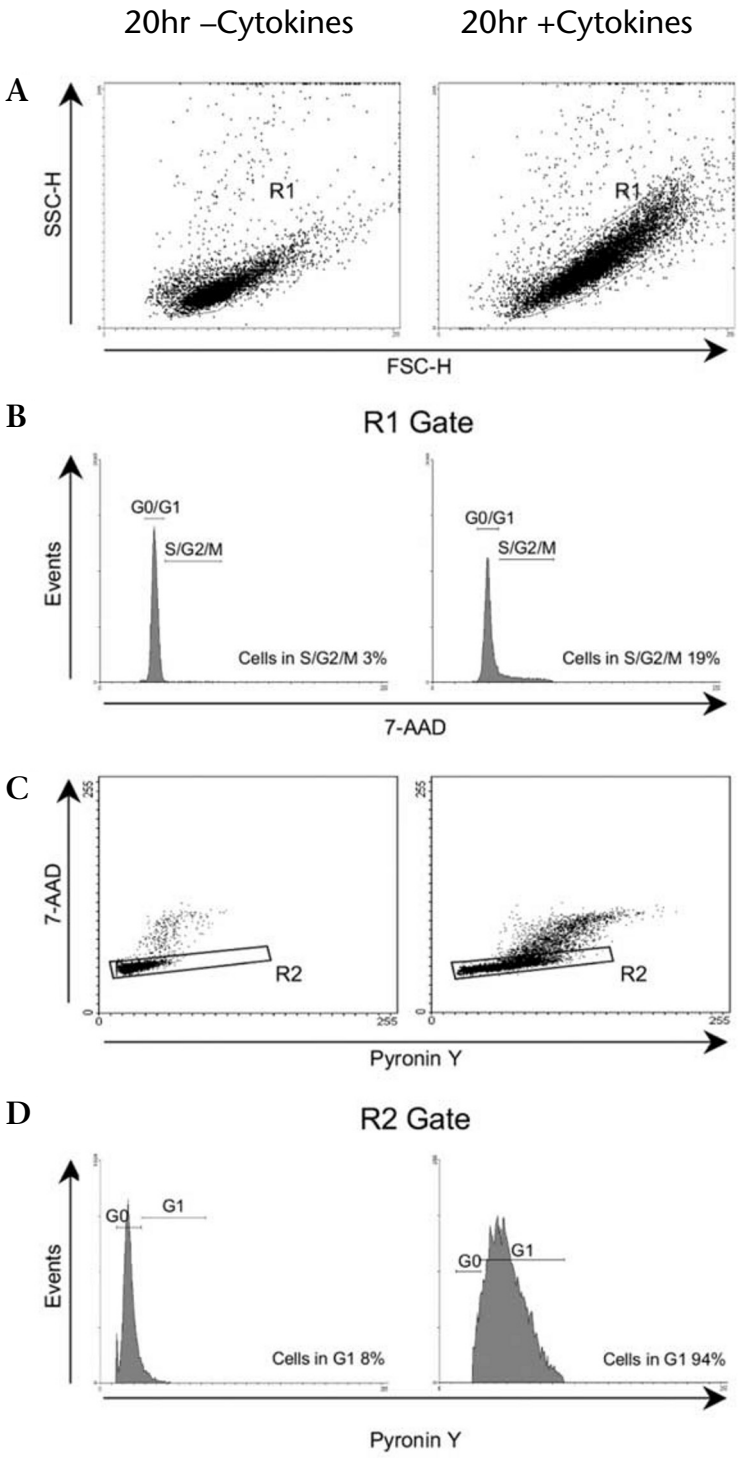

FIG. 2. Assessment of the cell cycle status of CD34+ cells after 20 hours of transduction in the absence or presence of cytokines. CD34+ cells were transduced in the absence or presence of cytokines for 20 hours, stained with 7-AAD and Pyronin Y, and analyzed by FACS for DNA and RNA content. Cells in $S/G_2/M$ have double the DNA of cells in G_0/G_1, and cells in G_1 have a higher RNA content than cells in G_0. (A) Dot plots showing physical parameters of all acquired events and the R1 gate used for the analysis. (B) Histogram of DNA staining of R1-gated cells. The proportion of cells in $S/G_2/M$ is increased upon exposure to cytokines. (C) Dot plots showing RNA staining (pyronin Y) versus DNA staining (7-AAD) of R1-gated cells and the R2 gate, which encompasses the G_0/G_1 population. (D) Histogram of RNA staining of R-2 gated cells plotted for Pyronin Y staining. The proportion of cells in G_1 is increased upon exposure to cytokines. One of two similar experiments is shown.

CD34+ cells with increasing concentrations of vector expressing the *GFP* gene under the control of the human *PGK* promoter. The transduction was carried out at a concentration of 5×10^5 cells/ml in serum-free medium in the absence or presence of a cytokine cocktail (20 ng/ml IL6, 20 ng/ml TPO, 100 ng/ml SCF, 100 ng/ml FLT3LG) for 20 hours. This cytokine cocktail was chosen because it was previously shown to maintain viability and provide for some expansion of SRC *in vitro* [35]. After transduction, cells from both conditions were maintained in liquid culture with cytokines and analyzed for *GFP* expression 5 days later, or plated in methylcellulose colony assay and scored for colony number and fluorescence 14 days later. There was a very steep increase in transduction efficiency between 1 and 5×10^7 transduction units (T.U.)/ml, both in total CD34+ cells (Fig. 1A) and CFC (Fig. 1B), after which a plateau was reached. At vector concentrations of ϑ 5×10^7 T.U./ml, the dose at which maximum transduction efficiency was achieved, the average transduction efficiency of CD34+ cells in the absence of cytokines was 44 ± 13% ($n = 8$), whereas when cytokines were present, a mean transduction efficiency of 65 ± 14% ($n = 7$) was achieved. The corresponding transduction efficiencies of CFC were 73 ± 18% ($n = 7$) and 85 ± 10% ($n = 7$) in the absence or presence of cytokines, respectively. In both cases, the difference between transduction efficiencies in the absence or presence of cytokines was statistically significant by paired Student's *t*-test (Fig. 1). It should also be noted that there was no difference in the numbers or types of colonies that grew from transduced or mock-transduced cells (data not shown), indicating that the vector did not inhibit or alter the ability of these cells to produce colonies in semisolid medium.

