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Transduction of Umbilical Cord Blood CD34+ NOD/SCID-Repopulating Cells by Simian Foamy Virus Type 1 (SFV-1) Vector

James R. Zucali,* Tina Ciccarone,† Vicky Kelley,* Jeonghae Park,† Calvin M. Johnson,† and Ayalew Mergia†,[1]

*Department of Medicine, College of Medicine, University of Florida, Gainesville, Florida 32610; and †Department of Pathobiology, University of Florida, Gainesville, Florida 32611-0880

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Foamy viruses are nonpathogenic retroviruses that offer unique opportunities for gene transfer into various cell types including hematopoietic stem cells. We used a simian foamy virus type 1 vector (SFV-1) containing a *LacZ* reporter gene with a titer of $1–5 \times 10^6$ viral particles/ml that was free of replication-competent retrovirus to transduce human umbilical cord blood CD34+ cells. Transduced CD34+ cord blood cells were transplanted into NOD/SCID mice and plated in serum-free methylcellulose culture to determine the transduction efficiency of human hematopoietic progenitor cells. A transduction efficiency of about 20% was obtained. At 6–10 weeks posttransplantation, human hematopoietic cell engraftment and marking were determined. Marrow from transplanted mice demonstrated human cell engraftment by the presence of human (CD45+) cells containing both CD19+ lymphoid and CD33+ myeloid cells. Serial sampling of NOD/SCID bone marrow revealed the presence of 6.7–14.0% CD45+ cells at 6 weeks posttransplant as compared to 3.6–27.2% CD45+ cells at 9–10 weeks posttransplant. Human progenitors examined from NOD/SCID bone marrow cells 9 weeks posttransplant revealed from 7.4 to 25.9% of the colonies exhibiting X-gal staining. Our study demonstrates the ability of a simian foamy virus vector to transduce the SCID-repopulating cell and offers a promising new gene delivery system for use in hematopoietic stem cell gene therapy.

Key Words: CD34+ cells; foamy virus; NOD/SCID.

INTRODUCTION

Foamy viruses are retroviruses that offer several unique features for gene transfer. These viruses are found in many mammalian species and appear to be ubiquitous. Foamy viruses have a broad host range and can efficiently be propagated in various cell types of several species. Others and we have shown that cultured epithelial and fibroblast cells as well as lymphoid and neural-originated cells support the growth of foamy viruses (Mergia *et al.,* 1996; Mikovits *et al.,* 1996). A foamy virus isolate from one species can infect several mammalian species (Hooks and Detrick-Hooks, 1981). In the infected animals the virus can be recovered from many organs including blood cells and brain tissue (Hooks and Detrick-Hooks, 1981). However, no disease has been correlated to foamy virus infection in naturally or experimentally infected animals (Flugel, 1991; Mergia and Luciw, 1991; Weiss, 1988). Animal caretakers accidentally infected with simian foamy virus also remain healthy while virus persists in the infected individuals (Callahan *et al.,* 1999; Heneine *et al.,* 1998; Schweizer *et al.,* 1997). These features of foamy viruses offer unique opportunities to develop an ideal vector system for human gene therapy.

Several versions of foamy virus vectors based on SFV-1 and human foamy virus (HFV) capable of gene transfer were developed (Mergia and Wu, 1998; Russell and Miller, 1996; Schmidt and Rethwilm, 1995; Wu *et al.,* 1998; Wu and Mergia, 1999). These vectors contain the 5′-untranslated leader region and the 5′ end of the *gag* gene which contain important *cis*-acting elements. Similar to other retroviruses, this region is complex, with specific sequences that have features of secondary structure. For retroviruses, the 5′-untranslated region is implicated in viral RNA genome encapsidation, diploid genome dimerization, and efficient *gag* translation (Berkowitz *et al.,* 1996; Linial and Miller, 1990; Miele *et al.,* 1996). Although the encapsidation signal for foamy viruses is unknown, the 5′-untranslated region serves for diploid genome dimerization and efficient *gag* translation (Erlwein *et al.,* 1997; Heinkelein *et al.,* 2000; Russell *et al.,* 2001). Recent studies on foamy virus vector construction suggest that additional sequences required for foamy virus vector transfer are found in the 3′ end of the *pol* gene (Erlwein *et al.,* 1998; Heneine *et al.,* 1998; Wu *et al.,* 1998). The role of these sequences in foamy virus replication and vector construction remains to be determined.

