High Levels of Transgene Expression Following Transduction of Long-Term NOD/SCID-Repopulating Human Cells with a Modified Lentiviral Vector

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ABSTRACT

Both oncoretroviral and lentiviral vectors have been shown to transduce CD34+ human hematopoietic stem cells (HSC) capable of establishing human hematopoiesis in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice that support partially human hematopoiesis. We and others have reported that murine stem cell virus (MSCV)-based oncoretroviral vectors efficiently transduced HSC that had been cultured ex vivo for 4-7 days with cytokines, resulting in transgene expression in lymphoid and myeloid progenies of SCID-engrafting cells 4-8 weeks post-transplantation. Although lentiviral vectors have been demonstrated to transduce HSC under minimal ex vivo culture conditions, concerns exist regarding the level of transgene expression mediated by these vectors. We therefore evaluated a novel hybrid lentiviral vector (GIN-MU3), in which the U3 region of the HIV-1 long terminal repeat was replaced by the MSCV U3 region (MU3). Human cord blood CD34+ cells were transduced with vesicular stomatitis virus G envelope protein-pseudotyped lentiviruses during a 48-hour culture period. After a total of 4 days in culture, transduced cells were transplanted into NOD/SCID mice to examine gene transfer and expression in engrafting human cells. Fifteen weeks post-transplantation, 37% ± 12% of engrafted human cells expressed the green fluorescence protein (GFP) gene introduced by the lentiviral vector. High levels of GFP expression were observed in lymphoid, myeloid and erythroid progenies, and in engrafted human cells that retained the CD34+ phenotype 15 weeks post-transplantation. This study provides evidence that lentiviral vectors transduced both short-term and long-term engrafting human cells, and mediated persistent transgene expression at high levels in multiple lineages of hematopoietic cells. ***Stem Cells*** *2001;19:247-259*

INTRODUCTION

Recombinant oncoretroviral vectors (RV) such as those based on Moloney murine leukemia virus (Mo-MLV) have been widely used during the past 20 years and are currently being tested in numerous clinical trials targeting hematopoietic stem cells (HSC). Freshly isolated human CD34+ progenitor cells are largely in the G_0/G_1 phase of cell cycle. Therefore, stimulating CD34+ cells into active cell cycling by cytokines prior to or during ex vivo transduction by RV is required. Using the nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse model as a surrogate assay for engrafting human HSC, we and others have found that a cytokine cocktail in a serum-free medium can maintain NOD/SCID repopulating cells (SRC). Under the conditions employed, myeloid/erythroid progenitor cells were expanded ~5-fold, lymphoid potential was preserved, and SRC were maintained (~2-fold reduction) after 7 days in culture [1]. These culture conditions also permitted efficient transduction of SRC. In addition, RV-mediated gene expression in transduced SRC and their

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progenies, a necessary next step for efficient gene therapy, has also been improved by using the long terminal repeats (LTRs) of viruses other than Mo-MLV. One such LTR is from the murine stem cell virus (MSCV), a genetically optimized RV based on MESV and Mo-MLV [2]. Using the MSCV-based MGIN RV packaged with either amphotropic or GALV envelopes, we reported high levels of transgene expression in the progeny of transduced human SRC in the bone marrow (BM) of transplanted mice [1, 3-4]. Similar results were reported by other investigators using RV similar to MGIN and the same NOD/SCID mouse model [5-7], and in a nonhuman primate transplantation model [8]. Moreover, it was recently reported that RV transduction of $CD34^+$ cells with the SCID-X1 gene followed by autologous transplantation resulted in clinical benefit in patients lacking the functional gene and suffering from lymphocyte deficiency [9]. These promising results encourage continuous use and improvement of RV transduction of HSC for various diseases. Despite this progress, however, it remains uncertain whether HSC gene transfer efficiencies currently achievable by RV are high enough for clinical indications in which the transduced cells do not have a growth advantage [9].

