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Comparison of Various Envelope Proteins for Their Ability to Pseudotype Lentiviral Vectors and Transduce Primitive Hematopoietic Cells from Human Blood

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Substantial effort has been invested in developing methodologies for efficient gene transfer into human, repopulating, hematopoietic stem cells. Oncoretroviral vectors are limited by the lack of nuclear mitosis in quiescent stem cells during *ex vivo* transduction, whereas the preintegration complex of lentiviral vectors contains nuclear-localizing signals that permit genome integration without mitosis. We have developed a flexible and versatile system for generating lentiviral vector particles and have pseudotyped such particles with amphotropic, ecotropic, feline endogenous virus (RD114) or vesicular stomatitis virus (VSV-G) envelope proteins. Particles of all four types could be concentrated ~ 100-fold by ultracentrifugation or ultrafiltration. RD114 or amphotropic particles were more efficient than VSV-G-pseudotyped particles at transducing human cord blood $CD34^+$ cells and clonogenic progenitors within that population. Amphotropic particles transduced cytokine-mobilized, human peripheral blood $CD34^+$ cells capable of establishing hematopoiesis in immunodeficient mice more efficiently than the other two types of particles. We conclude that the use of amphotropic pseudotyped lentiviral vector particles rather than the commonly used VSV-G-pseudotyped particles should be considered in potential applications of lentiviral vectors for gene transfer into this therapeutically relevant target cell population.

Key Words: gene therapy, stem cells, vector, lentivirus, oncoretrovirus, pseudotype, bone marrow, envelope, transduction, concentration

INTRODUCTION

The ability to transfer genes into repopulating hematopoietic stem cells *ex vivo* and to achieve expression of the transferred gene in their differentiated progeny following transplantation would create many therapeutic opportunities. Despite nearly two decades of effort, stem-cell-targeted gene transfer for therapeutic purposes has remained an elusive goal [1]. Only recently has success been achieved in patients with severe combined immunodeficiency secondary to a lack of a component, the common γ-chain, of a key receptor on lymphoid progenitors [2]. In this disorder, there is potent selection for gene-corrected cells, thereby overcoming the low transduction efficiency of repopulating stem cells.

Most efforts to transfer genes for therapeutic purposes into hematopoietic stem cells have focused on the use of murine oncoretroviral vectors [1]. Such vectors require mitosis of the target cell to allow integration of the viral genome [3], but hematopoietic stem cells are typically quiescent [1]. In addition, the receptor for the most commonly used particles that are generated with the oncoretroviral amphotropic envelope protein is expressed at low levels on primitive hematopoietic cells, thereby limiting transduction efficiency [4,5]. Improved transduction efficiencies have been achieved by using complex cytokine mixtures to activate stem cells *ex vivo* [6,7], by using alternative envelope proteins to generate vector particles [8,9], and by using retronectin to colocalized target cells and vector particles during *ex vivo* transduction [10]. These modifications have resulted in low levels of genetically modified bone marrow and blood cells with only a few animals having up to 10% marked cells following transplantation in large animal models [11–14]. These studies have been done using peripheral blood cells mobilized with stem-cell factor (SCF) or Flt-3 ligand and granulocyte-colony stimulating factor (G-CSF), whereas human studies that have used cells mobilized with G-CSF alone have resulted in lower efficiencies of gene transfer [15]. Thus, despite improvement in transduction conditions, the level of efficiency of stem-cell transduction with

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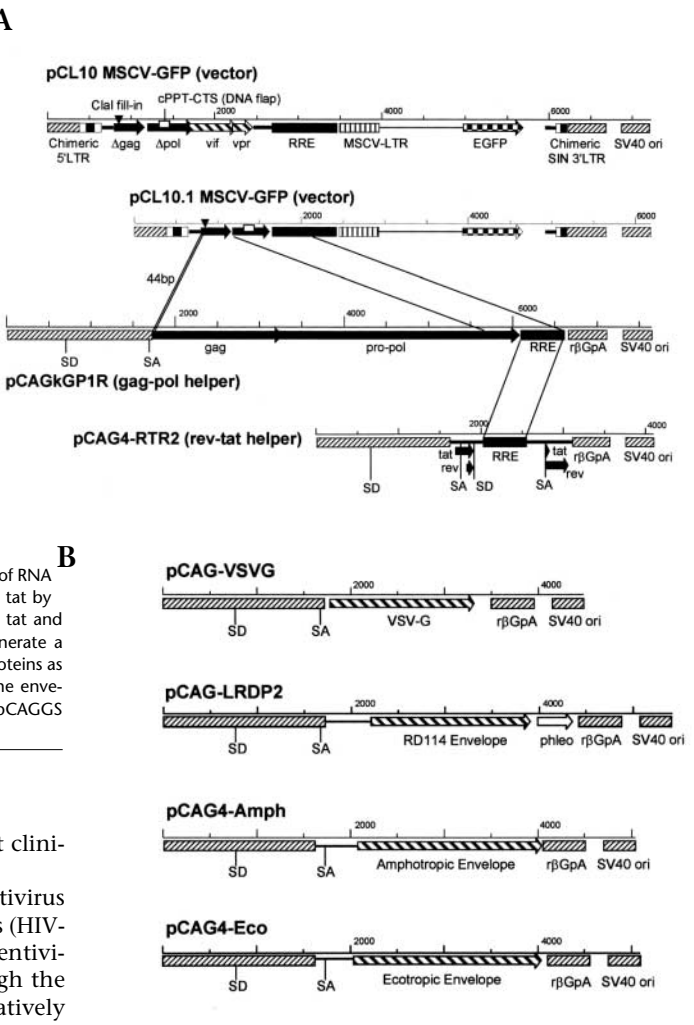