The fact that virus appears to be present in all tissues of infected animals suggests that the cell receptor for

[1] To whom reprint requests should be addressed at Department of Pathobiology, University of Florida, P.O. Box 110880, Gainesville, FL 32611-0880. Fax: (352) 392-9704. E-mail: mergiaa@mail.vetmed.ufl.edu.

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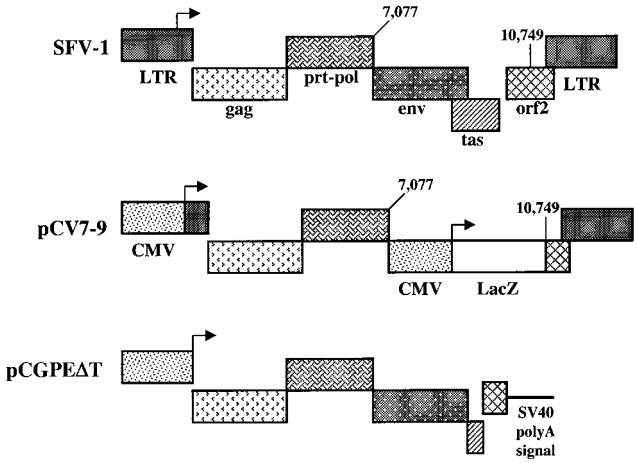

FIG. 1. Schematic representations of SFV-1 vector (pCV7-9) containing the *LacZ* reporter gene and packaging construct (pCGPEΔT) used to generate SFV-1 vector stocks. The provirus genome of SFV-1 is shown in the top panel. CMV is the promoter of the human cytomegalovirus immediate-early gene.

foamy virus is present virtually in all cells including nondividing cells. Transduction of nondividing cells by the HFV vector remains controversial. Bieniasz *et al.* demonstrated that foamy virus replication is cell cycle dependent (Bieniasz *et al.,* 1995). Others have shown that the foamy virus DNA can enter the nucleus of G1/S phase arrested cells (Saib *et al.,* 1997). However, the study found no viral gene expression. Although low efficiency, Russell and Miller were able to deliver and express a transgene into stationary cells using HFV vector and the efficiency of gene expression was higher than that of the MuLV vector (Russell and Miller, 1996). Our recent studies with SFV-1 vector, however, clearly demonstrated efficient gene transductions that are equivalent to the lentivirus vectors in a variety of nondividing cells (Mergia *et al.,* 2001). Because SFV-1 vector transduces a wide variety of vertebrate cells by integration of the vector genome and transduces stationary cultures efficiently, they may be more effective at transferring genes into the human hematopoietic stem cells. In support of this notion Vassilopoulos *et al.* reported that murine hematopoietic progenitors were transduced by a single HFV vector stock and that mice that received transduced murine bone marrow cells expressed the vector-encoded transgene in all major hematopoietic cell lineages (Vassilopoulos *et al.,* 2001). Others, however, indicated low-level expression of functional foamy virus receptor on hematopoietic progenitor cells (von Laer *et al.,* 2001). Here, we present the first report of efficient transduction of human CD34+ cells capable of engrafting NOD/SCID mice by a simian foamy virus vector containing the *LacZ* marker gene.

RESULTS

SFV-1 vector transduction of hematopoietic progenitors assayed *in vitro*

To evaluate gene transfer and expression in transduced human CD34+ cord blood cells, helper-free SFV-1 vectors expressing the *LacZ* reporter gene were made by transfecting SFV-1 vector and packaging plasmid into 293T cells. Transient high-titer vector was achieved in the SFV-1 system by replacing the U3 region with the human cytomegalovirus (CMV) immediate-early gene promoter (Fig. 1). Using this vector system, viral stocks with titers ranging from 1.0 to 5.0 $\times$ 10^6 transducing units/ml were obtained. Since our vector system lacks the *tas* gene, no replication-competent virus was generated (data not shown). This titer compares favorably with titers of amphotropic or GALV-pseudotyped oncoretroviral vectors reported in the past (Miller *et al.,* 1993).