Recently, attention has focused on vectors derived from lentiviruses like HIV-1, which have been shown to transduce a variety of slowly or nondividing cells including unstimulated $CD34^+$ cells [10-14]. Several groups demonstrated that lentiviral vectors (LV), but not RV, can transduce freshly isolated, nondividing subpopulations of human $CD34^+$ cells [15-17]. Even so, transduction efficiencies were much higher when $CD34^+$ cells were stimulated by cytokines for a brief period (<24 hours) prior to transduction or during an extended transduction (~24 hours) [16-18]. This is consistent with the notion that LV can efficiently transduce cells that have exited G_0 but have not entered the S phase. In contrast, RV integration can occur only after breakdown of the nuclear envelope concomitant with progression through the M phase of the cell cycle. For this reason, we decided to investigate whether LV may efficiently transduce long-term engrafting SRC.

Early versions of LV have several shortcomings. First, the promoters utilized in first generation LV vectors are not optimal for directing transgene expression in hematopoietic cells. Unlike the MSCV LTR, which directs a high level of transgene expression in most transduced cells, the HIV-1 LTR is weak or inactive in transduced human hematopoietic cells in the absence of the HIV-1-encoded activator Tat. The strategy to retain the tat gene [15, 17-18] raises a concern that Tat may have adverse effects on the biological properties of the transduced cells [19-21]. Other first and second generation LV do not express Tat in transduced cells but use an internal cytomegalovirus (CMV) promoter to direct transgene expression [10]. Although the CMV immediate early (IE) promoter performs well in many types of dividing cells in culture, it has been found to be less active in primary hematopoietic cells tested and in transgenic mice [22-24]. This may explain why transgene expression level was low in human hematopoietic progenies derived from SRC transduced with an LV vector containing the CMV IE promoter [10]. Since the MSCV LTR has been shown to function as an effective promoter in SRC [1, 3-7], we decided to evaluate a hybrid LV in which the HIV-1 LTR was replaced in part with sequences from the MSCV LTR.

Our preliminary data indicated that the U3 region of the MSCV LTR could function as a promoter in a variety of human cells in the context of the LV backbone. Using a set of vectors that contain the same reporter cassette in either an RV or LV backbone, we sought to address the following two questions: A) Is the modified hybrid LV capable of achieving efficient transduction of long-term SRC, in the absence of extended ex vivo pre-stimulation? and, B) Does the MSCV LTR in the hybrid LV mediate a high level of (and/or sustained) transgene expression in vivo comparable to that achieved in the native RV configuration we observed?

Based on our previous studies, we developed a 48-hour protocol of ex vivo culture (including gene transduction) under completely serum-free conditions where viral supernatants were collected, and isolated $CD34^+$ cells were exposed to vector particles for only 8 hours. We report here that 37% ± 12% of human cells that engrafted in the BM of recipient NOD/SCID mice expressed the green fluorescence protein (GFP) gene introduced by a hybrid HIV-1/MSCV LV at 15 weeks post-transplantation. High levels of GFP expression were observed in lymphoid, myeloid, and erythroid progenies.

Materials and Methods

Vector Construction

The RV MGIN (Fig. 1A) is based on MSCV and has been described previously [25]. In this vector, the enhanced GFP gene is coexpressed with the bacterial neomycin phosphotransferase (Neo^R) gene, linked by an internal ribosome entry site (IRES) from the encephalomyocarditis virus. The transcription of this reporter expression cassette GFP-IRES-Neo^R (GIN) is controlled by the MSCV LTR.

Several new LV containing the GIN reporter cassette from MGIN have been generated based on the backbone of the HIV-1-based vector pHR′CMV-LacZ [26] (provided by *Dr. Didier Trono*, University of Geneva Medical School; Geneva, Switzerland). Construction of the LV used in this study will be described elsewhere (*Y.-c. Bor* and *R.G.H.*, manuscript in preparation). Briefly, the vectors were constructed