In these experiments, a vector concentration of 5×10^7 T.U./ml corresponded to a multiplicity of infection (M.O.I.) of 100. However, when different M.O.I.s versus vector concentrations were tested, the critical parameter affecting transduction efficiency was found to be vector concentration [36] (data not shown). Increasing the vector concentration beyond 10^8 TU/ml led to a slight decrease in transduction efficiency. The MFI, which

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TABLE 1: Engraftment and transduction efficiency of SRC

	-Cytokines		+Cytokines	
Experiment	%CD45+	%GFP+	%CD45+	%GFP+
2	2.2	11	3.1	94
	na	na	0.7	67
	na	na	0.4	69
3	91	10	50	75
	71	35	69	49
5	4.4	89	4.5	95
	na	na	3.4	74
6	29[a]	47	13	83
	3.3	42	4	68
	37	81	49	73
8	2.1	70	0.9	74
	3.1	65	0.9	64
	1.4	61	3.1	99
	na	na	0.6	95
9	25[b]	47	63[c]	46
	12.1	9.1	11	70
10	nd	nd	9[d]	89
	nd	nd	9	79
	nd	nd	7	54
	nd	nd	43[e]	70
	nd	nd	31[f]	91
	nd	nd	28	85
Mean (± 2 SEM)	23 ± 17	47 ± 16[g]	18 ± 9	76 ± 6[g]

Shaded boxes indicate mice that were transplanted into secondary recipients. na, Data not available due to death of mice before analysis; nd, not done. Experiment number 10 was performed at a later time to obtain secondary transplants with the highest efficiency of gene marking. Thus, the "no cytokine" arm was not performed.
[a,b,c,d,e,f]Mice for which secondary transplants were successful.
[g]This difference is statistically significant, $P = 0.0004$.

increases with increasing average vector copy number in the transduced population, also reached a plateau, which was again higher when cytokines were present in the transduction medium (Fig. 1C).

Thus, it seems that the presence of a cytokine cocktail during the 20-hour transduction protocol significantly increases the transduction efficiency of both total CD34+ cells and CFC. To investigate further the effect of the cytokine cocktail on CD34+ cells, they were analyzed by costaining with 7-AAD and Pyronin Y (Fig. 2). After 20 hours, the percentage of cells in $S/G_2/M$ was 3% and 19% in the absence or presence of cytokines, respectively, indicating that, although there was some shift of cells into $S/G_2/M$, most cells (81%) remained in G_0/G_1, even in the presence of the cytokine cocktail. However, on further analysis of the G_0/G_1 population, it was found that 94% of this population was in late G_1 after 20 hours in cytokines, whereas only 8% were in late G_1 in the absence of cytokines (Fig. 2).

Effect of Cytokines on Transduction Efficiency and Repopulation Potential of SRC

We then assessed the transduction efficiency of SRC using the optimal vector concentration of 5×10^7 T.U./ml in the absence or presence of cytokines. We injected intravenously 2.5 to 4×10^5 transduced CD34+ cells into sublethally irradiated NOD-SCID mice, and 6–12 weeks later we analyzed the bone marrow for multilineage engraftment and *GFP* expression (Table 1). Under both conditions, very high levels of transduction were achieved: 47 ± 16% ($n = 12$) and 76 ± 6% ($n = 22$) of engrafting human CD45+ cells were GFP+ in the absence or presence of cytokines, respectively. This difference was highly statistically significant ($P < 0.0005$). FACS analysis of different subpopulations showed *GFP* expression within the progenitor (CD34+), B cell (CD19+), myeloid cell (CD13+ or CD14+), and erythroid cell (glycophorin A+) populations in equivalent proportions (Fig. 3). GFP+ cells also engrafted spleen and peripheral blood (data not shown). Notably, high levels of transduction and a significant difference between the two transduction protocols were equally evident if only the subset of mice showing a high level of human engraftment was examined. In these conditions, stochastic variation in transduction frequency due to very small numbers of engrafting SRC is reduced.

Bone marrow from transplanted mice was placed in a human-specific colony assay, and in all cases *GFP* expression was detected in myeloid, erythroid, and mixed-lineage colonies, further demonstrating maintenance of myeloid differentiation and function (Fig. 4A). PCR was carried out on individual bone-marrow-derived CFC that were either GFP+ or GFP– by fluorescence microscopy. Primers for human β-globin were used to confirm the presence of human DNA, and primers for *GFP* were used to demonstrate the presence or absence of vector sequences (Fig. 4B). In all cases, CFC that were fluorescent were positive for *GFP* sequences, whereas those that were not fluorescent were negative for *GFP* sequences. This suggested that there was no significant silencing of gene expression in CFC during the 6- to 12-week engraftment period and that transduction efficiencies of CFC that were determined by fluorescence microscopy were accurate. Overall, there was a concordance between the percentage of GFP+ bone marrow-derived CFC and the total percentage of GFP+ cells in the same graft, with a tendency towards a higher value in the CFC (data not shown). The latter phenomenon has been observed by others [28], but the significance of this remains unclear.

In light of the fact that the transduction frequency of the human graft was improved by the addition of the cytokine cocktail to the transduction medium, it was

