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Transduction of Human CD34^{+}CD38^{-} Bone Marrow and Cord Blood-Derived SCID-Repopulating Cells with Third-Generation Lentiviral Vectors

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The major limitations of Moloney murine leukemia virus (MoMLV)-based vectors for human stem cell applications, particularly those requiring bone marrow (BM) stem cells, include their requirement for mitosis and retroviral receptor expression. New vectors based upon lentiviruses such as HIV-1 exhibit properties that may circumvent these problems. We report that novel third-generation, self-inactivating lentiviral vectors, expressing enhanced green fluorescent protein (EGFP) and pseudotyped with vesicular stomatitis virus G glycoprotein (VSV-G), can efficiently transduce primitive human repopulating cells derived from human BM and cord blood (CB) tested by the SCID-repopulating cell (SRC) assay. Highly purified CD34^{+}CD38^{-} CB or BM cells were efficiently transduced (4–69%) and stably expressed in EGFP for 40 days in culture following infection for only 24 h without fibronectin, polybrene, or cytokines. Nonobese diabetic/severe combined immune-deficient (NOD/SCID) mice transplanted with transduced cells from either CB or BM donors were well engrafted, demonstrating maintenance of SRC during the infection procedure. Serially obtained femoral BM samples indicated that the proportion of EGFP+ cells within both myeloid and lymphoid lineages was maintained or even increased over time, averaging 42.3 ± 6.6% for BM donors and 23.3 ± 7.2% for CB at 12 weeks. Thus, the third-generation lentivectors readily transduce human CB and BM stem cells, under minimal conditions of ex vivo culture, where MoMLV-based vectors are ineffective. Since CB is inappropriate for most therapeutic applications, the efficient maintenance and transduction of BM-derived SRC during the short infection procedure are notable advantages of lentivectors.

Key Words: **stem cells; lentivirus; NOD/SCID; gene transfer.**

INTRODUCTION

Genetic manipulation of human hematopoietic stem cells (HSC) for therapeutic purposes holds promise. However, current transduction methodologies used in human trials result in levels of gene marking and expression within hematopoietic cells that are too low for therapeutic benefit. The major barrier remains the low efficiency of introducing the foreign gene into human long-term repopulating cells (1–4). An additional problem that has until recently plagued all research on human stem cells has been the inability to accurately assay, and therefore define, human repopulating cells. The development of the NOD/SCID xenotransplant system has permitted the detailed characterization of primitive human repopulating cells, termed SCID-repopulating cells (SRC) (5–7). The SRC assay is a key tool to improve our understanding of the signals that regulate the developmental program of HSC and to create efficient transduction methods (7–13).

Retroviruses remain the only practical means to permanently transduce stem cells. The major barriers to the use of MoMLV-based vectors include poor expression of retroviral receptors required for retroviral entry in the

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human stem cell compartment (14, 15) and the quiescent state of most HSC (16, 17). Integration of retroviral vectors requires mitotic activity; the intact nuclear membrane of quiescent cells prevents nuclear transport of the preintegration complex (18). Efforts to surmount these barriers have focussed on two major areas: attempts to promote stem cell proliferation without altering their repopulating and differentiation capabilities and development of new vector systems that are able to transduce quiescent cells. With optimization of every parameter of the *ex vivo* transduction procedure, significant progress has been made. Improvements include higher vector titers, serum-free infection with CH296 fibronectin fragment to increase the local multiplicity of infection (m.o.i.), cytokine combinations designed to induce modest SRC proliferation without differentiation, promoters that express well in hematopoietic cells, and reduced duration of *ex vivo* culture (8–13, 19). While original studies of SRC transduction showed low efficiency of gene marking (~0.1 to 3%) in only half of the engrafted NOD/SCID mice (7), the enhancements listed above routinely result in the vast majority of mice containing >20% marked human cells (8–13, 19). However, the necessary *ex vivo* culture required for MoMLV-based vectors likely has a negative effect on the survival and perhaps function of SRC (19–21) because we do not have a clear understanding of the *in vitro* requirements which induce stem cell proliferation without differentiation.