This foamy virus vector was used to transduce human cord blood CD34+ cells on fibronectin-coated plates. Since transgene expression immediately following transduction may not reflect the level of stable viral integration (Gallardo *et al.,* 1997; Haas *et al.,* 2000), colony-forming cell assays were performed to test the efficiency of stable gene transfer into clonogenic erythroid, myeloid, and mixed progenitor cells. Transduced SFV-1 cord blood CD34+ cells were plated in methylcellulose and mean transduction efficiencies determined. After staining for *LacZ* expression, we routinely obtained about 20% transduction efficiency (Table 1). Single as well as multilineage colonies stained positive for *LacZ* gene expression, indicating that transgene expression was maintained over 14 days (Fig. 2).

SFV-1 vector transduction in SCID-repopulating cells (SRCs)

Several studies have demonstrated that *in vitro* assays do not predict the *in vivo* engraftment activity of human hematopoietic stem cells. We decided therefore to use the SCID-repopulating cell assay with NOD/SCID recipient mice that had been previously irradiated at 3.0 cGy as a surrogate measure of gene transfer into engrafting human hematopoietic stem cells. We performed two in-

TABLE 1

Percentage β-gal+ Colonies Were Scored After 14 Days in Methylcellulose Cultures of SFV-1-Transduced CD34+ Human Cord Blood Cells

β-gal+ colony	Total colonies	%β-gal+ colonies
10	46	21.8
10	43	22.3
6	48	12.5
	Mean	19.2

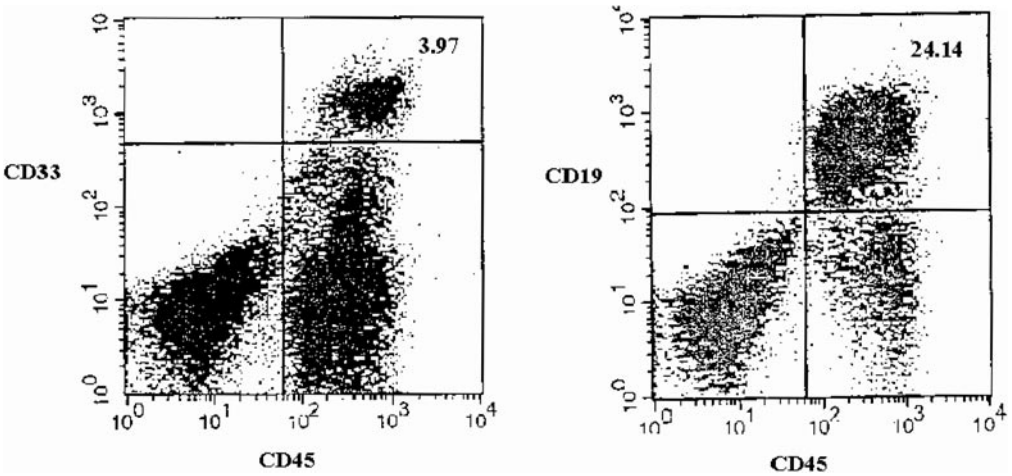

FIG. 2. Multilineage repopulation capacity of transduced SCID-repopulating cells. Cells from the bone marrow of a NOD/SCID mouse transplanted with *LacZ*-transduced human CD34+ cells and examined by flow cytometry 10 weeks after transplantation. The numbers in the upper right quadrants show the percentages of human CD33+ myeloid or human CD19+ lymphoid cells found within the CD45+ population. Quadrants were set according to isotype-matched negative control stainings.

dependent experiments to determine if transgene expression could be found in SRCs. In each experiment, 1–1.5 × 10^5 SFV-1-transduced CD34+ cells were transplanted into NOD/SCID recipient mice. Six to 10 weeks later, the presence of engrafted human cells expressing the human CD45 antigen within mouse bone marrow was analyzed by FACS. Figure 3 shows a typical FACS analysis of engrafted cells showing that the CD45+ cell population contained both human myeloid CD33+ cells and human lymphoid CD19+ cells.