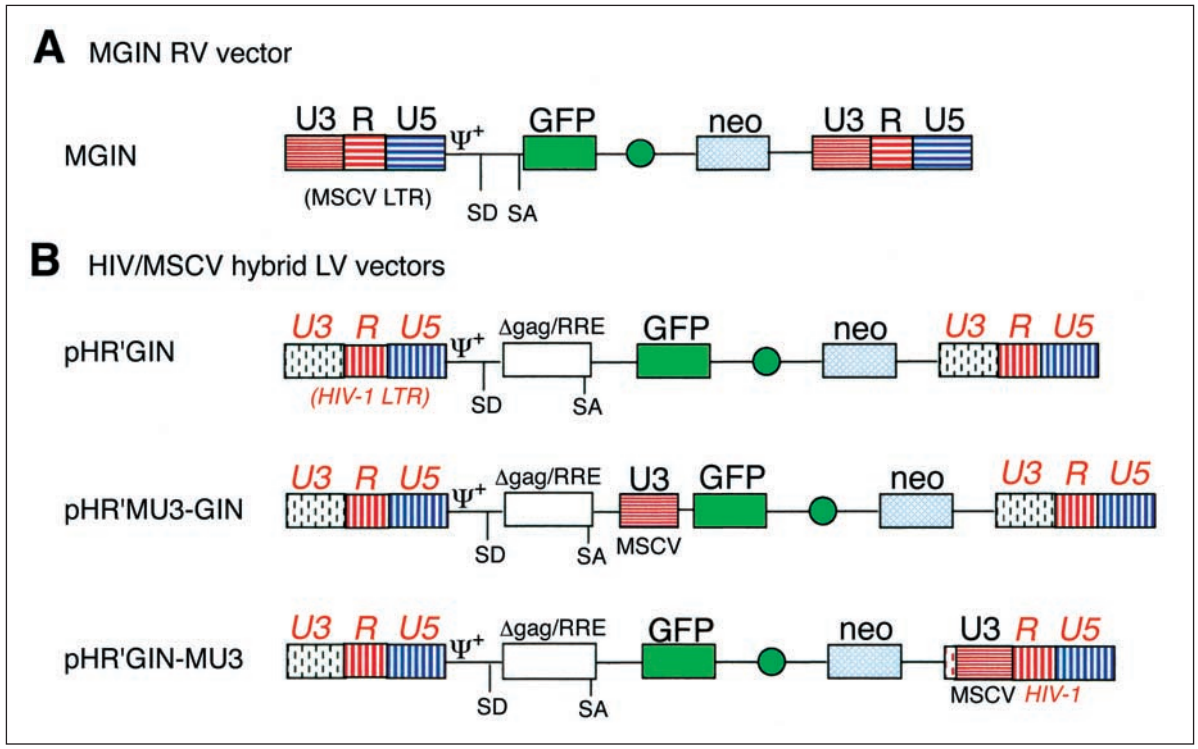

Figure 1. Schematic diagrams of the MGIN RV and HIV/MSCV LV vectors used in this study. *The MSCV-based MGIN vector containing the GFP and Neo^R selectable marker genes linked by an IRES (circle) is indicated as are novel HIV-1-based LV vectors containing the GFP-IRES-Neo^R (GIN) reporter cassette. In addition to the HIV-1 LTR regions (in italics), packaging signal (ψ^+), a truncated gag region containing the rev responsive element (Δgag/RRE) is included for high levels of LV production. In the vector pHR′MU3-GIN, the MSCV U3 (MU3) region was inserted as an internal promoter. In the vector pHR′GIN-MU3, the MU3 region was used to replace the U3 region (except the terminal 35 bp) of the 3′ HIV LTR. Note that the U3 regions of the 3′ LTRs of RV and LV vectors are transferred to the 5′ LTRs after reverse transcription and integration in transduced cells. Abbreviations: SD = splice donor; SA = splice acceptor.*

by standard polymerase chain reaction and subcloning techniques from the HIV-1-based pHR′CMV-LacZ vector and the MGIN RV. First, the CMV-lacZ expression cassette was replaced by the GIN cassette to derive the vector pHR′GIN in which no internal promoter exists (the U3 region of the HIV-1 LTR is the sole promoter). In pHR′MU3-GIN, the MSCV U3 region (MU3; nucleotides –266 to +30 relative to the U3/R boundary) was inserted into the pHR′GIN backbone, where it functions as an internal promoter to drive expression of the GIN reporter cassette. In pHR′GIN-MU3, the U3 region of the 3′ HIV-1 LTR, except for 35 bp at each end, was replaced with corresponding MU3 sequences (Fig. 1B).

Cell Lines, Media and Cytokines

Recombinant human c-kit ligand (KL = stem cell factor) was generously donated by Amgen (Thousand Oaks, CA; http://www.amgen.com). Thrombopoietin (Tpo) and Flt-3 ligand (FL) were purchased from Pepro Tech (Rocky Hill, NJ; http://www.peprotech.com). Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 culture media were purchased from GIBCO-BRL (Gaitherburg, MD; http://www.lifetech.com) and fetal bovine serum (FBS) from Hyclone (Logan, UT; http://www.hyclone.com). QBSF-60 serum-free medium was generously donated by Quality Biological (Gaithersburg, MD; http://www.quality-biological.com).