The second approach, aimed at the development of new vectors, has only recently met with success. HIV-1-derived vectors have been shown to transduce a variety of quiescent cell types (22–27). Central to this unique property of lentiviruses is the karyophilic determinants contained in their MA and Vpr proteins, which interact with the nuclear import machinery and mediate the active transport of the HIV preintegration complex through the nucleopore (28–30). Case *et al*. (31) showed that VSV-G pseudotyped lentiviral vectors, but not MoMLV vectors, can transduce nondivided hematopoietic progenitors and $CD34^+CD38^-$ cells in G_0 cell cycle status, even in the absence of growth factor stimulation and after only a brief exposure to lentivirus. Others have reported similar observations using *in vitro* assays and lentivirus vectors pseudotyped with VSV-G to transduce immature mobilized peripheral blood (PB) and CB cells (22, 24, 27, 32–34). However, these studies have not directly assayed the human stem cell; the critical question remains whether the hematopoietic repopulating cells have been transduced. Miyoshi *et al*. (35) have recently shown that human CB $CD34^+$ cells able to engraft NOD/SCID mice could be transduced with an early generation lentivector under conditions where MoMLV vectors were ineffective. However, for many gene therapy applications cells from adult donors are required, and prior studies with MoMLV have shown that adult BM cells are much more refractory to gene transfer than CB cells (1, 10, 36–38). The reduced transduction of BM-derived SRC is likely due to the fact that they have less proliferative capacity and different growth requirements than CB SRC, making them more difficult to both maintain and transduce during liquid culture. It is not known if lentivectors can efficiently transduce repopulating stem cells from adult BM.

The studies reported to date utilized first- and second-generation vectors, which contain various features to enhance efficacy and maintain safety. Third-generation vectors have additional safeguards, including deletion of the U3 region of the LTR making them self-inactivating (39) and packaging by a conditional expression system (40). It is important to evaluate this new generation of vectors for their ability to transfer genes into HSC. We evaluated various parameters of the transduction procedure with the aim of maximizing SRC transduction and minimizing the duration of *ex vivo* culture to ensure SRC survival. In addition, we compared the ability of the lentivector to transduce human lineage-depleted (Lin^-) $CD34^+CD38^-$ BM and CB-derived SRC, as prior studies using MoMLV vectors found that highly purified primitive cells from either source were more difficult to maintain and transduce and that CB-derived SRC were easier to transduce than those from BM (10, 36). We found that 24-h exposure to a third-generation HIV-derived vector achieved stable and efficient gene transfer into progenitor cells and SRC. Evaluation of the kinetics of repopulation of the transduced cells in the engrafted mice by BM sampling of NOD/SCID recipients indicated that the transduced SRC produced a human graft that was either stable or increased over time. These results illustrate the effectiveness of HIV-based gene transfer systems for transducing human HSC from BM or CB without compromising *in vivo* repopulating capacity.

MATERIALS AND METHODS

Sample collection and purification. CB and BM samples were obtained according to procedures approved by the Human Experimentation Committees of Mount Sinai Hospital and Princess Margaret Hospital (Toronto, Ontario, Canada), respectively. Mononuclear cells were obtained by centrifugation on Ficoll–Paque (Pharmacia, Uppsala, Sweden). Lineage depletion was performed using the StemSep immunomagnetic enrichment system (Stem Cell Technologies Inc., Vancouver, British Columbia, Canada) as previously described (41). The Lin^- cells (50–75% $CD34^+$) were stored at –170°C. To obtain the $CD34^+CD38^-$ cell population, we first purified Lin^-CD38^- cells with an antibody cocktail for $CD34^+CD38^-$ cell enrichment (Stem Cell Technologies Inc.). From this fraction, we sorted the $CD34^+CD38^-$ cells (resulting purity >90%) with a FACStar Plus (Becton–Dickinson, San Jose, CA) as described (20).

Flow cytometry. Flow cytometric analysis was performed using a FACScalibur Plus (Becton–Dickinson) as reported (12). Cells from transplanted NOD/SCID mice were assessed using anti-CD45–peridinin chlorophyll protein (PerCP), anti-CD34–phycoerythrin (PE), anti-CD19–PE, and anti-CD33–PE (all Becton–Dickinson). EGFP fluorescence was detected using detector channel FL1 calibrated to the FITC emission profile. During quadrant analysis, only levels of fluorescence that excluded >99% of isotype control events were considered to be specific.