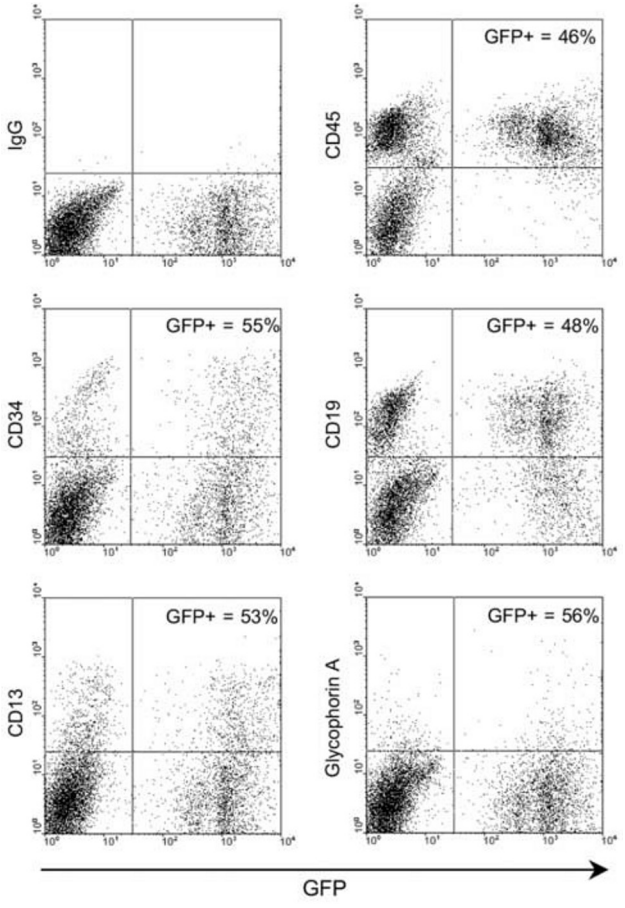

FIG. 3. Multilineage engraftment of a representative NOD/SCID mouse with *GFP*-expressing cells. Immediately following transduction, sublethally irradiated NOD/SCID mice were injected with 2.5 to 4 $\times$ 10^5 transduced CD34+ cells, and 6 to 12 weeks later the bone marrow was analyzed for the presence of multiple lineages of human hematopoietic cells and *GFP* expression. Bone marrow cells were stained with PE-conjugated isotype control (IgG) and antibodies for CD45 (total human cells), CD34 (primitive cells), CD19 (B lymphoid cells), CD13 (myeloid cells), and glycophorin A (erythroid cells). All lineages were found to contain equivalent proportions of GFP+ cells. FACS profiles from a single representative mouse are shown. The proportion of PE-positive cells that are GFP+ is indicated in the upper right quadrant.

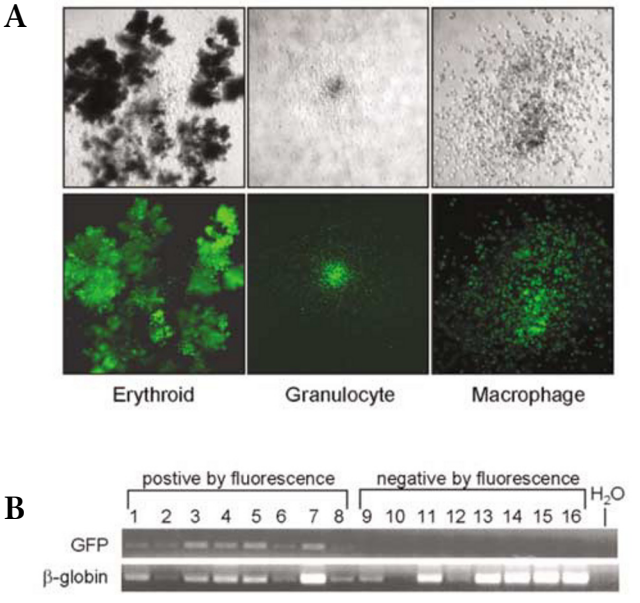

FIG. 4. PCR analysis for vector sequences in NOD/SCID mouse bone-marrow-derived human CFC. (A) Bone marrow from transplanted mice was plated in human-specific colony assay. GFP+ colonies of all types were detected. (B) Colonies from primary engrafted mice were plucked and analyzed by PCR for the presence of vector sequences. A representative experiment is shown, in which colonies 1–8 were GFP+ by fluorescence microscopy, whereas colonies 9–16 were GFP-negative by fluorescence microscopy. Colonies were also analyzed for human β-globin sequences to confirm the presence of sufficient human DNA for analysis. No GFP-negative colonies from primary mice were found to be positive for vector sequences by PCR, indicating that gene expression was not silenced *in vivo*.

important to determine whether this treatment influenced the total number or repopulating ability of SRC. In the experiments described above, there was not a statistically significant difference in the engraftment levels achieved by cells transduced under both conditions (Table 1). The mean percentage of CD45+ cells in mouse bone marrow was 23 ± 17% and 18 ± 9% in the absence or presence of cytokines, respectively ($P = 0.65$). To further investigate the effect of cytokines on engraftment of transduced cells, we performed limiting dilution experiments to determine the frequency of SRC after transduction under both conditions. The results of these experiments are shown in Table 2. We found that the frequency of SRC in populations of CD34+ cells transduced in either the absence or presence of cytokines was not significantly different. At a dose of 30,000 transduced CD34+ cells, one mouse in eight had multilineage engraftment and had human CFC in the bone marrow, both with and without cytokines present, indicating an equivalent frequency in the cell populations transduced under both conditions. When the frequencies were calculated using Poisson statistics [37], they were found to be 1 in 76,000 (95% confidence interval 35,087–166,683) and 1 in 80,000 (95% confidence interval 40,509–154,310) in the absence or presence of cytokines, respectively. In both cases, the proportion of mice engrafted and the engraftment levels achieved at low cell doses were similar. These results indicate that the short *ex vivo* exposure to cytokines increased the susceptibility of SRC to transduction by lentiviral vectors without inducing detectable changes in their number or engrafting capability relative to those transduced without cytokines present.

Secondary Transplants

To demonstrate that the transduced cells include those meeting the criteria of a repopulating stem cell, self-renewal must also be demonstrated. Performing secondary transplant experiments is one way to do this.

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TABLE 2: Engraftment of NOD/SCID mice injected with limiting numbers of transduced cells

		–Cytokines			+ Cytokines		
Dose		%CD45	%GFP+	CFC	%CD45+	%GFP+	CFC
$3/4 \times 10^5$	#1	29	47	+	13	83	+
	#2	3.3	42	+	4	68	+
	#3	37	81	+	49	73	+
	#4	2.1	70	+	0.9	74	+
	#5	3.1	65	+	0.9	64	+
	#6	1.4	61	+	3.1	99	+
	#7				0.6	95	+
1×10^5	#1	2.1	42	+	2.3	9	nd
	#2	2	33	+	14	82	+
	#3	1.3	17	+	0	0	-
	#4				0.8	21	+
	#5				2	74	+
	#6				18	96	+
3×10^4	#1	0.5	17	-	1.1	0	-
	#2	1	0	-	1.4	63	+
	#3	0.6	0	-	1.2	0	-
	#4	7.1	9.6	+	2.0	34	-
	#5	0.24	12	-	0	0	-
	#6	0	0	-	0	0	-
	#7	0	0	-	0	0	-
	#8	0	0	-	0	0	-