Human cell lines 293T (SV40 T antigen expressing embryonic kidney fibroblasts), HT1080 (fibrosarcoma) cells, and TE671 (rhabdomyosarcoma) cells were maintained in DMEM plus 10% FBS. Human 293T and TE671 cells were used to produce viruses after transfection, while the three adherent cell lines were target cells to monitor viral transduction. Human CD34$^+$ (suspension) TF1 cells (ATCC CRL-2003) were maintained in RPMI 1640 plus 10% FBS and 1 ng/ml GM-CSF (Amgen), and used as previously described to monitor hematopoietic cell transduction [3].

Viral Supernatant Production

Human 293T and TE671 cells were used as packaging cells after transfection to produce vesicular stomatitis virus G envelope protein (VSV-G)-pseudotyped LV and RV. The

293T cell line was transfected by a $CaPO_4$ method essentially as previously reported for its derivative BOSC23 cell line [25]. In addition to various LV or RV (MGIN), two more types of plasmids were used in the cotransfections. One, pMD.G, expresses VSV-G [26]. The other is a packaging plasmid expressing the respective gag/pol proteins of either HIV or Mo-MLV. For LV vectors, the plasmid pCMVΔR8.91 carrying the HIV-1 gag/pol, tat and rev genes was used [27]. Both pMD.G and pCMVΔR8.91 plasmids were generously provided by *Dr. Didier Trono*. For the MGIN RV vector, a plasmid (provided by *Dr. Gary Nabel*) expressing Mo-MLV gag/pol under the control of the CMV IE promoter was used [28]. The ratio of transducing vector, pMD.G and a gag/pol plasmid was fixed at 20 μg:15 μg:5 μg for 25×10^6 cells plated in a 15-cm plate. After overnight transfection, cells were cultured in fresh medium consisting of either DMEM + 10% FBS or QBSF-60 serum-free medium (for transducing $CD34^+$ cells subsequently). For large-scale viral production using 15-cm culture plates, 10 mM sodium butyrate was added to the medium after transfection to increase titers (by 20%-50%). The supernatants containing recombinant viruses were collected twice 36-60 hours after transfection. Collected supernatants were centrifuged or passed through a 0.45-μm filter (Millipore; Bedford, MA; http://www.millipore.com) to remove cellular debris. For transducing primary $CD34^+$ cells, pools of viral supernatants were concentrated by centrifugation (50,000 *g*) for 3 hours at 4ºC. Concentrated viruses in pellets were resuspended in QBSF-60 medium overnight at 4ºC.

Gene Transduction of Cell Lines and Titer Measurement

Aliquots of unconcentrated viral supernatants of both RV and LV were used to determine viral titers by transfer of G418 resistance or GFP transgene expression using 293T cells. 293T cells (1×10^5) were plated per well (in 2 ml medium) in 6-well plates 1 day prior to transduction. Various dilutions of viral supernatants were added onto cells in the presence of 8 μg/ml polybrene. After overnight incubation, fresh medium was added to replace the polybrene and virus-containing medium. For titer measurements based on transfer of the Neo^R gene, 1 mg/ml (active) G418 was added and G418-resistant colonies were counted after 10-12 days of selection. For titer measurements based on GFP transgene expression, transduced 293T cells were cultured for 3 days before the GFP signal was analyzed by fluorescence-activated cell sorting (FACS). The two assays gave essentially similar values for titers, measured as transducing units per ml of supernatants.

Adherent TE671 and HT1080 cells were transduced as for 293T cells. Hematopoietic TF1 cells were transduced as previously described [3]. Briefly, 2×10^5 TF1 cells in 1 ml were mixed with 1 ml of viral supernatants and 8 μg/ml polybrene (Sigma; St Louis, MO; http://www.sigma-aldrich.com) in a 5-ml tube. The transduction mixture was then centrifuged (1,800 *g*) for 4 hours at 32ºC to 35ºC. After this spinoculation procedure, the cells were resuspended in fresh medium and cultured for 3 days before FACS analysis.