Lentivector production. Replication-defective self-inactivating HIV-based vectors expressing EGFP under the control of an internal promoter, the immediate-early human cytomegalovirus (CMV) or that of the phosphoglycerokinase (PGK) gene, were pseudotyped with VSV-G and

packaged by a conditional expression system that only uses a fractional set of HIV-1 genes and provides enhanced biosafety (23, 39, 40). Briefly, the VSV-G pseudotyped lentivector was generated by transient cotransfection of the transfer vector constructs pRRL-SIN-CMV-GFP and pRRL-SIN-PGK-GFP (39) with the VSV-G expressing construct pMD.G and the third-generation packaging constructs pMDLg/pRRE and pRSVRev (40) into 293T cells. High-titer viral vector stocks were prepared by ultracentrifugation. The functional titers of viral vectors determined by infection of HeLa cells were between 1.2×10^8 and 1.2×10^9 transduction units (TU)/ml. The overall gene transfer efficiencies were very similar in cells transduced with the two variant vectors. However, the vector containing the PGK promoter resulted in slightly more homogenous EGFP expression compared to the CMV promoter (data not shown).

Infection protocol. Infections were carried out in flat-bottom 96- or 24-well plates (Nunc, Burlington, Ontario, Canada). Sorted $CD34^+CD38^-$ cells were deposited at a density of $1.0–4.0 \times 10^4$ cells per well in a 96-well plate in 0.1–0.2 ml of medium. Lin^- CB cells were seeded at $0.5–1.0 \times 10^5$ cells in 1 ml per well in a 24-well plate. Culture medium (CM) consisted of the serum-free medium X-VIVO 10 (BioWhittaker, Waldersville, MD) supplemented with 1% bovine serum albumin (Stem Cell Technologies Inc.) and 2 mM L-glutamine (Gibco BRL, Burlington, Ontario, Canada). The cells were transduced at m.o.i. between 12 and 450 for 24 h in the presence or absence of cytokines and incubated at 37°C and 5% CO_2. The virus concentration during the transduction period was $1.5 \times 10^6–8.8 \times 10^7$ particles/ml. The cytokine mixture included 20 ng/ml IL-6 and 100 ng/ml stem cell factor (SCF, kindly provided by Amgen, Thousand Oaks, CA). After a 24-h infection, the cells were harvested and washed twice.

In a preliminary experiment, Lin^- CB cells were incubated in the presence of 200 ng/ml Flt-3 ligand (FL, Immunex, Seattle, WA), 200 ng/ml SCF, 10 ng/ml granulocyte–colony-stimulating factor (G-CSF) and IL-6 (all Amgen), and 20 ng/ml thrombopoietin (TPO, kindly provided by Kirin Brewery, Tokyo, Japan). After 24 or 48 h of prestimulation, the lentivector was added to the culture at a m.o.i. of 45 for 5 or 24 h.

Culture of transduced populations to assess transgene expression in vitro. After infection and prior to further incubation, cells were washed twice to eliminate residual virus which might increase the transduction level during the culture. Sorted $CD34^+CD38^-$ cells were seeded at 1×10^3 cells per well of a 96-well plate in 0.2 ml of the CM plus 10 ng/ml IL-6, 10 ng/ml G-CSF, 100 ng/ml SCF (all Amgen), and 100 ng/ml FL (Immunex). Lin^- CB cells were seeded in a 24-well plate (1×10^4 cells per well in 1.0 ml of medium). At different time points, an aliquot of the cells was analyzed for CD34 and EGFP expression by flow cytometry.

Analysis of SRC by NOD/SCID mouse repopulation. Transduced cells were injected iv into irradiated (3.5 Gy) NOD/SCID mice using a slightly modified version of our standard protocol (7). Upon the transplantation of low numbers of cells ($<10^5$ cells/mouse), mice were coinjected with irradiated (20.0 Gy) accessory cells (10^6 Lin^+ cells/mouse) and received two ip injections of human IL-3, SCF, and granulocyte–macrophage colony-stimulating factor (GM-CSF, 6 μg each; Amgen) on day 2 and day 4 posttransplantation (42). At periodic intervals following transplantation, BM samples were aspirated from the femur as described (21, 43). At the end of the experiment, mice were sacrificed and PB, spleen, and BM were analyzed by flow cytometry for the presence of human cells. Cells were double-labeled with anti-human CD45–PerCP in combination with anti-CD34–PE, anti-CD19–PE, or anti-CD33–PE antibodies. As controls of nonspecific fluorescence, cells were labeled with conjugated nonspecific isotype antibodies. Additionally, cells from nontransplanted NOD/SCID mice were labeled with anti-CD45, anti-CD34, anti-CD19, and anti-CD33 antibodies.