Engraftment of secondary recipients was successful in 6 of 11 mice transplanted (Table 1). In a first set of experiments, the engraftment levels of the secondary recipients were 0.82% (28% GFP+), 0.81% (64% GFP+), and 2.6% (31% GFP+) for mouse a, b, and c, respectively. In a second experiment we used *B2m*$^{-/-}$ NOD/SCID mice solely as secondary recipients to improve engraftment and obtained the following engraftment levels: 3% (22% GFP+), 11% (72% GFP+), and 3% (30% GFP+) for mouse d, e, and f, respectively. FACS analysis of the bone marrow of a representative primary-secondary mouse pair from the first set of experiments is shown in Fig. 5A. Secondary engraftment was multi-lineage and GFP-positive human CFC were detected in the BM of secondary mice. We performed PCR on colonies derived from secondary mouse bone marrow. Again, all colonies that appeared positive by fluorescence microscopy also scored as positive by PCR (Fig. 5B). The presence of GFP+ SRC in primary mice 6 weeks post-transplant suggested that self-renewal occurred within the transduced SRC population, when transduction had been performed in both the presence and the absence of cytokines.

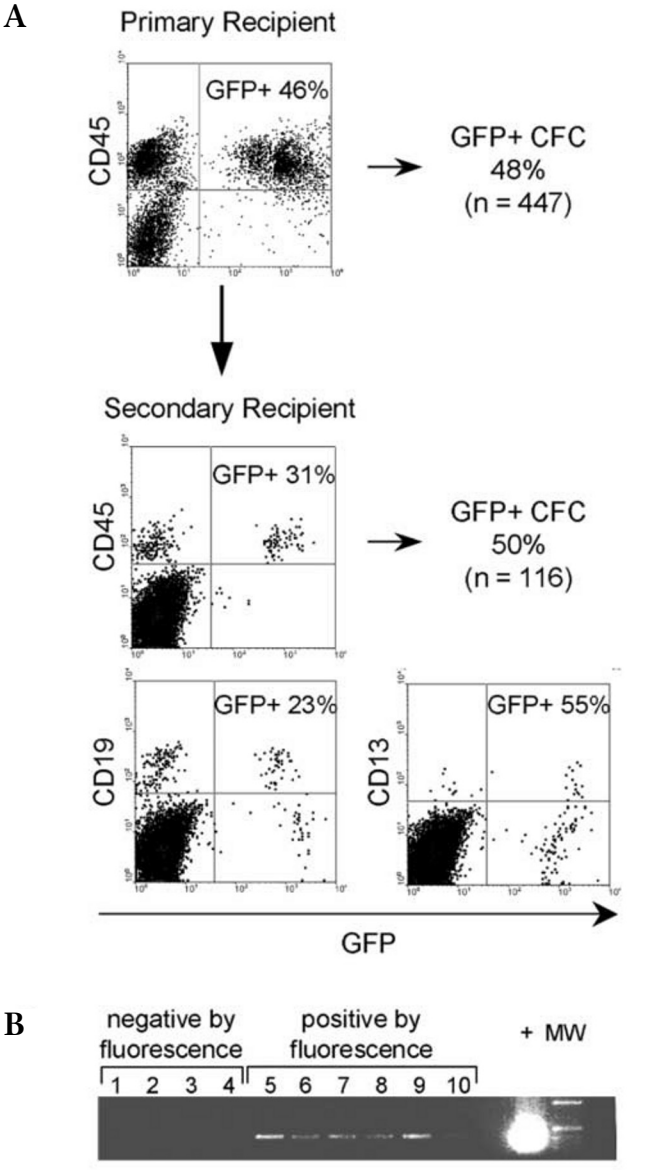

FIG. 5. Engraftment of secondary recipients with transduced cells. Whole bone marrow from primary recipients taken 6 weeks after injection with transduced human CB CD34+ cells was injected into sublethally irradiated secondary recipients. (A) A representative FACS analysis from a primary-secondary mouse pair, in which the primary had 63% human CD45+ cells, of which 46% were GFP+, and the secondary had 2.6% CD45+ cells, of which 31% were GFP+. Both lymphoid (CD19+) and myeloid (CD13+) lineages were detected in the GFP+ population, as well as GFP+ CFC. (B) Colonies from the secondary mouse were analyzed by fluorescence and PCR. Colonies 1–4 were negative by fluorescence microscopy, colony 5 was dim fluorescent and colonies 6–10 were bright fluorescent. All fluorescent colonies were GFP-positive by PCR. MW, molecular weight marker; +, positive control.

Linear Amplification-Mediated PCR Analysis of Vector-Integration Sites

Genomic DNA was extracted from the total bone marrow cells and individual hematopoietic colonies grown from five pairs of primary and secondary mice engrafted by SRC