Assays for Replication-Competent LV Viruses

Absence of replication-competent LV viruses was determined by two independent methods. MU3-transduced and G418-selected TE671 cells were cultured for ~50 cell doublings. Supernatants were collected from confluent monolayers of TE671 cells that constitutively expressed GFP and Neo^R. The presence of infectious viral particles in supernatants (undiluted) was assayed using 293T cells as per titer measurement. No Neo^R-resistant colonies were detected. In addition, the absence of HIV-1 gag (p24) proteins in supernatants collected from MU3-transduced TE671 cells was confirmed by enzyme-linked immunosorbent assay (ELISA). The ELISA assay readily detects HIV-1 gag proteins as low as 1.6 pg or 7.8 pg/ml (ZeptoMetrix Corporation; Buffalo, NY; http://www.zeptometrix.com).

Human $CD34^+$ Cell Transduction

Normal human cord blood (CB) $CD34^+$ cells were purchased from either AllCells (San Mateo, CA; http://www.purecell.com) or Poietics/BioWhittaker (Gaithersburg, MD; http://www.biowhittaker.com/). $CD34^+$ cells were purified by immunomagnetic selection (Miltenyi Biotec; Auburn, CA; http://www.miltenyibiotec.com), then cryopreserved. The purity of $CD34^+$ cells provided by both suppliers was usually >95%. One day before transduction, cryopreserved CB $CD34^+$ cells were thawed and resuspended at $1\text{-}2 \times 10^5$ cells/ml in QBSF-60 medium containing human KL (100 ng/ml), FL (100 ng/ml) and Tpo (10-20 ng/ml) (KFT medium). For transduction, fresh or previously frozen vector supernatants in QBSF-60 medium were mixed at a 1:1 ratio with KFT medium containing CB $CD34^+$ cells in the presence of 8 μg/ml polybrene. The multiplicity of infection (MOI) ranged from 30 to 80. The transduction mixture was then centrifuged (1,800 *g*) for 4 hours at 32°C to 35°C. After this spinoculation procedure, the cells were washed once and cultured in KFT medium. The transduction procedure was repeated the following day. The cells then were cultured for another 2 days in the same medium before being harvested for in vitro and in vivo assays.

In Vitro Assays for Progenitor Cells

Colony-forming cells (CFC) including BFU-E, colony-forming-unit-granulocyte/monocyte (CFU-GM) and colony-forming unit-mixed (CFU-mix) in transduced $CD34^+$ cell populations were evaluated by plating 1×10^3 transduced cells in 1 ml methylcellulose medium containing human KL, GM-CSF, interleukin 3, and erythropoietin (MethoCult H4434,

StemCell Technologies; Vancouver, Canada; http://www.stemcell.com) in triplicates. A Nikon inverted fluorescence microscope (Melville, NY; http://www.nikonusa.com) was employed to examine total and green fluorescent colonies, as described [1, 25]. The presence of human CFC in harvested BM cells (10^5) from transplanted NOD/SCID mice was also assayed using the same methylcellulose medium. Under conditions that selectively support human CFC, no mouse BFU-E, CFU-GM or CFU-mixed colonies of >50 cells were formed [1, 29].

Human-Mouse Chimera Assay

Immunodeficient NOD/LtSz-scid/scid (NOD/SCID) mice, originally obtained from Jackson Laboratory (Bar Harbor, ME; http://www.jax.org), were bred and housed in the animal facility at Johns Hopkins Oncology Center. All animals were kept and handled under pathogen-free conditions and fed with sterile food and acidified water following the protocols approved by the Johns Hopkins Animal Care and Use Committee. For transplantation, 6-10-week-old mice received 300 cGy irradiation using a ^{137}Cs gamma-irradiator (84 cGy/minute). The transduced human cells were suspended in 500 µl QBSF-60 medium and transplanted into mice by lateral tail vein injection. Six to 15 weeks after transplantation, mice were sacrificed and BM samples were harvested as previously described [1, 29-30].

Flow Cytometry and Antibodies

R-Phycoerythrin (PE)-conjugated purified mouse anti-human CD34 (clone 8G12/HPCA-2), CD19 (SJ25C1), CD13 (L138), and isotype control antibodies were purchased from Becton Dickinson (San Jose, CA; http://www.bd.com). PE-Cychrome 5 (CYC-5)-conjugated mouse anti-human CD45 and an isotype control antibody were purchased from PharMingen (San Diego, CA; http://www.pharmingen.com). A FACSort (Becton Dickinson) flow cytometer equipped with an Argon laser tuned at 488 nm was used for FACS analysis. Green fluorescence from GFP was detected in the FL1 emission channel [1, 25].