Gene transfer into different subpopulations of human primitive ($CD45^+CD34^+$), myeloid ($CD45^+CD33^+$), and B lymphoid ($CD45^+CD19^+$) cells was determined by flow cytometric measurement of EGFP fluorescence.

Statistical analysis. Data are presented as means ± SE. The significance of differences between groups was determined by using Student's *t* test.

RESULTS

Lentivector Transduction of Hematopoietic Progenitors Assayed in Vitro

Although prior studies suggest that cell cycling may not be important for lentivector transduction, cytokine stimulation might still improve transduction of quiescent cells for reasons other than mitotic induction. In preliminary experiments, purified Lin^- CB cells were incubated in serum-free medium in the presence or absence of SCF, FL, G-CSF, IL-6, and TPO in various combinations of prestimulation (0 to 48 h) and lentiviral vector exposure times (5 to 24 h) and analyzed daily for 6 days (data not shown). A significant percentage of $EGFP^+$ cells (5%) were obtained without prestimulation and with only 5 h of exposure. The 24-h infection without prestimulation, representing a good combination of high gene transfer (15%) and short culture time, was selected for all subsequent experiments. Unless indicated, cytokines were not used because transduction efficiency was not significantly improved. The percentages of $EGFP^+$ cells in the presence and absence of cytokines were 20.2 ± 4.8 and 16.6 ± 2.6, respectively ($n = 6$).

The stability of EGFP expression within transduced Lin^- CB and sorted $CD34^+CD38^-$ BM or CB cells was determined by culturing cells following the 24-h infection procedure (m.o.i. was 12 to 220). Flow cytometric analyses indicated that at different times postinfection, the number of cells expressing EGFP was maintained and/or increased over 35 days of analysis (Fig. 1). There was a trend toward increased gene transfer in BM (Fig. 1A) compared to CB cells (Fig. 1B), but this difference was not significant. Lentivirus m.o.i., or the presence of SCF and IL-6 in the 24-h transduction procedure, had no apparent effect on gene transfer efficiency or stability. SCF and IL-6 were evaluated because the long-term goal was to assess transduction of SRC and prior studies found that these cytokines can enhance SRC engraftment by

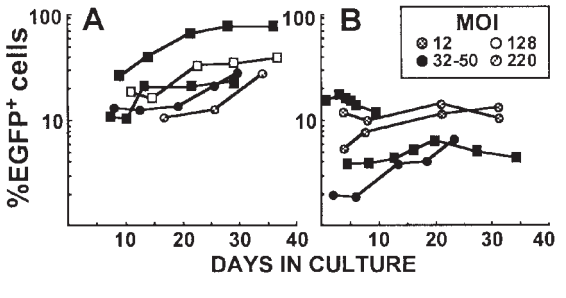

FIG. 1. Kinetics of EGFP expression in cultured cells. Cells were transduced with the lentivector for 24 h in the presence (squares) or absence (circles) of IL-6 and SCF and placed into suspension cultures for up to 35 days. The viral m.o.i. in transduction cultures ranged from 12 to 220 (see inset). *Y*-axis: percentage of $EGFP^+$ cells analyzed by flow cytometry. Each line represents a separate experiment. (A) $CD34^+CD38^-$ BM-transduced cells. (B) Lin^- and $CD34^+CD38^-$ CB-transduced cells.