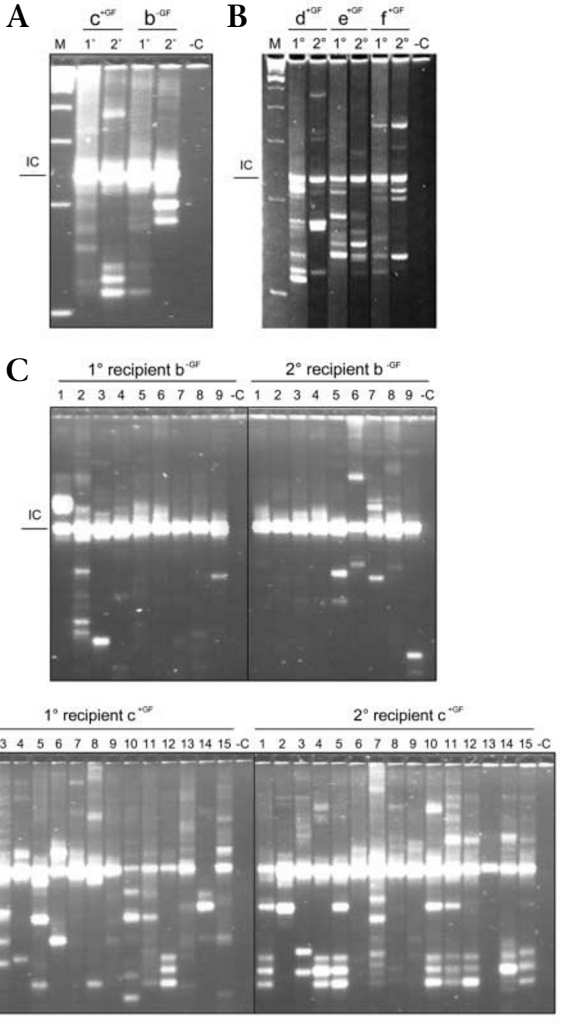

FIG. 6. Vector integration analysis in bone marrow (BM) and BM-derived human colonies. (A and B) LAM-PCR on BM DNA from primary (1°) and secondary (2°) mouse recipients engrafted with human cells transduced in the presence (c^{+GF}, d^{+GF}, e^{+GF}, f^{+GF}) or absence (b^{-GF}) of cytokines. Please note the presence of an internal vector control fragment (IC, 239 bp) in each sample that results from amplification of the proviral 5′-LTR. -C, negative control analysis on 1 μg of untreated mouse BM DNA. (C and D) LAM-PCR on individual BM-derived human colonies from a primary and related secondary recipient engrafted with cells transduced in the absence (C) or in presence (D) of cytokines, respectively. (D) CFC-GM 12 from the 1° c^{+GF} mouse and CFC-GM 1, 10, 11, CFC-M 4, 12, CFC-G 15, and BFC-E 5 from the related 2° mouse showed the same vector integration pattern. –C, 750 ng of non-transduced DNA.

transduced with or without cytokines (Table 1, experiments 9 and 10). Using a recently developed linear amplification-mediated PCR (LAM-PCR) [38,39] that permits the identification and sequencing of single proviral-genomic fusions, we mapped the vector integration pattern and monitored the clonal activity of transduced SRC in the mice (Fig. 6). Multiple vector integration bands were observed in the bulk bone marrow cells of the primary mice examined, suggesting polyclonal engraftment by multiple transduced SRC. Fewer bands were observed in the secondary mice with apparent propagation of selected bands from the cognate primary mouse, suggesting self-renewal and oligoclonal secondary engraftment by SRC (Figs. 6A and 6B). The sequences of some individual integration sites were obtained and direct matches to the human genome sequencing database were established (Table 3). The above interpretations were confirmed by analyzing individually a panel of human colonies grown in methylcellulose from the bone marrow of the mice at the time of sacrifice (Figs. 6C and 6D). Polyclonal and oligoclonal engraftments were proved in the primary and secondary mice, respectively, by the identification of multiple versus few repeated vector integration patterns in the different CFC. Notably, most individually examined CFC displayed several integration bands indicating transduction by a relatively high copy number of vector. This was more evident in the mice engrafted by cells transduced in the presence of cytokines (c^{+GF} mice).

Self-renewal of transduced SRC in the primary mice was directly proven when the same vector integration pattern was found in individual CFC from the primary and corresponding secondary mice. This was most clearly shown by a multiple integration pattern found in more than half the CFC, of both myeloid and erythroid types, from the secondary mouse and also detected in 1 of 15 CFC analyzed from its cognate primary mouse (CFC 12 from primary mouse c^{+GF} and CFC 1, 4, 5, 10, 11, 12, 15 from secondary mouse c^{+GF}; Fig. 6D). This clone appeared to carry at least five copies of the vector with a distinguishing triplet of small bands providing a signature marking. Minor differences in the amplification pattern of this clone were observed between different reactions, in particular for the extent of amplification of the high-molecular weight bands, presumably because of the high number of simultaneous integrations and the efficient competition by the smaller size bands. The clonal identity of the same "triplet" integration pattern identified in different samples was proven by direct PCR sequencing of the same integration site from LAM-PCR DNA amplicons of the same size isolated from the bone marrow and individual CFC of the primary and secondary mice (Table 3). Other candidate matches were observed in the primary-secondary pairs of these and other mice engrafted with cells transduced with or without cytokines, but were not further analyzed in this study.

DISCUSSION

Here we have validated a protocol for efficient gene transfer into human cord blood HSC by optimizing experimental parameters and proving the stem cell features of the transduced cells. As previously shown, lentiviral vectors efficiently transduce SRC [25–31]. In the optimized conditions used here, a human graft stably expressing the

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TABLE 3: Sequence information of unique vector integration sites identified in bone marrow and CFC DNA from primary and secondary recipients

Mouse[a]	Sample	Proviral/Genomic Sequence (5′-3′)[b]	Length[c]	Score	E-Value[d]	Locus[e]
c2° +GF	BM	*ATCTCTAGCA*ATCTCTACTTCACA CCATTTACAAAAATT**CCTAACTGCTG**	29	58	2e-07	Chr. 9q34
c2° +GF	BM	*ATCTCTAGCA*GGAAGTCCACAAT T**CCTAACTGCTGTGCCATGAATTCAGA**	14			
c2° +GF	BM	*ATCTCTAGCA*GGTGGAAGGATTGCT TGAGCCCANAAGTTCGAGGCTGTAG	251	478	e-132	Chr. 19
b2° -GF	BM	*ATCTCTAGCA*AACAGTAAGATGTTA AGATACTGAAAAATATTGTAGCCAA	86	163	3e-38	Chr. 1
c1° +GF	CFC	*ATCTCTAGCA*GGAAGTCCACAAT T**CCTAACTGCTGTGCCATGAATTCAGA**	14			
c2° +GF	CFC	*ATCTCTAGCA*GGAAGTCCACAAT T**CCTAACTGCTGTGCCATGAATTCAGA**	14			

[a]Letters refer to mice listed in Table 1, 1° and 2° denote primary and secondary recipients.
[b]50 nucleotides are shown in 5′-3′ orientation for each sequenced proviral-genomic fusion DNA. The DNA sequence corresponding to the last 10 nucleotides of the proviral DNA is indicated in italics. Linker sequences are presented in bold if the flanking genomic DNA is smaller than 40 nucleotides. Please note that only sequences > 20 bp have been taken into consideration for alignment search with the human genome sequencing data base ('Blast Search'; NCBI).
[c]Length of the genomic DNA.
[d]Expect value.
[e]Lentiviral vector integration locus in the human genome.