We further examined gene transduction into long-term engrafted human cells by terminating transplanted NOD/SCID mice at 9–10 weeks posttransplantation. In addition, the presence of transduced human colony-forming progenitor cells within the bone marrow of transplanted NOD/SCID recipient mice was assayed subsequently (Tables 2 and 3). In the first experiment (Table 2), anywhere from 3.6 to 27.3% of the bone marrow cells analyzed were labeled with anti-CD45 antibody at 9 weeks following transplantation. When these cells were placed in a colony assay, the percentage of *LacZ*+ human colonies ranged from <0.1 to 20% of the total colonies observed. These *LacZ*+ colonies included CFU-GM, CFU-Meg, BFU-E, and CFU-mix, indicating that multiple lineages could be transduced by this SFV-1 vector. No *LacZ*+ colonies were seen in control cultures plating cells obtained from NOD/SCID mice receiving nontransduced human CD34+ cells. This experiment was repeated using 6 more transplanted NOD/SCID mice (Table 3). In this study, bone marrow cells obtained from 4 of the 6 mice showed detectable human cell engraftment of greater than 1.0% CD45+ cells at 10 weeks following transplantation. The percentage of colonies staining positive for *LacZ* ranged from 11.8 to 25.9%. Once again all of the different colony types were observed staining blue, indicating multilineage expression of the transgene. The numbers of engrafted human progenitors containing the *LacZ* reporter gene were in good correlation with the

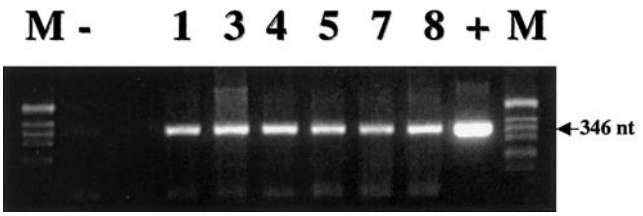

FIG. 3. PCR analysis of DNA obtained from the bone marrow cells of 6 NOD/SCID mice receiving *LacZ*-transduced human CD34+ cells shown in Table 3. Each Lane contained DNA amplified with primers specific for *LacZ* gene. Lanes show negative (−) and positive (+) controls and six DNA samples from bone marrow of 6 NOD/SCID mice. Positive control is DNA-amplified from vector pCV7-9. The size of the PCR product is given in nucleotide (nt). M represents a 1-kb ladder size marker.

TABLE 2

Experiment 1: Analysis of Bone Marrow Cells Obtained from NOD/SCID Mice Transplanted with *LacZ*-Transduced CD 34^+ Cord Blood Cells

Animal	Time of analysis	% CD 45+	% β-gal+ colonies
A1	6 weeks	6.7	NA[a]
B2	6 weeks	14.0	NA
B3	6 weeks	9.0	NA
B4	6 weeks	9.8	NA
B5	6 weeks	13.8	NA
A1	9 weeks	3.6	<0.1
B2	9 weeks	20.2	11.5
B3	9 weeks	20.6	19.5
B4	9 weeks	27.3	20.0
B5	9 weeks	16.4	7.4

[a] NA, not applicable.

TABLE 3

Experiment 2: Analysis of Bone Marrow Cells Obtained from NOD/SCID Mice at 10 Weeks Following Transplantation with β-gal- Transduced CD 34+ Cord Blood Cells

Animal #	%CD 45+	%β-gal+ colonies
1	8.7	20.2
3	20.9	25.9
4	13.0	11.8
5	0.9	<0.1
7	1.0	<0.1
8	21.0	18.5

percentage of CD45+ cells analyzed, suggesting that functional multipotential human hematopoietic stem cells can be readily transduced by SFV-1 vectors and repopulate in NOD/SCID mice.

Transduction of human CD34+ cells with *LacZ* was confirmed using standard polymerase chain reaction (PCR) and reverse transcription (RT) PCR amplification in both DNA and RNA extracts obtained from the bone marrow of the transplanted NOD/SCID mice (Figs. 3 and 4). A signal representing the presence of DNA sequences specific for β-galactosidase was seen in all six samples obtained from experiment 2 (Fig. 3). Furthermore, total RNA obtained from the bone marrow cells of each of these same NOD/SCID mice was analyzed by RT-PCR for the presence of β-galactosidase-specific RNA (Fig. 4). A signal representing the presence of β-gal-galactosidase RNA was also seen for each sample obtained from the NOD/SCID mice.