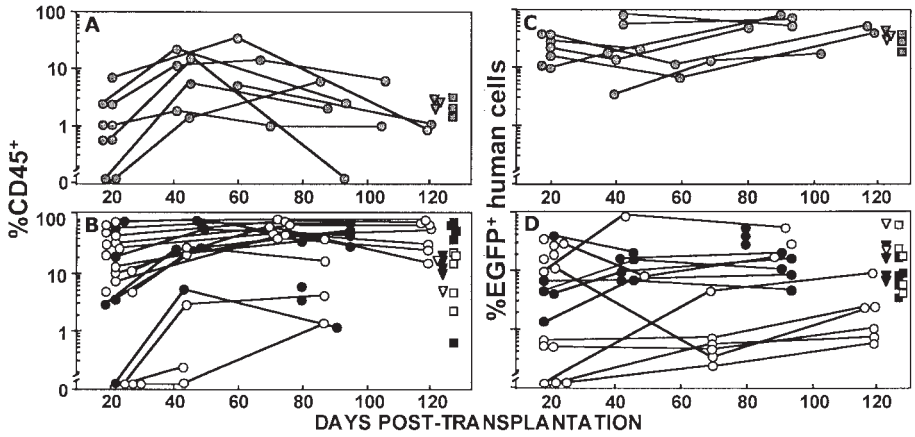

FIG. 2. Kinetics of human engraftment (A, B) and gene transfer efficiency (C, D) of individual NOD/SCID mice transplanted with transduced Lin– CB cells (open symbols), sorted CD34+CD38– BM (gray symbols), and CD34+CD38– CB cells (closed symbols). BM was periodically sampled from each recipient at the time indicated and analyzed by flow cytometry. Data from spleen (squares) and PB (triangles) collected at the end of the experiment are also presented.

upregulating the chemokine receptor CXCR4 (44). Thus, this treatment may enable better detection of potentially transduced SRC.

Lentivector Transduction of SRC

Prior studies utilizing MoMLV-based vectors have clearly shown that methods that efficiently transduce progenitors assayed *in vitro* may be much less effective for primitive repopulating cells (7). Moreover, culture conditions used during *ex vivo* culture often result in progenitor expansion but loss of repopulating cells (7, 12, 19, 20). Thus, it is crucial to determine if the optimized lentivector transduction methods described above are also applicable for human SRC. BM and CB cells were transduced for 24 h (with or without SCF/IL-6) and injected, at cell doses ranging from 1 to 4 $\times$ 10^4 for CD34+CD38– cells and 1 to 3 $\times$ 10^5 for Lin– cells, into conditioned NOD/SCID mice.

To follow the engraftment kinetics of the transduced cells in transplanted recipients, individual mice were analyzed at monthly intervals using femoral BM sampling to determine the proportion and characteristics of human CD45+ hematopoietic cells (Figs. 2A and 2B). Mice transplanted with CD34+CD38– BM cells showed an increase in the percentage of CD45+ cells from days 20 to 40 (2.5 to 8.4%, respectively), declining by the end of the experiment (120 days, mean of 1.7%; Figs. 2A and 3A). In contrast, mice transplanted with CB cells had higher levels of engraftment at 20 days posttransplantation (23%) that were maintained (38–45%) until the end of the study (Figs. 2B and 3A). There was no effect of cytokine addition on the engraftment levels with either CB or BM cells (Table 1). These kinetics are consistent with prior reports showing that uncultured CB produces a more extensive and stable graft than BM-derived cells (45, 46). These data demonstrate that SRC from both tissues are well maintained during the brief infection procedures.

High levels of gene-marked human cells were found in all engrafted NOD/SCID mice. In general, the proportion of EGFP-expressing cells in most BM- and CB-transplanted mice increased or was maintained over time (Fig. 3B and Table 2). Interestingly, kinetic analysis of individual mice revealed several different patterns in the contributions that marked cells made to the overall human graft (Figs. 2C and 2D). In most cases, the proportion of gene-marked cells increased to a maximum and remained at this level. In some mice, the EGFP+ cell percentage fell and subsequently increased, while in others the percentage declined with time. Overall, mice transplanted with BM cells showed an increase in gene-marked cell frequency from 18% at 20 days posttransplantation to 42% at 80–120 days (Fig. 3B and Table 2). Similar kinetics were observed in CB-transplanted mice (from 9 to 23%; Fig. 3B and Table 2). Addition of SCF and IL-6 during the infection procedure had no effect on SRC transduction efficiency (Table 1). Moreover, the percent-

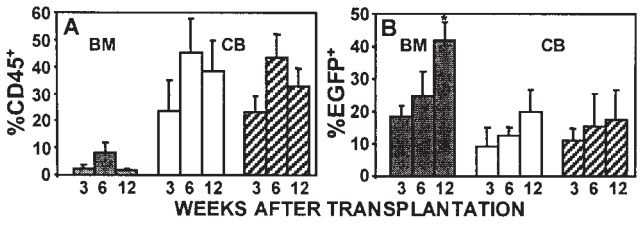

FIG. 3. EGFP+ cells are maintained or increased within the human graft. Each bar represents the mean ± SE level of human engraftment (A) and gene transfer efficiency (B) in mice transplanted with transduced CD34+CD38– BM (gray bars, $n = 9$) and CD34+CD38– CB (open bars, $n = 8$) cells and Lin– CB cells (striped bars, $n = 11$). *Statistically different from data corresponding to day 20 posttransplantation ($P < 0.05$).