FIG. 1. (A) The lentiviral vector system. Several modifications were introduced to improve vector production and/or enhance safety as the vector genome was assembled to derive pCL10. The 5′ LTR is chimeric in that the U3 region of the HIV-1 LTR is replaced with the CMV enhancer. A deletion in the U3 region of the 3′ LTR renders it self-inactivating; in addition, a portion of the R region and the U5 region have been removed and replaced with the rabbit β-globin gene polyadenylation site to enhance safety and improve the efficiency of vector production. An insertion into the GAG-POL coding sequence was achieved by filling in the *Cla*I site, creating a frameshift and a termination codon. The central polypurine tract (cPPT) and central termination sequence (CTS) were added to improve vector production; the vif and vpr coding sequences present in pCL10 were eliminated from pCL10.1. The expression plasmid pCAGGS [43] was used to construct the cassettes expressing HIV structural and regulatory proteins and various envelope proteins. This expression plasmid contains a powerful chimeric CMV enhancer/β-actin promoter, the β-globin large intron (which ensures splicing of most transcripts), the rabbit β-globin polyadenylation sequence (which includes an RNA stability element in the 3′-untranslated region), and the SV40 origin of replication (ori) to increase plasmid copy number in 293T cells. The accessory proteins, tat and rev, were expressed together on a plasmid separate from that expressing the GAG/GAG-PRO-POL-precursor polyprotein. The RRE element was included to enhance nuclear to cytoplasmic transfer of RNA molecules encoding gag/pol and to modulate expression of rev and tat by fostering transport of unspliced transcripts. It was positioned in the tat and rev plasmid to ensure that homologous recombination did not generate a plasmid encoding both the HIV structural and functional accessory proteins as shown in the diagram. (B) Envelope expression plasmids. Each of the envelope coding sequences was subcloned into the expression plasmid, pCAGGS [43].

oncoretroviral vectors remains inadequate for most clinical applications.

More promising are retroviral vectors of the lentivirus class typified by the human immunodeficiency virus (HIV-1) [16]. In contrast to oncoretroviral vectors, the lentiviral vector preintegration complex can move through the nuclear membrane without mitosis [3] and it is relatively stable [17], increasing the window of opportunity for genome integration. Another advantage of lentiviral vectors is their ability to transfer and express complex genetic elements, most specifically a genomic globin gene with associated regulatory elements necessary for high-level expression [18]. Previous efforts to use oncoretroviral vectors for globin gene transfer had been largely unsuccessful [1]. Beginning with the report that lentiviral vectors based on HIV-1 were superior to traditional oncoretroviral vectors at introducing genetic information into primitive human hematopoietic cells from cord blood that establish hematopoiesis in immunodeficient mice [19], several reports have described transduction of primitive cord blood or bone marrow cells with such vectors [20–24]. However, transduction of G-CSF-mobilized primitive hematopoietic cells from peripheral blood, the preferred clinical target, has not been evaluated.

Most lentiviral vector preparations have relied on the use of the envelope protein from VSV-G to generate vector particles [19–24]. However, lentiviral vectors based on the HIV-1 internal components can be pseudotyped with a variety of envelope proteins [25,26]. Such pseudotyped vector particles share the desirable feature of VSV-G-pseudotyped particles in that they can be concentrated by ultracentrifugation or ultrafiltration.

Our studies were focused on evaluating lentiviral vectors that were generated with various envelope proteins for their ability to transduce primitive human hematopoietic cells. Of specific interest to us was the envelope protein of feline endogenous virus (RD114), which we have already shown yields oncoretroviral vector particles with enhanced efficiency at transducing primitive hematopoietic cells in human umbilical cord blood [9]. The lentiviral vector system we developed was genetically engineered to capitalize on features shown by others to enhance safety with respect to generation of replication-competent retroviruses during vector production and to enhance the efficiency of vector

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TABLE 1: Comparison of lentiviral vector systems

	Helper	Envelope	Vector	Titer on HeLa (TU/ml)
St. Jude I	pCAGkGP1R, pCAG4-RTR2	pCAG-VSVG	pCL10.1 MSCV-GFP	4.0×10^{7}[a]
1st generation[16]	pCMVΔR8.2	pMD-G	pRRL ppt PGK GFP W sin[33]	4.3×10^{6}
2nd generation[28]	pCMVΔR8.91	pMD-G	pRRL ppt PGK GFP W sin[33]	2.2×10^{6}
2nd generation	pCMVΔR8.91	pMD-G	pHR'sin cPPT SEW[b]	4.5×10^{6}
3rd generation[29]	pMDLg/p-RRE, pRSV-rev	pMD-G	pRRL ppt pGK GFP W sin[33]	3.1×10^{6}

[a]The mean for seven preparations was $4.0 \times 10^{7} \pm 1.6 \times 10^{7}$.
[b]Provided by Adrian Thrasher.

particle generation. Our results indicate that lentiviral vectors are relatively efficient at transducing repopulating stem cells present in human peripheral blood following G-CSF administration and suggest that vector particles pseudotyped with the amphotropic envelope protein are useful for transferring genes into this clinically relevant target population.

RESULTS

Characterization of the Vector System

A series of HIV-based lentiviral vector systems have been developed that introduce features to enhance safety and improve the efficiency of vector particle production [27]. In first-generation systems, all of the HIV structural, regulatory, and accessory proteins are encoded on a single helper plasmid, and the vector genome contains intact long terminal repeats (LTRs) [16]. In second-generation systems, several accessory proteins have been eliminated [28], whereas in third-generation systems only rev among the nonstructural proteins is retained. In the latter, the rev coding sequences are expressed from a separate helper plasmid, and the HIV LTRs are modified to eliminate tat dependence during vector production (5′ LTR) and to render vector LTR-dependent transcription self-inactivating upon integration (3′ LTR) [29–31]. Our system, designated St. Jude I (Fig. 1), is modeled after third-generation vector systems and modified to incorporate the central polypurine tract and central termination signal [32–34] into the vector genome. The vector contains the enhanced green fluorescent protein (EGFP) gene under the control of the murine stem-cell virus (MSCV) promoter in a configuration that expresses well in murine and nonhuman primate bone marrow transplant models [35,36]. Unlike other third-generation systems, tat is generated in our system during vector production on the basis of its ability to enhance subsequent transduction of $CD34^{+}$ cells. The efficiency of generation of vector particles with our system compares favorably to the efficiency of first-, second-, and third-generation systems developed by others when compared directly in our laboratory (Table 1).

Efficient Transduction of Murine Repopulating Hematopoietic Cells with Lentiviral Vector Particles Pseudotyped with the Ecotropic Envelope Protein

We sought to establish whether our lentiviral vector encoding EGFP could be pseudotyped with the oncoretroviral ecotropic envelope protein and concentrated by ultracentrifugation. Ecotropic pseudotyped particles were packaged efficiently with titers of conditioned medium similar to that of VSV-G-pseudotyped vectors. In addition, the ecotropic vector preparation could be concentrated ~ 100-fold by ultracentrifugation (data not shown). Vector preparations of both pseudotypes transduced long-term hematopoietic repopulating cells as reflected by the presence of substantial percentages of genetically modified cells of all hematopoietic lineages in the peripheral blood of mice 3–5 months after transplantation (Table 2).