DISCUSSION

In this study, we have shown that a vector developed from simian foamy virus can efficiently transduce human CD34+ hematopoietic progenitors obtained from human umbilical cord blood. This study also shows that NOD/SCID recipients of transduced CD34+ human hematopoietic cells express the vector-encoded transgene in all hematopoietic cell lineages analyzed. Transduction at the human hematopoietic stem cell level was suggested by the long-term transgene expression in transplant recipients. This is in contrast to the silencing problem of transgene expression over time reported for murine leukemia virus (MLV) vectors (Challita and Kohn, 1994; Palmer *et al.,* 1991). We did not observe significant silencing in our experiments which demonstrated the presence of X-gal staining human colony formation in the bone marrow of NOD/SCID mice 10 weeks after transplantation. We also observed relatively high transduction efficiencies (about 20%) which are comparable to those reported using human lentiviral based vectors (Miyoshi *et al.,* 1999).

Current gene delivery systems are mainly based on retroviruses, adenovirus, adeno-associated virus (AAV), and nonviral methods such as liposome-mediated gene transfer (Robbins *et al.,* 1998; Romano *et al.,* 2000; Smith, 1999; Wolfe *et al.,* 1999; Wu and Ataai, 2000). The problem with nonviral vectors is that the transfer of DNA to the nucleus is a very inadequate process *in vivo* (Callahan *et al.,* 1999; Heneine *et al.,* 1998; Sandstrom *et al.,* 2000; Schweizer *et al.,* 1995, 1997). Adenovirus vectors do not have the machinery necessary to integrate into the host genome (Robbins *et al.,* 1998; Romano *et al.,* 2000; Zhang and Russell, 1996). Although the current AAV vectors are capable of integration into the host cell genome, the efficiency of vector integration appears to vary with the cell type targeted (Robbins *et al.,* 1998; Romano *et al.,* 2000; Zhang and Russell, 1996). Since the life cycle of retroviruses involves integration of the genome into the host DNA, these viruses provide an efficient means of gene delivery. Several of the gene therapy studies using retroviral vectors are based on the murine leukemia virus (MuLV) (Anderson, 1998; Gunji *et al.,* 2000; Morgan and Blaese, 1999; Mountain, 2000). These vectors, however, appear to be inefficient *in vivo* (Culver *et al.,* 1990, 1991; Morgan *et al.,* 1994; Naldini *et al.,* 1996; Woffendin *et al.,* 1994). Vectors based on lentiviruses such as HIV are promising since they can infect both dividing and nondividing cells (Akkina *et al.,* 1996; Naldini *et al.,* 1996). Although the feasibility of lentiviruses in human gene therapy is still being exploited using animal models, lentivirus vectors suffer from the potential risk of causing

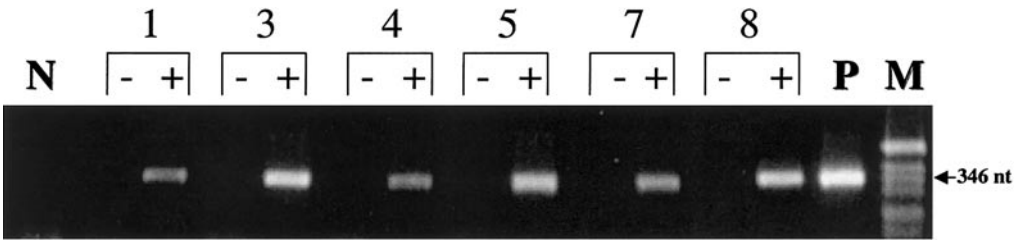

FIG. 4. RT-PCR analysis of RNA obtained from the bone marrow cells of 6 NOD/SCID mice receiving *LacZ*-transduced human CD34+ cells shown in Table 3. Each Lane contained DNA amplified with primers specific for *LacZ* transcripts. +/− equals positive or negative for the addition of reverse transcriptase. Lanes show negative (N) and positive (P) controls and the six RNA samples from bone marrow of 6 NOD/SCID mice. Positive control is DNA-amplified from vector pCV7-9. The size of the PCR product is given in nucleotide (nt). M represents a 1-kb ladder size marker.