TABLE 1
Human Engraftment and Gene Transfer Efficiency in Mice Transplanted with Cells Transduced in the Presence or Absence of SCF and IL-6

	+SCF/IL-6		-SCF/IL-6	
Graft type	$CD45^+$ (*n*)	$CD45^+EGFP^+$	$CD45^+$ (*n*)	$CD45^+EGFP^+$
$CD34^+CD38^-$BM	5.3 ± 2.3 (6)	32.0 ± 8.9	12.4 ± 10.7 (3)	36.7 ± 14.5
$CD34^+CD38^-$CB	37.2 ± 12.3 (8)	26.0 ± 6.8	ND	ND
Lin^-CB	37.3 ± 14.2 (4)	26.0 ± 4.9	36.8 ± 6.8 (7)	19.1 ± 11.3

Note. Data are presented as means ± SE; (*n*) number of mice analyzed.

age of marked cells in human grafts was not affected by lentivector m.o.i. within the ranges of m.o.i. tested (data not shown). These data demonstrate that SRC from highly purified BM and CB can be readily transduced with the third-generation lentivectors under conditions (i.e., short-term culture without cytokine stimulation) that would not permit transduction with MoMLV-based vectors.

To determine if the differentiation capacity of the gene-marked SRC was affected by lentivector transduction, multilineage expression of the transgene was investigated. Figure 4 shows a representative analysis of the B-lymphoid and myeloid distribution of human cells in the BM, spleen, and PB of a mouse transplanted with transduced CB cells. The human ($CD45^+$) graft within the BM, spleen, and PB of this mouse contained $EGFP^+$ cells within the $CD34^+$, B-cell ($CD19^+$), and myeloid ($CD33^+$) lineages. A detailed analysis of the lympho-hematopoiesis of a cohort of NOD/SCID mice transplanted with transduced cells is shown in Table 2. These data confirm that SRC from purified CB and BM cells were transduced and retained multilineage differentiation capacity. Moreover, a high proportion (up to 56%) of myelo-erythroid progenitors in the BM of engrafted mice contained the transgene detected by PCR (data not shown). $EGFP^+CD34^+$ or $CD33^+$ reconstitution was significantly higher at early stages of engraftment compared to the gene-marked B-cell population, while in the long term the $EGFP^+CD19^+$ reconstitution was predominant demonstrating the differential kinetics of myeloid and lymphoid lineage development from the engrafted SRC. The same pattern was observed with unmarked cells (data not shown). The percentages of $EGFP^+$ cells in the $CD34^+$, $CD19^+$, and $CD33^+$ populations were not significantly different, suggesting that there is no restriction on the generation of transduced cells among any lineage nor a restriction in gene expression (data not shown). In every experimental animal, the human hematopoietic engraftment was multilineage and EGFP was found in each lineage through the experimental period (3–12 weeks; Table 2) suggesting that transduction by third-generation lentivectors does not impair the developmental program of the SRC.

DISCUSSION

In this report, we provide evidence that a safety-enhanced third-generation lentivector efficiently transduces primitive human stem cells found within highly purified $CD34^+CD38^-$ BM and CB samples, overcoming several significant barriers encountered with MoMLV-based vectors. The SRC assay was used to assess stem cell transduction since this assay provides the most rigorous test of stem cell function. High levels (up to 56%) of gene-marked myelo-erythroid progenitors and mature myeloid and lymphoid human cells were detected in repopulated NOD/SCID mice, indicating that SRC were transduced and that lentivector transduction did not

TABLE 2
Lympho-Hematopoietic Reconstitution of NOD/SCID Mice with Lentivector-Transduced Cells