GFP transgene with up to 95% frequency repopulated NOD/SCID mice. However, if scoring only transgene expression frequency, it is difficult to distinguish between oligoclonal engraftment by few residual transduced SRC and polyclonal engraftment by a better-preserved pool of SRC transduced to significant frequency. Similarly, Southern blot analysis of bone marrow cells for integrated vector DNA may not distinguish between polyclonal engraftment and oligoclonal engraftment by SRC transduced with multiple vector copies. Using linear-amplification mediated PCR (LAM-PCR) to identify vector integration sites in human CFC panels from the transplanted mice, we showed multiple integration patterns indicating polyclonal engraftment by transduced SRC. LAM-PCR also showed that relatively high numbers of vector integrations per cell were achieved in SRC.

One purpose of this study was to determine the maximal transduction achieved in different experimental conditions to identify the critical factors. Whether such high levels of gene transfer would be appropriate for future clinical applications remains to be established. A dose response experiment revealed a plateau in transduction frequency of CD34+ cells and CFC at less than 100%. The addition of cytokines raised this plateau significantly, but a population of non-transduced cells remained. These results may indicate that a subset of cells is non-permissive to transduction under the conditions used, or that the ratio between infectious and total vector particles (vector infectivity) limits the transduction frequency achieved by a single vector exposure. As expected by the Poisson distribution of randomly occurring independent events, the average number of integrations per cell increased with the transduction frequency. This was first shown to occur by the increasing mean GFP fluorescence intensity (MFI) per CD34+ cell with higher vector doses. The MFI also reached a plateau, indicating that vector infectivity was limiting transduction, or that there was a toxic effect on the target cells at the highest vector concentrations. As previously reported [36], vector concentration and not MOI was one critical parameter driving transduction efficiency. The high vector concentrations used for maximal transduction were not detrimental to the cells and, when compared with the experimental conditions used in other published works [26–31,40], are much more similar than the calculated MOI. Moreover, we used only one incubation with the vector, as opposed to multiple vector hits, prolonged culture, and spinnoculation, procedures that all increase the actual infection rate (and not the calculated MOI) and may also be detrimental to the cells.

The influence of cytokines on SRC transduction by lentiviral vectors has remained controversial, with some studies reporting no effect of cytokines [26–31,40]. Here we used a cytokine combination previously shown to induce moderate SRC expansion [35] and found that its addition during the short *ex vivo* incubation with the vector did not affect the engraftment level or the SRC frequency but seemed to enhance the SRC susceptibility to transduction. This observation has implications for understanding the mechanism of transduction of lentiviral vectors and is relevant for the design of future clinical trials with this new gene transfer system. Although lentiviral vectors can integrate into the chromatin of non-dividing cells such as neurons, fibroblasts, and hepatocytes [41–43], resting T-lymphocytes have been shown

to be resistant to lentiviral infection [33,34,44–46]. Cycling of T lymphocytes through $S/G_2/M$, however, is not required, as stimulation into G_1 phase is sufficient to allow productive infection to occur [47,48]. It is conceivable that cells of different type and/or in different conditions display a range of susceptibility to transduction, with the quiescent G_0 T-lymphocytes at the lower end of the spectrum. A large proportion of CB-derived CD34+ cells remained in G_0/early G_1 at the end of the 20-hour transduction period without cytokines, whereas most of the cells had been activated into late G_1 phase by exposure to the cytokines. Although the untreated cells were transduced to high frequency, the triggering of the cell cycle correlated with an increase in transduction efficiency. We could not measure the cell cycle status of the few SRC contained in the CD34+ population, but we know from previous studies that the cytokine combination used induces moderate *ex vivo* expansion of SRC [35]. As we also observed an increase in transduction efficiency of SRC upon addition of cytokines, we suggest that SRC in the G_1 phase of the cell cycle are more susceptible to transduction than resting cells. However, it remains to be determined whether the efficient transduction of cytokine-untreated, CB-derived SRC is due to a large fraction of these cells being in G_1 or to a less efficient but productive transduction of cells in G_0. Although we analyzed too few samples to draw any conclusion, the lower number of vector integrations observed in CFC from mice engrafted by SRC transduced without cytokines would suggest the latter case.

Others have studied the effect of stimulating HSC into various phases of the cell cycle on their ability to repopulate. Wilpshaar *et al*. [49] found that CB CD34+ cells in G_0 or G_1 had similar NOD/SCID reconstitution potential. However, Glimm *et al*. [50] found that SRC reside exclusively in the G_1 population, and not in the G_0 or in the $S/G_2/M$ populations, after 5 days of culture with certain cytokines. Our results indicate that a 20-hour exposure of CB SRC to SCF, FLT3LG, TPO, and IL6 did not compromise self-renewal and NOD/SCID repopulating ability, consistent with the expectation that no cell would transit through $S/G_2/M$ in this short time. However, given the relatively brief duration of observation permitted by the NOD/SCID system and the fact that studies with SRC derived from sources other than CB reported an engraftment advantage of resting cells [16], caution should be exerted in extrapolating our results to future clinical applications as a possible decrease in long-term repopulation capacity may offset the advantage obtained with the increased transduction efficiency in the presence of growth factors.

One of the most stringent criteria to identify a HSC is its ability to self-renew. This is typically demonstrated by engraftment of secondary recipients, indicating the maintenance of the stem cell pool in the primary mouse, as reported for transduced SRC [13,28]. However, this is an indirect demonstration and does not rule out the possibility that different cells that were present in the initial inoculum, some of which remained quiescent throughout the primary engraftment period, repopulated primary and secondary recipients. To truly demonstrate self-renewal, one must prove that the engrafted populations from the primary and secondary mouse have arisen from a common precursor by clonal analysis. The previous studies used retroviral integration to show pluripotency [51] and to track clonal activity of SRC in primary NOD/SCID recipients [13], providing evidence for multilineage differentiation from individually transduced SRC in the transplanted mice. These studies, however, did not prove conclusively self-renewal of SRC because the authors could not perform clonal analysis in secondary recipients. Our findings now confirm and extend these works. By identification of the same vector integration pattern in CFC from primary and secondary mice, we proved that human primitive hematopoietic cells originating committed progenitors at the time of sacrifice of the primary mice propagated themselves to the secondary host in which they were still generating committed progenitors of both myeloid and erythroid types 6 weeks thereafter. This is the first direct demonstration, to our knowledge, that transduced human SRC self-renew in the engrafted mice and provides a long-sought validation both of the experimental model and of the new gene transfer system. We showed *GFP* expression in both lymphoid and myeloid human populations of the engrafted mice and performed the integration site analysis on the total bone marrow and individual myeloid CFC. Because lymphoid cells were the predominant human cells in the marrow of the engrafted mice and the vector integration pattern amplified from the total bone marrow was similar to that obtained from the majority of myeloid CFC in the c^{+GF} secondary mouse (Fig. 6), we suggest a common origin both of lymphoid and myeloid lineages from the same transduced SRC.