Transduction of Human Cord Blood $CD34^{+}$ Cells with a Lentiviral Vector Pseudotyped Various Envelope Proteins

Our initial data suggested that the RD114 and amphotropic envelope proteins were less efficient at generating infectious lentiviral vector particles than the VSV-

TABLE 2: Lentiviral vector mediated gene transfer into murine repopulating hematopoietic cells

Blood lineage	% GFP positive cells	
	VSV-G[a]	Ecotropic[a]
	n = 5	*n* = 6
RBC	69 (35–97)	61 (45–74)
Myeloid Cells	78 (50–97)	85 (77–94)
Platelets	68 (25–96)	73 (64–90)
B-Cells	74 (62–89)	59 (48–70)
T-Cells	68 (61–77)	41 (26–52)

[a]Analysis of the mice which received the cells transduced with the VSV-G pseudotype was performed at 19 weeks and those which received cells transduced with the ecotropic pseudotype at 12 weeks.

TABLE 3: Transduction of cord blood CD34+ cells with pseudotyped lentiviral vectors

			%GFP+(N)			
Vector/envelope	Transduction method[a]	Vector concentration[b]	No prestimulation		Prestimulation	
PCL10/VSV-G	VCM[c]	4.3×10^5	7.3	(2)	37.9	(2)
	VCM	1.3×10^7	17.7	(3)		-
	VCM	3.4×10^7	64.0	(1)	62.0	(1)
	Preload[d]	1 -	4.2	(5)	28.6	(2)
PCL10/RD114	VCM	4.2×10^5	67.0	(5)	88.0	(2)
	VCM	5.2×10^5	70.0	(3)		-
	VCM	2.0×10^6	89.0	(1)	77.0	(1)
	Preload	1 -	36.1	(13)		-
PCL10/Amph	VCM	2.0×10^6	89.0	(1)	77.0	(1)
MSCV/RD114	VCM	4.3×10^5	-	-	67.0	(1)
	Preload	1 -	0.6	(3)	52.0	(1)

[a]Two different cytokine combinations were used in individual experiments as indicated in the Materials and Methods.
[b]Vector concentrations expressed as infectious particles/ml of culture medium were based on titering of transducing particles on HeLa cells.
[c]Cells were transduced in vector containing medium (VCM) for 12 hours.
[d]Cells were transduced in retronectin-coated plates which had been incubated with vector containing culture medium as described [9].

G envelope protein (Table 3). Titering of serial dilutions on HeLa cells indicated that the titers of VSV-G preparations usually exceeded those of the RD114 or amphotropic pseudotyped preparations by > 10-fold. We considered the possibility that vector particle production was more nearly equivalent among the various envelope proteins and that the VSV-G particles were merely more efficient at transducing HeLa cells. This hypothesis was tested with VSV-G and RD114 envelope-pseudotyped particles on cord blood CD34+ cells at various concentrations of vector particles or after preloading vector particles onto retronectin. Indeed, the RD114-pseudotyped particles were superior to the VSV-G pseudotype on these cells at various concentrations of vector particles (Table 3 and Fig. 2). In several individual experiments including those included in Table 3, without prestimulation the percentage of CD34+ cells transduced with VSV-G particles was 13.7 ± 16.9 versus 51.6 ± 20.8 with RD114 particles ($P < 0.0001$), whereas with prestimulation, the corresponding values were 28.9 ± 18.2 versus 80.0 ± 7.6 ($P < 0.0001$). In preliminary experiments, particles pseudotyped with the amphotropic envelope protein also seemed potentially superior to VSV-G particles at

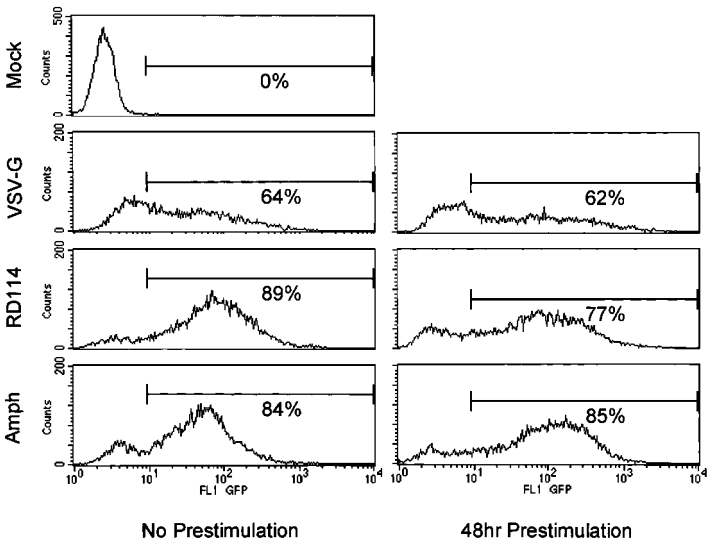

FIG. 2. FACS analysis of transduced cord blood CD34+ cells. Target cells were transduced with a fixed volume (0.5 ml) of conditioned medium containing vector particles of the defined pseudotyped specificity: VSV-G, RD114 or amphotropic (Amph) envelopes, as indicated on the vertical axis. Cells transduced without prestimulation were immediately exposed to vector particles. At the end of 12–15 hours, the cells were transferred to fresh cytokine-containing medium and cultured for a total of 96 hours. Prestimulated cells were incubated in cytokines for 48 hours, exposed to vector particles for 12 hours, and replated in cytokine-containing medium without vector particles to a total of 96 hours *ex vivo*. The multiplicities of infection for the VSV-G, RD114, or amphotropic pseudotyped particles were 10^7, 10^6, or 10^6 infectious particles, respectively, as determined by titering these preparations on HeLa cells.