disease in humans. Vectors based on animal lentiviruses have been implicated as an alternative to HIV vectors for human gene therapy. However, the recent report that FIV can infect cynomolgus macaques (*Macaca fasicularis*) with clinical signs, including depletion of CD4+ cells and weight loss, that are consistent with FIV infection indicates the risk associated with the usage of animal lentivirus-based vectors for human gene therapy (Johnston *et al.,* 2001). As an alternative to lentivirus, the SFV-1 vector system may provide a safe and efficient means of gene delivery, satisfying current concerns with existing retrovirus vectors. We previously established that SFV-1 vectors transduce nondividing cells efficiently, implicating that quiescent nondividing cells such as human stem cells are prone to SFV-1-mediated gene transfer. Similarly, expression of cellular SFV receptor molecules on hematopoietic stem cells may also account for the improved transduction efficiency although the expression of these receptors is not known. Our finding of efficient transduction of human SCID-repopulating cells in this report strengthens the notion that foamy virus vectors are ideal for human gene therapy. Future studies will determine whether vectors based on nonpathogenic foamy viruses may be a safe alternative for use in human gene therapy.

MATERIALS AND METHODS

SFV-1 vector system

The construction of the SFV-1 vector plasmid pVC7-9 is described elsewhere (Wu and Mergia, 1999). In pCV7-9, the minimal human cytomegalovirus immediate-early gene promoter was fused to the transcription start site located in the R region of the 5′LTR to direct high-level vector transcription from +1 of the SFV-1 genome (Fig. 1). The CMV promoter used was the *Bam*HI-*Sac*I fragment of pLNCX at position 2798–3516 (Miller and Rosman, 1989). The vector contains SFV-1 sequences from the beginning of the R to the end of the *pol* gene, the polypurine track (PPT), and the 3′LTR. To monitor for the efficiency of transduction, a *LacZ* gene under the control of a CMV promoter is placed between the *pol* gene and the PPT. Packaging plasmid (pCGPEΔT) is derived by deleting the *tas* gene from pCGPETH, which contained the structural genes *gag, pol,* and *env,* and *tas* downstream from the CMV promoter (Park and Mergia, 2001; Wu and Mergia, 1999). Splice donor of the 5′SFV-1 is provided between the CMV promoter and the *gag* gene in pCGPEΔT. The SV40 poly(A) signal sequence is supplied at the 3′ end. SFV-1 vector was produced by transient cotransfection of each vector plasmid pCV7-9 and packaging plasmid pCGPETH with LipofectAMINE transfection reagent (Gibco-BRL) as described previously (Park and Mergia, 2001). The amounts of SFV-1 vectors produced were titered on fresh 293T cells plated at a density of 2.5×10^4 in 24 well plates. Forty-eight hours after infection, cells were stained for β-galactosidase activity and positive cells were counted.

Cell culture

Human umbilical cord blood CD34+ cells (>94% CD34+) were obtained from BioWhittaker (Gaithersburg, MD). CD34+ cells (5×10^5) were transduced 2–3 times for 5 h each in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% FBS (Hyclone, UT) containing 100 ng/ml human Flt-3 ligand, 100 ng/ml human stem cell factor, and 100 ng/ml human thrombopoietin (Tpo) (Peprotech, Rocky Hill, NJ) on two wells of a fibronectin-coated 24-well tissue culture plate under spinoculation conditions (1000 rpm) with an SFV-1 vector ($2–5 \times 10^6$ viral particles) containing the *LacZ* reporter gene. In between transductions, the CD34+ cells were washed and cultured in IMDM supplemented with 20% FBS containing the same concentration of growth factors as described for the transduction. Following the last transduction, $1.0–1.5 \times 10^5$ transduced CD34+ cells were transplanted by lateral tail vein injection into each of 4–5 sublethally irradiated NOD/SCID mice as described below. Transduced cells were also plated in serum-free methylcellulose culture (Methocult SF^{BIT} H4436, StemCell Technologies, Vancouver, BC) in the presence of 20 ng/ml human Flt-3 ligand, 50 ng/ml human stem cell factor, 20 ng/ml human GM-CSF, 20 ng/ml human IL-3, 20 ng/ml human IL-6, and 3 units/ml human erythropoietin (Epo) for 14 days at which time colonies (>50 cells) were scored. Colony morphology was determined by examining colonies under an inverted microscope. All hematopoietic growth factors were obtained from Peprotech. Briefly, cultures were stained for the presence of β-galactosidase activity by incubating at 37°C overnight with 5-bromo-4-chloro-3-indoyl-β-D-galactopyranososide (X-gal) at pH 8.2 (Morshead *et al.,* 2002). Staining for X-gal at this pH gave no nonspecific staining in the control culture. Colonies were monitored and scored for the appearance of blue staining using an inverted microscope.