Another crucial issue for validating gene therapy is maintenance of gene expression *in vivo*. *GFP* expression was stable to high levels in the human grafts of all primary and secondary mice examined by FACS and we found a strong correlation between fluorescence of the hematopoietic colonies from primary and secondary mice inspected at the microscope and detection of vector sequences by PCR. Furthermore, clonal analysis proved stable gene expression in the multilineage progeny of individual self-renewing SRC over the 14-week span of the experiments. These findings are in agreement with recent studies on lentiviral-vector mediated transgenesis in which expression of a transduced *GFP* gene was maintained throughout the differentiation of embryonic stem cells and the development of mice and rat embryos [52,53].

Human primitive hematopoietic progenitors with the features of self-renewing, repopulating stem cells were

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transduced to very high frequency by the improved lentiviral vectors and the optimized protocols described here. Validation of these results in surrogate models allowing longer follow-up and with HSC from other sources will enable the design of gene therapy protocols possibly alleviating the requirement for *in vivo* selection of transduced HSC or for toxic preconditioning regimens. Coupled with the recently reported ability to deliver complex transcriptional regulatory elements and to achieve therapeutic levels of gene expression in transgenic mouse models of thalassemia and sickle cell disease [54,55], lentiviral vectors may soon become fundamental tools in HSC-based gene therapy.

MATERIALS AND METHODS

Cells. HeLa and 293T cells were maintained in Iscove's modified Dulbecco's medium (IMDM; Sigma Chemical Co., Milan, Italy) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Inchinnan, Scotland), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Mononuclear cells from human cord blood (CB) (scheduled for discard according to an Institution-approved protocol) were obtained by gradient centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden) and CD34+ cells were isolated using the Miltenyi MACS CD34+ progenitor cell isolation kit (Miltenyi Biotec, Gladbach, Germany). CD34+ cells were frozen in IMDM with 10% dimethylsulfoxide, 50% FBS, and stored in liquid nitrogen.

Vector production. Vector stocks were prepared and concentrated as previously described [56]. Briefly, 293T cells were transfected with the transfer vector plasmid, pRRLsin-18.PPT.hPGK.eGFP.Wpre [25], the VSV-G envelope-encoding plasmid pMD.G [19], and the packaging plasmid CMVΔR8.74 [56]. The supernatant was harvested, ultracentrifuged, and the vector pellet resuspended in a small volume of PBS/0.1% BSA. Vector titer was determined by adding serial dilutions to 10^5 HeLa cells in a Costar six-well plate (Corning Inc, Corning, NY) and determining the proportions of *GFP* expressing cells by FACS analysis (FACSCalibur, Becton Dickinson Immunocytometry Systems, San Jose, CA) 72 hours later.

Transduction. CD34+ cells were thawed and pooled, viable cells were counted, and they were incubated at a concentration of 5×10^5 cells/ml with the indicated concentration of vector for 20 hours at 37°C and 5% CO_2, in serum-free medium (IMDM containing 1% BSA, 10 μg/ml bovine pancreatic insulin, 200 μg/ml human transferrin, 10^{-4} M 2-mercaptoethanol, and 2 mM L-glutamine; StemCell Technologies, Vancouver, Canada) in the absence or presence of cytokines (20 ng/ml recombinant human (rh) IL6, 100 ng/ml rhSCF, and 100 ng/ml rh-FLT-3 ligand (rhFLT3LG; all from PeproTech Inc., Rocky Hill, NH), and 20 ng/ml rhTPO (Amgen, Thousand Oaks, CA).

Assessment of transduction efficiency in total cells and colony forming cells (CFC). Transduced cells were washed with IMDM and viable cells were counted. We plated 10,000 to 50,000 cells in IMDM/10% FBS with 20 ng/ml rhIL3, 20 ng/ml rhIL6, and 100 ng/ml rhSCF. After 5 days cells were assessed for %GFP+ by FACS. Transduced cells were also plated at a density of 800 cells/ml in methylcellulose medium (0.92% methylcellulose, StemCell Technologies, 30% FBS, 2 mM L-glutamine, 10^{-4} M 2-mercaptoethanol, 1% bovine serum albumin in IMDM) containing 3 U/ml rh-erythropoietin (Cilag, Milan, Italy), 10 ng/ml rh-granulocyte-macrophage colony-stimulating factor (Peprotech), 10 ng/ml rhIL3 (Peprotech), and 50 ng/ml rhSCF, and were scored by light and fluorescence microscopy 14 days later.

Transplantation of mice. NOD/LtSz scid/scid (NOD/SCID) mice [57] were obtained from the British Columbia Cancer Research Center and maintained according to approved protocols. We sublethally irradiated (350 cGy) 8- to 10-week-old mice 24 hours before intravenous injection of transduced CD34+ cells. Cells were transduced with 5×10^7 T.U./ml. The mice were maintained with water containing 120 μg/ml ciprofloxacin and 8 mM HCl and killed 6 to 13 weeks later. Blood, spleen, and bone marrow cells were retrieved for FACS analysis. In addition, bone marrow cells were plated in methylcellulose colony assays, at doses ranging from 50,000 to 250,000 nucleated cells/ml, as described above. For DNA analysis, cells were pelleted and extracted by phenol/chloroform. When secondary transplants were performed, the maximum number of cells obtained from a primary mouse (ranging from 10 to 40 million) was injected intravenously into a single secondary mouse. When indicated, β2 microglobulin deficient (*B2m*$^{-/-}$) NOD/SCID mice (obtained as above) were used as secondary recipients.