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TABLE 4: Transduction of human peripheral blood CD34+ cells derived NOD/SCID mice repopulating cells

	Vector concentration of transduction medium[a]	PB-CD34+ cells		Progenitor derived colonies		NOD/SCID human cells		
Envelope	(HeLa UT/ml)	%GFP + mean (Min–Max)	No. of exp.	%GFP+ Min–Max	No. of exp.	% CD45+ Mean (Min–Max)	%GFP+(CD45) Mean (Min–Max)	No. of mice
No Prestimulation								
Mock	–	0	2	0	2	31 (4–81)	0 (0-0)	6
LD cytokines[b]	–	0	1	0	1	34 (15–62)	0 (0-0)	4
36hr in culture	–	0	1	0	1	22 (19–27)	0 (0-0)	3
VSV-G	2.3×10^7	19 (10–28)	2	16 (5–26)	2	32 (2–71)	4.7 (2.2–7.2)[d]	7
RD114	1.3×10^6	27 (25–29)	2	19 (12–25)	2	29 (2–55)	1.6 (0.4–2.7)	8
Amphotropic	$1.3–1.7 \times 10^6$	44 (36–52)	2	45 (37–52)	2	13 (2–27)	11.6 (1.0–17.6)[d]	6
LD cytokines[b]	1.3×10^6	33	1	36	1	54 (37–63)	4.3 (2.0–5.8)	5
36hr in culture[c]	$1.6–4.8 \times 10^6$	24 (13–36)	2	26 (12–39)	2	13 (2–33)	5.0 (2.5–10.0)	4
Prestimulation								
Mock	–	0	2	0	2	17 (2–34)	0 (0-0)	5
VSV-G	2.3×10^7	44 (30–58)	2	47 (32–61)	2	5 (1–13)	9.2 (3.0–17.6)[e]	6
RD114	1.3×10^6	56 (45–66)	2	59 (47–70)	2	24 (2–62)	1.0 (0.5–1.7)	7
Amphotropic	1.7×10^6	70 (63–77)	2	74 (68–79)	2	17 (3–32)	16.0 (10.8–20.7)[e]	8

[a]Transduction was performed in 67% conditioned medium + 33% X-VIVO10 containing 1% BSA, 300 ng/ml SCF, 300 ng/ml Flt3-L, and 50 ng/ml IL-6 for 12 hours except for i ndicated experiments. In all experiments, 3×10^6 cells were transduced on retronectin-coated 10-cm tissue culture dish in total volume of 15 ml medium.
[b]Cells were transduced in low dose (LD) cytokines (SCF 50 ng/ml and IL-6 25 ng/ml).
[c]Transduced in X-VIVO10 + BSA 1% for 36 hours using vector preparations concentrated by ultracentrifugation.
[d]The mean and standard deviation for VSV-G was 4.7 ± 1.9 and for amphotropic 11.0 ± 7.7; this difference is statistically significant ($P < 0.05$).
[e]The mean and standard deviation for VSV-G was 4.7 ± 1.9 and for amphotropic 16.0 ± 3.2; this difference is statistically significant ($P < 0.02$).

transducing cord blood CD34+ cells (Table 3 and Fig. 2). Control experiments with oncoretroviral (MSCV) vector particles pseudotyped with the RD114 envelope protein confirmed a relatively high level of transduction, but only after prestimulation of the target cells for 48 hours in medium containing high concentrations of cytokines (Table 3). Two different cytokine concentrations were used in various experiments, as indicated in the notes to Table 3, without significant effect on transduction efficiency (data not shown).

Transduction of G-CSF-Mobilized Primitive Hematopoietic Cells from Peripheral Blood with Lentiviral Vector Particles Pseudotyped with Various Envelope Proteins

Our success at transducing cord blood cells with lentiviral vector particles pseudotyped with the RD114 or amphotropic envelope proteins prompted us to evaluate the efficiency of transduction of G-CSF-mobilized, peripheral blood hematopoietic cells. The primitive cells in the mobilized population have generally been more refractory to transduction with oncoretroviral vector particles than primitive hematopoietic cells from cord blood [37]. Our results suggest that G-CSF-mobilized peripheral blood CD34+ cells and the clonogenic progenitors within this population were more efficiently transduced with lentiviral vector particles pseudotyped with the RD114 or amphotropic envelope proteins than with particles pseudotyped with the VSV-G envelope protein (Table 4). Prestimulation of the target CD34+ cells (1×10^5/ml) in high concentrations of cytokines (SCF, 300 ng/ml; Flt-3 ligand, 300 ng/ml; interleukin-6 (IL-6), 50 ng/ml) for 48 hours before vector particle exposure increased transduction efficiency by about twofold with each vector pseudotype. The efficiency of transduction of CD34+ cells was about twofold higher over various concentrations of vector particles when the vector production system included tat (data not shown).

One advantage of VSV-G-pseudotyped particles is their stability and ability to be concentrated by ultracentrifugation. We found that RD114 (these studies) and we and others [25] have shown that amphotropic pseudotyped particles could also be concentrated about 100-fold by ultracentrifugation or ultrafiltration, with recovery of 80–100% of infectious particles (data not shown). Although concentration of the vector particles increased experimental flexibility, we found that the frequency of transduction of peripheral blood CD34+ cells by a single exposure to vector particles could not be increased beyond 70–80% by increasing vector concentration (data not shown); this observation is analogous to results reported by others [17]. In these experiments, the cell concentration was constant at 2.5×10^4 cells/ml and the vector concentration was varied.