Results

Evaluation of Hybrid LV Containing the MSCV LTR Promoter

We tested a set of HIV-based LV containing different transcriptional promoters carrying the same GFP-IRES-Neo^R (GIN) reporter cassette as in the MGIN RV (Fig. 1). The U3 region of the MSCV LTR (MU3) was inserted into the HIV vector as an internal promoter or was used to replace the promoter elements within the U3 region of the HIV-1 LTR in the vectors pHR′MU3-GIN and pHR′GIN-MU3, respectively. For comparison, an LV without an internal promoter (pHR′GIN) and an MSCV-based MGIN RV were also used (Fig. 1). In preliminary experiments, VSV-G-pseudotyped LV and RV vectors were evaluated using human HT1080 cells to measure titers and levels of transgene expression. All four vectors had similar titers and transduced HT1080 cells at similar levels (Table 1). However, the intensities of GFP transgene expression in transduced HT1080 cells were different. The pHR′GIN vector, lacking an internal promoter and dependent on the HIV U3 region to drive transgene expression, was found to be the weakest. This observed weak activity of the HIV U3 promoter in the absence of Tat is consistent with previous reports [18]. The insertion of the MU3 region into pHR′GIN as an internal promoter (pHR′MU3-GIN) slightly increased transgene expression. However, when the MSCV LTR was inserted into the U3 region of the HIV LTR (in pHR′GIN-MU3), replacing the promoter region of the HIV U3 element, the level of transgene expression increased >3-4-fold over pHR′GIN or pHR′MU3-GIN. It is unclear why the level of transgene expression mediated by MU3-GIN was lower than by GIN-MU3. One possibility is that the functioning HIV U3 promoter present in the pHR′MU3-GIN (but absent in pHR′GIN-MU3) may interfere with the internal MU3 promoter in transduced cells. Therefore, we decided to use pHR′GIN-MU3 (abbreviated as GIN-MU3 hereafter) for all subsequent comparisons to the well-defined MGIN RV.

Table 1. Gene transfer and expression mediated by LV in comparison with the MGIN RV

Vector type	Vector name	% GFP$^+$ cells	Relative intensities of GFP signals
LV	pHR′GIN	87	34
	pHR′MU3-GIN	94	57
	pHR′GIN-MU3	92	149
RV	MGIN	87	258

HT1080 cells were used as target cells to evaluate VSV-G-pseudotyped LV and RV. Four days after transduction, the percentage of GFP$^+$ cells (above background) and the mean fluorescence intensities of GFP$^+$ cells were analyzed. The relative intensities of GFP transgene expression were normalized (dividing by the value of background fluorescence). Therefore, the value for the mock-transduced cells is defined as 1.

In Vitro Assessment of Gene Transfer on Hematopoietic Cells

To evaluate gene transfer and expression in transduced human cells by the GIN-MU3 hybrid LV in comparison with the MGIN RV, we produced VSV-G-pseudotyped viruses in parallel by transfecting human 293T cells. The average titer was 1-2 $\times$ 10^6 TU/ml for GIN-MU3 LV supernatants and 0.5-0.8 $\times$ 10^6 TU/ml for MGIN RV supernatants. The latter titer is comparable to the titers of amphotropic or GALV-pseudotyped MGIN vectors from our best stable packaging