Mice

All transplants were performed with EMV30^{null} NOD/SCID mice (Serreze *et al.,* 1995), originally purchased from Jackson Labs (Bar Harbor, ME) and bred in our animal facility. Four- to 5-week-old male and female NOD/SCID mice were used. Animals were housed under specific pathogen-free conditions in filter-top cages and handled in sterile cross flows. Sterilized food and water were given *ad libitum.* One to 3 h before grafting, the animals were subjected to total body irradiation at a dose of 3.0 cGy. Radiation was delivered in a Gamma Cell 40 small animal irradiator (Atomic Energy of Canada, Ottawa, Canada) equipped with two opposing cesium 137 γ-ray sources providing a homogenous dose distri-

bution to the animals. Following irradiation, mice were infused with 1.0–1.5 $\times$ 10^5 transduced human CD34+ cells by tail vein injection. Mice received sterile food and acidified sterile water and were injected with 1 μg human G-CSF intraperitoneally every other day for a total of four injections following transplantation.

Analysis of transplanted NOD/SCID mice

At 6–10 weeks posttransplantation, human hematopoietic cell engraftment and marking were determined. Serial bone marrow sampling for flow cytometry was obtained from NOD/SCID mice as described by Verlinden *et al.* (1998). Bone marrow cells were also obtained from the femurs, tibias, and humori of mice that were sacrificed by cervical dislocation by flushing with IMDM + 5% FBS. Red blood cells were lysed by a 5-min exposure with a hypotonic ammonium chloride solution. The remaining nucleated cells were then prepared for flow cytometry analysis, methylcellulose culture, or DNA and RNA extraction for PCR and RT-PCR analysis. Briefly, 0.5–1.0 $\times$ 10^6 cells were stained with FITC-labeled CD45 antibody (Clone H130), PE-labeled CD19 antibody (Clone HIB19), or PE-labeled CD33 antibody (Clone WM53) obtained from BD Pharmingen (San Diego, CA) and analyzed using a FACSort machine (Becton Dickinson, San Jose, CA) to determine the level of human cell engraftment. FITC-labeled mouse IgG1 and PE-labeled mouse IgG1 (Clone MOPC21) were used as controls and subtracted from the CD45-positive and CD19- or CD33-positive values. Bone marrow cells (1.0 $\times$ 10^5) obtained from each NOD/SCID mouse were also plated in serum-free methylcellulose with human cytokines as described above. Following 2 weeks of culture, the colonies were stained and both X-gal-positive and X-gal-negative colonies were enumerated.

Polymerase chain reaction

Genomic DNAs were constructed from bone marrow cells by using a QIAamp DNA Mini kit (Qiagen, Inc., Valencia, CA) as instructed by the manufacturer and the DNAs were analyzed by PCR. Briefly, 25 ng of genomic DNA was amplified using a *Taq* polymerase and 40 pmol of each primer. Primers for PCR were 5′-GAT TGG TGG CGA CGA CTC CTG-3′ (forward, AYM86) and 5′-CCG AGT TTG TCA GAA AGC AGA CCA-3′ (reverse, AYM87B) selected from the 3′ end of the coding sequence of the *LacZ* gene region. The polymerase chain reaction was performed for 30 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 2 min, and polymerization at 72°C for 1.5 min. Ten microliters of each of the amplified products was electrophoresed on a 1.2% agarose gel.

Reverse transcription-polymerase chain reaction

Total RNA was isolated from bone marrow cells using the Ambion RNA extraction kit (Austin, TX) and subsequently treated with RNAse-free DNAse. Half a microgram of RNA was added to the reverse transcriptase reaction using the Ambion RT-PCR kit and the AYM87B primer as described by the manufacturer. The PCR was performed for 30 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 2 min, and polymerization at 72°C for 1.5 min with the addition of an opposing primer (AYM86). Ten microliters of each of the products was electrophoresed on a 1.2% agarose gel.

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