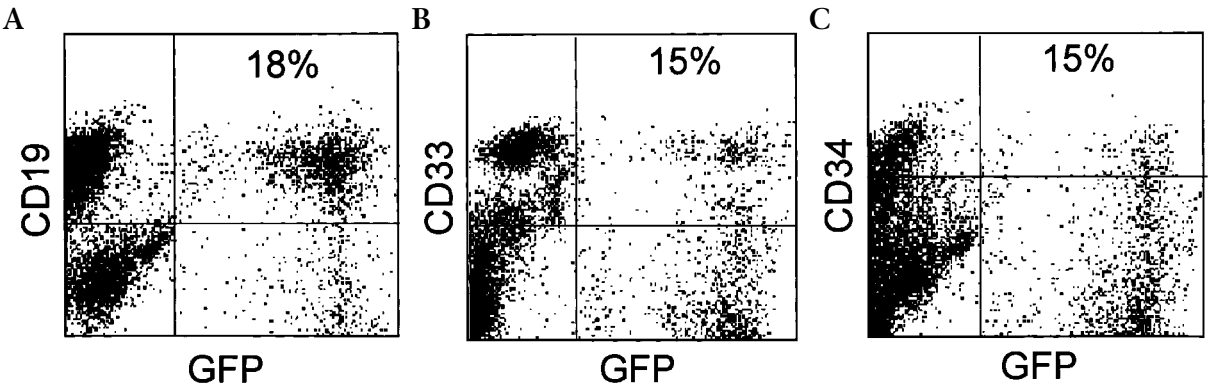

FIG. 3. Multilineage expression of the EGFP marker in human hematopoietic cells after transplantation of transduced human cells into immunodeficient mice. NOD/SCID mice transplanted with genetically modified, G-CSF-mobilized human peripheral blood CD34$^+$ cells transduced with lentiviral vector particles pseudotyped with the amphotropic envelope protein were killed after 6 weeks, and bone marrow cells were analyzed for transduced (A) B-lymphoid (CD19–PE), (B) myeloid (CD33–PE), or (C) primitive (CD34–PE) cells defined as human by expression of the human CD45 marker. In this representative animal, 18% of the B lymphocytes, 15% of the myeloid cells, and 15% of the CD34$^+$, immature cells within the human CD45$^+$ population were positive for EGFP.

The various pseudotyped lentiviral vector particles were also tested for their ability to transduce cells capable of establishing human hematopoiesis in immunodeficient (nonobese/severe combined immunodeficient (NOD/SCID) strain) mice. Significant transduction of this target population was observed with VSV-G and amphotropic pseudotyped particles (Table 4). The frequency of transduction, as reflected by the proportion of genetically modified (GFP$^+$) human hematopoietic cells (CD45$^+$) in bone marrow 6 weeks after transplantation, was about twofold higher after 48 hours of prestimulation (Table 4). Cells of both the myeloid and lymphoid lineages were GFP$^+$, as were primitive cells expressing the CD34 antigen (Fig. 3). Under identical conditions of transduction without prestimulation, the proportion of human CD45$^+$, EGFP$^+$ cells was higher in animals that received cells transduced with amphotropic particles compared with VSV-G particles (11.6 ± 7.7 versus 4.7 ± 1.9, $P < 0.05$). Similarly, the average proportion of human CD45$^+$ cells that were EGFP$^+$ in the mice that received prestimulated cells transduced with amphotropic vector particles was significantly higher than in mice that received cells transduced with VSV-G particles (16.0 ± 3.2 versus 9.2 ± 5.6, $P < 0.02$). Although the proportion of human CD45$^+$ cells was somewhat lower with prestimulated cells transduced with VSV-G particles, the percentage of CD45$^+$, EGFP$^+$ cells did not correlate with engraftment (data not shown). The mean values of genetically modified myeloid (CD33$^+$) and primitive (CD34$^+$) cells from animals that received cells transduced with amphotropic particles exceeded the values from animals that received cells transduced with VSV-G particles, although the results were not statistically significant (data not shown). The proportion of genetically marked B lymphocytes (CD19$^+$) analyzed separately was significantly higher in animals that received cells transduced with amphotropic particles (8.5 ± 5.4 versus 16.4 ± 5.0, $P < 0.02$).

DISCUSSION

Our studies have focused on comparing the ability of lentiviral vectors pseudotyped with various envelope proteins to transduce primitive human hematopoietic cells. Vector particles bearing the VSV-G, RD114, ecotropic, or amphotropic envelope proteins could be efficiently generated and effectively concentrated using a state-of-the-art lentiviral vector system with a self-inactivating, HIV-based vector. The particles pseudotyped with the VSV-G envelope protein had a 10-fold higher titer on HeLa cells than particles pseudotyped with the amphotropic envelope protein, but amphotropic and RD114-pseudotyped particles transduced human CD34$^+$ and progenitor cells from umbilical cord blood or the blood of adult volunteers following G-CSF administration as efficiently as the VSV-G-pseudotyped particles. The amphotropic particles were superior at transducing the cells within the CD34$^+$ cell population from adult peripheral blood capable of establishing human hematopoiesis in immunodeficient mice.

Our results indicate that lentiviral vectors pseudotyped with either the VSV-G or ecotropic envelope proteins transduced mouse repopulating cells with transduction efficiencies (Table 2) higher than previously reported [38,39]. The lentiviral vector system offers considerable experimental flexibility with respect to vector design in that it is capable of transferring complex genetic elements that can be designed to achieve lineage-specific expression [18,40], a goal that has been difficult to achieve with conventional oncoretroviral vectors. Most work in murine systems using conventional oncoretroviral vectors has relied on the derivation of high-titer producer clones, a process that can take weeks to months to achieve. In contrast, the lentiviral vector system relies on the production of vector particles following plasmid transfection of 293T cells, allowing the transition from recombinant plasmid to

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vector particles to be achieved over only several days. Thus the lentiviral vector system offers considerable experimental advantages over conventional oncoretroviral vectors in mouse model systems.

The higher relative efficiency of amphotropic versus VSV-G-pseudotyped lentiviral vector particles at transducing primitive human hematopoietic cells was unexpected. The receptor for the amphotropic envelope protein is known to be expressed at limiting low levels on such cells [4,5], whereas the VSV-G-pseudotyped particles are thought to use a receptor comprising a ubiquitously distributed phospholipid [41]. However, a recent direct comparison found equivalent transducing efficiencies for VSV-G versus amphotropic envelope-pseudotyped oncoretroviral vector particles when tested on primitive human hematopoietic cells from cord blood, bone marrow, or peripheral blood [42]. Of interest is a recent report that G-CSF-mobilized, primitive human hematopoietic cells, analyzed in a model designed to evaluate T-lymphopoiesis, were somewhat more efficiently transduced with VSV-G-pseudotyped particles than with amphotropic particles [22]. Despite the high efficiency with which RD114-pseudotyped oncoretroviral vector particles transduced primitive hematopoietic cells from cord blood [9], such particles transduce cytokine-mobilized, NOD/SCID repopulating hematopoietic cells from human peripheral blood poorly (P.F.K., unpublished data). Lentiviral vectors bearing this envelope protein also transduced early hematopoietic cells from human peripheral blood less effectively than amphotropic particles (Table 4). Certainly, there are many other influences besides receptor density on the transduction efficiency of various target populations, and our results indicate that amphotropic envelope particles should be considered in using lentiviral vectors for transduction of human stem cells.