Graft type		3 weeks	6 weeks	>12 weeks
$CD34^+CD38^-$ BM	$CD45^+$	2.5±1.2	8.4±3.4	1.7±0.6
	$CD45^+EGFP^+$	18.6±4.5	24.6±7.7	42.3±6.6*
	$CD34^+EGFP^+$	12.8±3.6	6.9±3.6	3.8±1.7
	$CD19^+EGFP^+$	3.8±3.4	8.6±1.7	26.6±6.4
	$CD33^+EGFP^+$	18.1±4.2	3.5±1.5	6.4±2.0
$CD34^+CD38^-$ CB	$CD45^+$	23.5±11.6	45.3±14.2	38.5±11.2
	$CD45^+EGFP^+$	9.1±5.8	9.8±1.0	23.3±7.2
	$CD34^+EGFP^+$	5.2±3.2	3.1±0.9	3.7±2.0
	$CD19^+EGFP^+$	ND	5.9±1.1	16.5±6.0
	$CD33^+EGFP^+$	ND	2.3±0.3	7.5±4.2
Lin^-CB	$CD45^+$	25.8±5.9	43.4±8.8	32.9±6.6
	$CD45^+EGFP^+$	14.0±4.1	19.4±16.1	22.4±10.9
	$CD34^+EGFP^+$	17.1±5.5	9.4±7.7	4.1±1.8
	$CD19^+EGFP^+$	2.7±0.9	21.7±16.7	18.2±11.5
	$CD33^+EGFP^+$	15.7±4.8	11.3±10.7	6.3±3.9

Note. The proportion of human ($CD45^+$) and gene-marked human ($CD45^+EGFP^+$) cells in the BM of 28 NOD/SCID mice was examined. The proportion of gene-marked immature ($CD34^+EGFP^+$), B lineage ($CD19^+EGFP^+$), and myeloid ($CD33^+EGFP^+$) cells within the $CD45^+$ population was also determined.

*Statistically different from data corresponding to mice analyzed at week 3 ($P<0.05$).

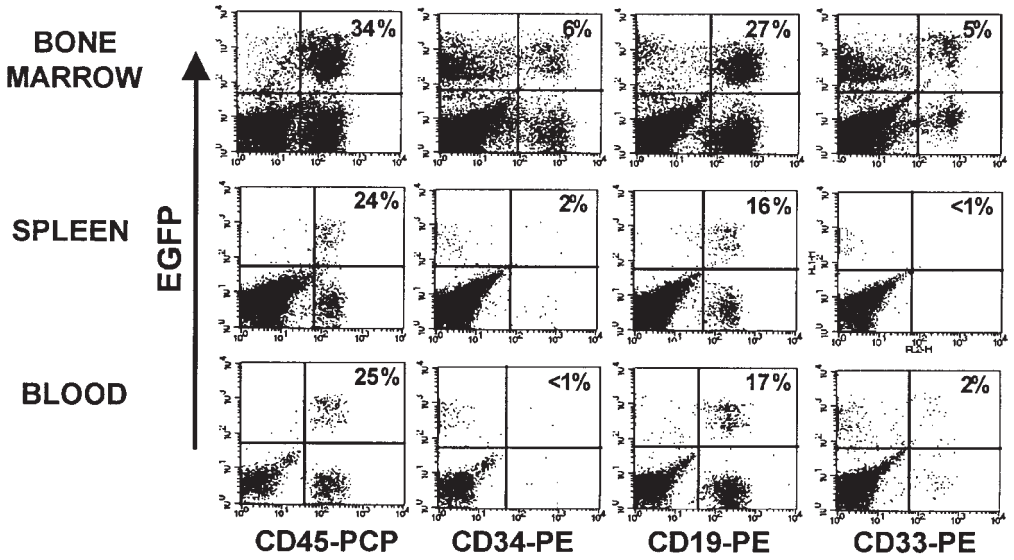

FIG. 4. Multilineage repopulation capacity of transduced SRC. BM, spleen, and PB of a representative mouse transplanted with transduced Lin⁻ CB cells were examined at 90 days posttransplantation. These data are representative of 28 mice analyzed. Dot plots show analysis of cells labeled with anti-CD45–PerCP plus anti-CD34–PE, anti-CD19–PE, or anti-CD33–PE antibodies to determine the proportion of human cells within each lineage. Quadrants were set according to isotype-matched negative controls.

compromise their multilineage differentiation potential. Because efficient transduction was achieved in the absence of proliferation-enhancing cytokines, it is probable that the primitive CD34⁺CD38⁻ cells remained in a quiescent G_0 state during transduction (22–27). Previous reports have indicated that lentivectors infect nondividing cells, but some cell types apparently require exposure to cytokines to permit efficient transduction (27). Our results suggest that some primitive repopulating hematopoietic cells do not have this requirement. In several experiments SCF and IL-6 were included in short-term transduction cultures to determine if the engraftment of transduced SRC was improved due to the increased expression of CXCR4, as was reported (41). The gene transfer efficiency was not enhanced, and the absence of a significant effect upon engraftment levels suggests that the repopulation capacity of SRC was not altered. Depending on the efficiency of virus preparation, the m.o.i. varied between experiments. The infectivity (TU/ng) and concentration of the virus were also variable, ranging from 2.3×10^4 to 9.5×10^4 TU/ng and 1.5×10^6 to 8.8×10^7 particles/ml, respectively. The gene transfer efficiency was not substantially altered by increased m.o.i., infectivity, or concentration of the virus suggesting that even the lowest m.o.i. was sufficient for effective transduction. The fact that gene transfer efficiency averaged 42 and 23% (upon BM or CB transplantation, respectively) irrespective of the m.o.i. implies the existence of a subpopulation of SRC that is refractory to lentivector transduction under the conditions described here. Future studies will attempt to characterize the properties of SRC that mediate this resistance.