FACS analysis of mouse tissues. Cells from mouse tissues were treated with 7% ammonium chloride (StemCell Technologies) to lyse red cells, resuspended in phosphate-buffered saline (PBS) with 2% FBS, 5% human serum, and 5% mouse serum, and incubated on ice for 10 minutes. Cells were then divided into 100 μl aliquots and 10 μl of each of the following human-specific phycoerythrin-conjugated antibodies were added: anti-CD45, anti-CD34, anti-CD19, anti-CD14, or anti-CD13, and IgG isotype control (DAKO, Glostrup, Denmark). After 30 minutes on ice, cells were washed, stained with 2 μg/ml propidium iodide, resuspended in PBS/2% FBS, and flow cytometric analysis was performed. A gate was set to exclude at least 99.9% of cells labeled with the isotype control, and the % of antibody-positive/GFP-positive cells was determined after excluding nonviable cells. We scored 10,000 events.

Determination of SRC frequencies. The frequency of SRC in a population of cells was determined by injecting cohorts of mice with ~ threefold dilutions of transduced cells. After 6 weeks the bone marrow was analyzed and a mouse was scored as positive if both myeloid and lymphoid lineages were detectable by FACS (> 0.5% CD45+ cells) and human CFC were detectable by methylcellulose culture. The frequency of SRC was then calculated from the proportions of negative mice in each cohort, using the L-calc software program (StemCell Technologies), which uses Poisson statistics and the method of maximum likelihood [37].

Staining of CD34+ cells for cell cycle analysis. Cells were washed with PBS, and resuspended in 0.5 ml staining buffer (0.1 M phosphate-citrate, 0.15 M NaCl, 5 mM sodium EDTA, 0.5% BSA, pH 4.8 with 0.02% saponin). We added 10 μl 1 mg/ml 7-aminoactinomycin D (7-AAD) and incubated cells at room temperature for 20 minutes. Cells were then moved to ice, incubated for 10 minutes, and then 0.5 μl of 1 mg/ml Pyronin Y was added, and cells were incubated on ice for a further 10 minutes. Cells were then analyzed by FACS for DNA and RNA staining. Up to 100,000 events were acquired.

PCR on mouse BM-derived human colonies. Individual, well-isolated colonies were plucked into 0.5 ml of PBS, vortexed, left at room temperature for 30 minutes, then vortexed again and centrifuged at 5000 rpm for 5 minutes. Pellets were resuspended in 20 μl of lysis buffer (20 mM Tris HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.45% Tween-20, 0.45% Nonidet P-40, and 1.5 mg/ml proteinase K) and incubated at 56°C for 1 hour and 100°C for 15 minutes, followed by phenol chloroform extraction and resuspension in 30 μl of H_20. PCR was carried out on 5 μl of the preparation. Two primer sets were used, one specific for human β-globin (5′-GGGCAAGGTGAACGTGGATGA-3′ and 5′-CCATCACTAAAGGCACCGAGC-3′) and one specific for *GFP* (5′-GCAGAAGAACGGCATCAAGGT-3′ and 5′-GAACTCCAGCAGGACCATGTGA-3′). Amplification was performed with AmpliTaq Gold (Perkin Elmer, Branchburg, NJ) in 2.5 mM $MgCl_2$ for β-globin and 1.5 mM for *GFP*. DNA was denatured at 95°C for 10 minutes, then amplified with 30 cycles of 95°C for 30 seconds, 56°C for 1 minute, and 72°C for 1 minute, and a final single cycle of 72°C for 7 minutes. PCR products were analyzed by agarose gel electrophoresis, yielding a 307-bp band for β-globin and a 202-bp band for *GFP*. Only colonies that were β-globin positive were scored for the presence of *GFP* sequences. An improved protocol yielding higher sensitivity was used to analyze colonies from secondary mice. We used 25 pmoles of two previously described *GFP* primers [25] giving a 417-bp band with AmpliTaq Platinum (Gibco BRL) in 1.5 mM $MgCl_2$. PCR conditions were one cycle

of 94°C for 2 minutes, then 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds.

Lentiviral vector (3′-LTR) integration site analysis. Integration site analysis was performed on DNA isolated either from total bone marrow (BM) or individual BM-derived human colonies. BM-DNA (1 μg) or colony DNA samples (3–5 μl) were prepared as described above and served as templates for further analysis. The genomic-proviral junction sequence was identified using a recently developed linear amplification-mediated (LAM)-PCR. Full details of LAM and ligation-mediated (LM) integration site analysis have been described [38,39] and will be reported elsewhere (M.S. *et al.*, manuscript submitted). In brief, linear amplification of target DNA was performed by repeated primer extension with an LTR-specific 5′ biotinylated primer (5′-GAGCTCTCTGGCTAACTAGG-3′ or 5′-GAACCCACTGCTTAAGCCTCA-3′) and *Taq* polymerase (Qiagen, Valencia, CA; 100 cycles of amplification with addition of fresh enzyme after 50 cycles). Amplified fragments of target DNA were enriched by magnetic tag selection of the labeled extension primers (Dynal, Oslo, Norway). A second DNA strand of each enriched target sequence was synthesized after random hexanucleotide priming with Klenow DNA polymerase (Roche Diagnostic, Indianapolis, IN). Double-stranded DNA was then specifically digested with the four-cutter enzyme *Tsp*509I that cuts within the genomic DNA at high frequency. The length of each fragment was therefore dependent on the distance of the vector insertion site from the next specific restriction endonuclease recognition sequence. A double-stranded asymmetric linker cassette was ligated to the restriction sites by T4 DNA ligase. The resulting ligation products were amplified by nested exponential PCR using oligonucleotide cassette-specific forward primers and LTR-specific reverse primers. The final products were separated on a Spreadex high-resolution gel (Elchrom Scientific, Cham, Switzerland). In each sample, a common fragment of 239 bp resulted from the cutting of *Tsp*509I within the vector in the linear amplicon from the 5′-LTR (the LTR-specific 5′ biotinylated primer annealed to both LTR) and it was used as an internal vector control of the procedure. For verification of the origin and potential identity of specific integration site amplicons, PCR sequencing was performed on amplicons isolated by DNA size fractionation through electrophoresis.

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