In developing our system, we tested various transcriptional elements to achieve high-level expression of the components of the packaging system, including GAG/POL, rev and tat, the envelope protein, and the vector genome. Specifically, the cytomegalovirus immediate early (CMV-IE), Rous sarcoma virus (RSV) LTR, and the HIV-1 LTRs were tested for each plasmid with a synthetic intron or the human β-globin large intron for splicing. Overall, we have found that the CAGGS plasmid, which includes a powerful, chimeric CMV enhancer/β-actin promoter, the β-globin large intron, the rabbit β-globin 3′-untranslated region and polyadenylation sequence,and the simian virus-40 (SV40) origin of replication [43], gave the highest efficiency of particle production (data not shown). Initially, we used the rev and tat cDNAs to express these proteins from separate expression plasmids but found that high levels of these proteins reduced vector production. Accordingly, we developed a plasmid in which expression of these proteins is under the negative feedback of rev by virtue of its capacity to promote nuclear to cytoplasmic translocation of unspliced, nonfunctional transcripts (Fig. 1). Thus as rev accumulates, the level of tat and rev expression is downmodulated. Inclusion of tat in our system increased titers about twofold (data not shown), presumably because tat interacts with the 5′ end of the CMV promoter-driven vector transcript and increases its accumulation. Our vector also includes the central termination sequence and central polypurine tract, which together comprise the "flap" sequence that enhances transduction efficiency with lentiviral vectors [32–34]. We found that a minimal fragment of 120 bp containing these sequences was not as effective at increasing titer as a larger fragment that also includes a previously described enhancer [44]. Though useful for many experimental purposes, the St. Jude I system may be amenable to further optimization by a systematic evaluation of various vector and helper plasmid combinations and testing of various promoters.

Recent studies have identified pseudotransduction and episomal gene expression as being potential artifacts in the evaluation of gene transfer with lentiviral vectors [17]. Pseudotransduction is passive transfer of marker protein synthesized in vector-producing cells; it occurs within the first 24–48 hours and is unlikely in our studies because of the 48 hours of culture following transduction before the target $CD34^+$ population was scored by fluorescence-activated cell sorting (FACS). Furthermore, there was a close correlation between results obtained with the $CD34^+$ cell population and the progenitors within that population that were scored after 14 days in culture (Table 4). Because the preintegration complex of lentiviral vectors is relatively stable, gene expression without integration has been documented for 7 or more days post transduction [17]. Again, because transduction efficiency into clonogenic progenitors correlates with results obtained with bulk CD34 cells, episomal expression in our experiments seems unlikely and is excluded in the case of amphotropic and VSV-G-pseudotyped particles by the transduction of repopulating cells assayed in NOD/SCID mice (Table 4).

Continued refinements of transduction protocols have improved the efficiency of gene transfer into repopulating stem cells with oncoretroviral vectors as assayed in nonhuman primate models [8,11–14]. Whether the use of lentiviral vectors will result in even greater transduction efficiencies in the clinical context is currently unknown. Several studies have clearly established that lentiviral vectors are superior to oncoretroviral vectors at transducing primitive hematopoietic cells from umbilical cord blood without prestimulation with cytokines [19–24]. Also, lentiviral vectors seem superior at transferring complex vector genomes with multiple regulatory elements necessary to achieve high-level, tissue-specific expression [18,40]. Our data support the conclusion that lentiviral vectors can be efficiently packaged with a variety of envelope proteins and concentrated to enhance experimental versatility [25,26]. Despite limitations noted with use of amphotropic oncoretroviral vector particles [4,5], this

pseudotype seemed superior to the more commonly used VSV-G pseudotype in our experiments with lentiviral vectors in which cytokine-mobilized primitive human hematopoietic cells from adult normal volunteers were used to evaluate gene transfer efficiency.

Most work with lentiviral vectors has focused on the use of primitive hematopoietic cells from human umbilical cord blood [19–24]. The more clinically relevant target population, however, are the repopulating cells present in human peripheral blood following cytokine administration. There is a growing experience with the use of G-CSF-mobilized stem cells for reconstitution following allogeneic bone marrow transplantation, and it would seem that this target population is the preferred one for future gene therapy trials. Coadministration of SCF along with G-CSF yields a population of stem cells that are more amenable to transduction with conventional oncoretroviral vectors in primate models than cells mobilized with G-CSF alone or with G-CSF and Flt-3 ligand [45], but SCF is currently not available for use in humans. Thus, our observations indicating that lentiviral vectors pseudotyped with the amphotropic envelope protein can be used to successfully transduce primitive cells following G-CSF administration to human volunteers represents potentially significant progress toward the development of gene therapy approaches. Future work will focus on optimizing transduction of this population by a systematic evaluation of various parameters including the cytokines used for prestimulation and the number of exposures to vector particles during *ex vivo* transduction.

Materials and Methods

Plasmid construction. The vector plasmids (Fig. 1) were constructed as follows. The 5′ LTR is a chimera consisting of the CMV enhancer (nt 544–924: GenBank acc. no. X03922) and the HIV LTR promoter, followed by the R and U5 regions (nt 377–634, acc. no. K03455). The 3′ LTR consists of parts of the U3 and R regions of the HIV-1 LTR (nt 9086–9120 and nt 9521–9621, acc. no. K03455) and the polyadenylation signal from the rabbit β-globin gene (rβGpA). The 5′ LTR is followed by the HIV-1 leader sequence and a part of the coding sequences for GAG up to nt 1147 (acc. no. K03455). To minimize the probability of transmission of functional GAG/POL coding sequences in the event of homologous recombination during vector production, we eliminated the *Cla*I site (nt 830), creating a shift in the reading frame and a termination codon. The vectors also have a segment of envelope sequence (nt 7637–8404, acc. no. K03455) that includes the rev response element (RRE) and downstream sequences including the polypurine tract (nt 8898–9085, acc. no. K03455). pCL10 has a 1512-bp *Pst*I (blunt)–*Hin*dIII (blunt) fragment from pRtatpEGFP [46], which includes nt 1420–1443 and nt 4554–6031 from the HIV genome (acc. no. K30455) connected by the linker sequence CGCGGATCAG inserted 5′ to the RRE (*Eco*NI site). pCL10.1 contains a 477-bp *Eco*RI (blunt)–*Nde*I (blunt) fragment from pRtatpEGFP instead, eliminating the vif and vpr coding sequences that are included in the larger fragment from pRtatpEGFP in the pCL10 vector. The *Eco*RI–*Nde*I fragment also includes nt 4649–5125 from the HIV genome (acc. no. K03455), containing the central polypurine tract and the central termination sequence (cPPT-CTS), which maximize transduction efficiency of vector preparations [32–34]. This fragment also contains an enhancer sequence [44]. The vectors also contain the full-length MSCV LTR and leader-(GAG sequences from MGirL22Y [35,36] as an internal promoter element. Although the LTR includes a polyadenylation signal and splice donor site, northern blot analysis of RNA extracted from transfected 293T cells did not show evidence of transcription termination within the MSCV-LTR, nor did Southern blot analysis of DNA from transduced HeLa cells show evidence of genome rearrangement.