Integration and expression of transgenes within HSC do not guarantee long-term expression of the transgene in their differentiated progeny (27, 32–34, 47). In our experiments, transduced BM and CB cells grown in liquid cultures for 35 days maintained or exceeded the starting proportion of EGFP expression *in vitro* (Fig. 1). Case *et al.* (31) have also reported the stable transduction of quiescent human CD34⁺CD38⁻ BM cells by HIV-1-based lentiviral vectors. In their study, the gene transfer efficiency to the extended long-term culture initiating cell (ELTC-IC) was 9.2 ± 5.2%, showing the lentiviral vector as clearly superior to an MoMLV-based vector for transduction of this primitive hematopoietic progenitor. In the studies reported here, we established that the repopulating cells within the CD34⁺CD38⁻ cell fraction from BM were efficiently transduced. This finding represents an important advancement of lentivector methodology since prior experience showed that purified quiescent cells, particularly those derived from adult BM, were difficult to transduce with the culture conditions required for MoMLV vectors. In addition, serial sampling of the recipients' BM showed that the vector continued to be expressed over time. The percentage of EGFP⁺ cells increased in BM-transplanted mice and was maintained in CB-transplanted mice. Transgene expression was continually detected in multiple lineages as analyzed by cytometry for differentiation markers (CD34, CD19, CD33) on cells of hematopoietic organs (BM, spleen, PB) and in myelo-erythroid progenitor assays of BM. These results indicate that lentivectors are capable of transducing long-term repopulating cells with high proliferative and differentiative potential and maintaining *in vivo*

long-term expression in the multilineage differentiated progeny.

The serial aspiration technique also provides insight into the contribution of marked cells to the human graft over time. In the vast majority of cases, the proportion of marked cells increased or remained stable over 3 months. In several cases, however, the pattern is mixed, including situations where the frequency of gene-marked cells declines between the first and second month but increases again by the third month (Figs. 2C and 2D). These findings contrast with the results of our recent studies of CB cells transduced with MoMLV vectors under optimized conditions (3 days of culture with a complex mixture of cytokines; manuscript in preparation). In these MoMLV vector experiments the level of gene marking was comparable; however, approximately 60% of mice showed a continuous decline of marked cells over 3 months or a temporary rise followed by a decline. Although several interpretations are possible, we believe that the most likely explanation is that these patterns reflect the fact that some of the transduced SRC have short-term repopulation potential while others have long-term repopulation capacity. In this interpretation, a declining proportion of marked cells in the human graft would be the consequence of repopulation by transduced short-term SRC, while maintenance or an increase in the proportion of gene-marked cells would reflect repopulation by transduced long-term SRC. Thus, comparison of the pattern of engraftment between lentivector and MoMLV transduction procedures suggests that the lentivector procedure is more compatible with the support and transduction of long-term repopulating cells. Current studies are focused on confirmation of this interpretation by combining the kinetic analysis with detailed clonality studies to examine the fates of individual transduced SRC.

The data presented here demonstrate that lentivectors can overcome several key problems which limit the effectiveness of MoMLV-based vectors for HSC gene transfer. Efficient transduction of SRC is achieved within 24 h of culture in defined serum-free medium without cytokine stimulation. A major consequence of this short exposure time is the preservation of SRC, which permits even BM-derived SRC to be transduced. The efficient transduction of adult BM stem cells by lentivectors opens important avenues of clinical application, since most diseases require the use of adult hematopoietic stem cell sources. Moreover, the minimization of the handling procedure of highly purified HSC, combined with efficient transduction of SRC and our ability to follow their fate by periodic *in situ* BM sampling, creates new possibilities for the understanding of the biology of human HSC.

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