The GAG-POL helper plasmid, pCAGkGP1R, was assembled by inserting the coding sequences for GAG-POL and the RRE element (nt 112–4454 and nt 6976–7501, acc. no. M15654) into the expression plasmid, pCAGGS [43] (Fig. 1). The oligonucleotide segment that includes the GAG-POL initiation codon, ATG, was changed to match the Kozak consensus sequence in an effort to optimize expression. The sequence, ATAG, was inserted after the GAG-POL open reading frame to interrupt the vpr coding sequences with a stop codon. The rev-tat helper plasmid, pCAG4-RTR2, was assembled by inserting two fragments from the HIV genome (nt 5145–5514 and nt 6976–8019, acc. no. M15654) that encode the rev and tat exons and the RRE into pCAGGS. The sequence, TTGATATC, was inserted between the two fragments to place a stop codon into the vpu open reading frame. The *Xpa*I–*Eco*RI fragment, which contains the rabbit β-globin IVS2 splice acceptor site, was deleted from pCAGGS before assembly of the rev-tat helper plasmid.

The envelope expression plasmids were assembled as follows: 1) pCAG-VSV-G by inserting a blunt-ended *Xho*I fragment from pL VSV-G [47], which encodes the VSV-G envelope protein into pCAGGS; 2) pCAG-LRDP2 by inserting a blunted *Xho*II–*Sal*I fragment from the plasmid RDF [48], which encodes the RD114 envelope protein into pCAGGS; and 3) pCAG4-Amph by inserting a *Xba*I–*Hpa*I fragment from pPAM3 [49], which encodes the amphotropic envelope protein into pCAGGS, and removing the *Xba*I–*Eco*RI fragment that contains the rabbit β-globin IVS2 splice acceptor sequence. Further details regarding these constructions are available on request.

Vector production. Vector preparations were produced by cotransfection of 293T cells with a mixture of four plasmids using the calcium phosphate precipitation method. Briefly, 2–4 × 10^6 293T cells were seeded onto 10-cm^2 tissue culture dishes 24 hours before transfection. 293T cells were maintained in D10 medium (DMEM containing 10% (v/v) FBS, penicillin G (50 units/ml), and streptomycin (50 μg/ml)) and incubated at 37°C in 5% CO_2. The four-plasmid mixture consisted of 10 μg pCAGkGP1R, 2 μg pCAG4RTR2, 2 μg envelope plasmid, and 10 μg vector plasmid (either pCL10 or pCL10.1), proportions that had been empirically shown to maximize vector particle production. The plasmids were mixed and diluted to a total volume of 450 μl with double-distilled water, after which 50 μl of 2.5 M $CaCl_2$ were added. Subsequently, 500 μl 2× HBSS (280 mM NaCl, 1.5 mM Na_2PO_4, 50 mM Hepes, pH 7.05) were added dropwise while vortexing the mixture, after which 10 ml of DMEM containing 10% FBS, penicillin G (50 units/ml), and streptomycin (50 μg/ml) were added. The culture medium from each 10-cm^2 plate containing 293T cells was replaced with this D10–DNA–$CaPO_4$ mixture, after which the cells were incubated at 37°C in 5% CO_2 for 18 hours. Subsequently, the calcium phosphate–DNA mixture was removed and the 293T monolayer was washed twice with 5 ml PBS and then overlaid with fresh D10.

The medium conditioned by vector-producing cells was harvested 24 hours later, cleared by low-speed centrifugation, and filtered through 0.2-mm pore size cellulose acetate filters. Aliquots were quick-frozen and kept at –80°C until the time of use. All vector preparations were assayed for replication-competent retrovirus by exposure of HeLa and $293T^+$ cells to conditioned medium, followed by assay of culture medium for p24 as described [46]. Before use, the vector stock was thawed in a room temperature water bath over a few minutes and immediately used for transduction of target cells. Preliminary titers were determined by transfection of HeLa cells. Serial dilutions of each vector preparation were added to 10^5 HeLa cells growing in a monolayer in six-well plates in a total volume of 2 ml of D10 containing Polybrene (6 μg/ml). After 12–18 hours, the medium was changed to fresh D10 and culture was continued for another 2–3 days. Transfection efficiency was estimated by FACS analysis for EGFP expression and the titer calculated from a linear range. The p24 levels were determined on aliquots of each vector preparation by an ELISA (Coulter, Miami, FL) in accordance with the manufacturer's instructions.

Vector concentration. Vector preparations that were intended for concentration were derived by conditioning serum-free medium using either UltraCULTURE (BioWhittaker, Walkersville, MD) or X-VIVO 10 without phenol red (BioWhittaker). The serum-free medium was added after the

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overnight transfection and PBS wash of producer cells. After conditioning, the medium was harvested, clarified by low speed centrifugation, and filtered through 0.2-μm pore size cellular acetate filter. Concentration by ultracentrifugation was achieved by spinning the conditioned medium for 90 minutes at 25,000 rpm at 4°C using a Beckman SW28 rotor. The pellet was suspended in serum-free medium by repipetting and frozen for subsequent use. Concentration by ultrafiltration was achieved using Centricon Plus-80 filters according to the manufacturer's instruction (Millipore, Bedford, MA).

Lentiviral transduction and transplantation of murine bone marrow cells. Donor bone marrow cells were harvested from the femurs and tibias of C57/B16J mice (Jackson Laboratories, Bar Harbor, ME) after treatment with 150 mg/kg 5-fluorouracil (Pharmacia, Kalamazoo, MI) 48 hours earlier. Cells were placed into DMEM culture medium containing 20% FBS (Hyclone, Logan, UT) and 20 ng/ml murine IL-3, 50 ng/ml human IL-6, and 60 ng/ml murine SCF (all obtained from R&D Systems, Minneapolis, MN) for 24 hours. Cells were then collected and resuspended at 2×10^6 cells/ml in DMEM containing "2×" concentrations of the above ingredients. Cells were then mixed with an equal volume of conditioned medium containing ecotropic or VSV-G-pseudotyped viral vector at a multiplicity of infection of 2–4 (based on GFP transfer to NIH3T3 cells) and placed into Retronectin-coated 10-cm dishes not treated for tissue culture according to the manufacturer's specifications. After 18 hours had passed, vector diluted with fresh medium was reapplied, and this application was repeated 12 hours later. Cells were harvested after a total of 3 days in culture, washed with PBS, and injected into 6- to 10-week-old, female HW80 (B6.C-Tyrc H1b Hbbd/By) recipient mice (Jackson Laboratories) irradiated with 1100 cGy. Recipient peripheral blood red cells, platelets, and leukocyte subsets were analyzed for GFP expression by FACS analysis as described [36] at 12 and 19 weeks post transplantation.

Hematopoietic cell processing, culture, and transduction. Human umbilical cord blood was obtained from placentas following uncomplicated births. Low-density mononuclear cells were recovered by centrifugation on Histopaque-1077 (Sigma, St. Louis, MO) and $CD34^+$ cells were recovered from this mononuclear preparation using a CD34-specific magnetic selection system according to instructions provided by the manufacturer (Miltenyi Biotech, Bergisch Gladbach, Germany) with a final purity of ~ 90%. Peripheral blood $CD34^+$ cells were obtained from healthy adult volunteers on a protocol approved by the Institutional Review Board of St. Jude Children's Research Hospital. After informed consent, volunteers received four daily injections of G-CSF (Filgrastin; Amgen, Thousand Oaks, CA) at a dose of 10 μg/kg to a total maximum dose of 480 μg, subcutaneously. When 24 hours had passed since the last dose of G-CSF, a double volume peripheral blood apheresis was performed and $CD34^+$ cells were recovered from the mononuclear cell preparation by immunoselection using the CliniMACS selection system (Miltenyi Biotech) according to the manufacturer's instructions. The selected fractions contained a median of 94% (range 91–97%) of $CD34^+$ cells.

$CD34^+$ cells were transduced overnight (no prestimulation) or prestimulated with multiple cytokines for 48 hours in culture before transduction. Prestimulated cells were cultured in X-VIVO 10 containing 1% BSA and SCF (300 ng/ml), Flt3 ligand (Flt3L; 300 ng/ml), and recombinant human interleukin-6 (rhIL-6; 50 ng/ml) at a final cell density of $1–2 \times 10^5$/ml. Preliminary experiments were done at lower concentrations of cytokines (SCF 50 ng/ml and IL-6 25 ng/ml) (Tables 3 and 4). At the end of 48 hours, cells were recovered by centrifugation and resuspended in fresh medium containing cytokines at a final cell density of $1–2 \times 10^5$/ml. Cells that did not receive prestimulation were immediately put into transduction medium. All transductions were performed on Retronectin (TAKARA Shuzo, Otsu, Shiga, Japan)-coated tissue culture plates (20 μg/cm^2). After 12 hours, the cells were harvested for injection into NOD/SCID mice or resuspended in fresh medium at a concentration of 2×10^5 cells/ml and cultured to a total of 96 hours *ex vivo* before analysis for EGFP expression by FACS or for progenitors by clonogenic colony assay.

Assay of repopulating cells in immunodeficient mice. The immunodeficient mice [50] used for these experiments were the nonobese diabetic/severe combined immunodeficiency (NOD.CB17-PRKDCscid/J) strain. We housed 8- to 10-week-old animals in sterile microisolator cages containing sterile bedding and supplied with sterile food and acidified water. Using a ^{127}Cs source, the animals were sublethally irradiated (325 cGy) 12–15 hours before receiving human hematopoietic infusions. Each animal received cells derived from purified peripheral blood $CD34^+$ cells after expansion of 3×10^6 input cells during 12 or 60 hours in culture. The mice were killed 6 weeks following injection of human cells, and bone marrow was harvested for flow cytometric analysis.

Assays for gene transfer. Gene transfer into $CD34^+$ cells was assayed using FACS to detect expression of the EGFP gene. Cultured cells were washed in PBS containing 2% heat-inactivated FBS, stained with mouse anti-CD38 (clone HB-7) and anti-CD34 (clone 8G12) monoclonal antibodies conjugated to phycoerythrin (PE) or allophycocyanin (APC), respectively, washed, and fixed using PBS containing 2% paraformaldehyde. Both monoclonal antibodies were obtained from Becton Dickinson (San Jose, CA). Three-color flow cytometry was carried out and the data were analyzed using the CellQuest software package (Becton Dickinson).

Gene transfer into clonogenic progenitors was evaluated by plating transduced $CD34^+$ cells into Methocult GF (H4434 Stem Cell Technologies, Vancouver, BC, Canada). After culturing of 1000 transduced $CD34^+$ cells in 35-mm culture dishes (1 ml of semisolid medium per plate) at 37°C in a 5% CO_2 humidified atmosphere for 10–15 days, the total and EGFP-positive colonies were enumerated using phase contrast/fluorescence microscopy.

To assay for gene transfer into cells capable of establishing human hematopoiesis in immunodeficient (NOD/SCID) mice, bone marrow from animals 6 weeks after injection was subjected to FACS analysis using conjugated antibodies against human surface antigens as follows: 1) human hematopoietic cells, CD45–APC; 2) B lymphocytes, CD19–PE; 3) myeloid cells, CD33–PE; and 4) primitive cells, CD34–PE. These antibodies were obtained from PharMingen (San Diego, CA). We mixed $5–10 \times 10^5$ bone marrow cells with rat anti-mouse CD16/CD32 FC block (clone 2.4G2; PharMingen) to reduce nonspecific antibody binding, and then incubated them with saturating amounts of the conjugated antibodies. Cells from each animal were also stained with appropriate conjugated, isotype-matched, control antibodies obtained from Becton Dickinson or PharMingen. After incubation, cells were resuspended in red cell lysis buffer (0.83% ammonium chloride solution) and washed twice in PBS containing 2% FCS. In all experiments, cells stained with the isotype control antibody were used to set the quadrant markers such that the negative quadrant contained at least 97% of the control cells. The percentage of engrafted human cells was determined by CD45 positivity, and lineage marker and EGFP expression were determined on the $CD45^+$ gated population.

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