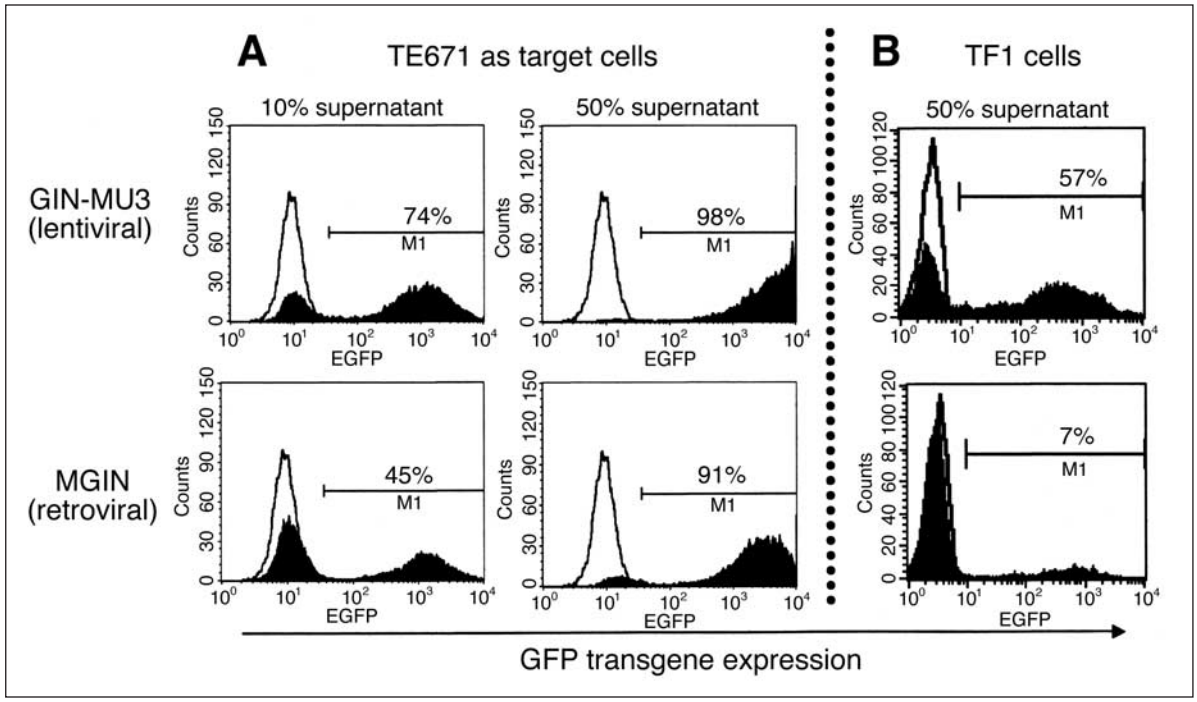

Figure 2. LV- and RV-mediated gene transfer and expression in human cell lines. *VSV-G-pseudotyped LV and RV were collected from transiently transfected 293T cells and used to transduce (A) TE671 adherent cells or (B) hematopoietic TF1 cells in suspension. Transduced cells were harvested 4 days after a single round of transduction with either 10% or 50% vector supernatants and analyzed by FACS. Background green fluorescence of mock-transduced cells is illustrated by unfilled histograms. The percentages of transduced cells expressing GFP are indicated. EGFP = enhanced GFP transgene expression.*

producer lines (data not shown). In addition, we developed a protocol to collect viral supernatants in serum-free medium (QBSF-60) which was used subsequently to transduce CD34$^+$ cells. The titers of collected viruses were essentially the same in QBSF-60 as in DMEM plus 10% FBS.

We further compared gene transfer and expression of the two vectors in another adherent human cell line (TE671) and in a human hematopoietic progenitor cell line (TF1). Various amounts of unconcentrated viral supernatants (10%-50% of total culture medium) were added. Three to 5 days later, transduced cells were analyzed for GFP expression by FACS (Fig. 2). After a single round of transduction, 74% and 98% of TE671 cells were GFP$^+$ when 10% and 50% (v/v) of GIN-MU3 LV supernatants were used, respectively. In contrast, only 45% and 91% of TE671 cells were GFP$^+$ when 10% and 50% of MGIN RV supernatants were used, respectively. The higher transduction efficiency of TE671 cells by the GIN-MU3 vector is consistent with the fact that the titers were two- to threefold higher than the GIN-MU3 vector. Similar results were observed with TF1 hematopoietic cells (57% versus 7%, Fig. 2B), and the overall transduction efficiency was lower than for TE671 cells. Although the VSV-G-pseudotyped GIN-MU3 vector gave two- to eightfold higher gene transfer efficiency (i.e., percentages of GFP$^+$ cells), the resulting transgene expression intensities were similarly high for the two vectors. We concluded that the MU3 region is fully functional as a promoter in the LV backbone. Stable transgene expression mediated by GIN-MU3 was observed after transduced TE671 or TF1 cells divided for >50 cell doublings.

Transduction of CB CD34$^+$ Cells

To directly compare the efficiencies of the two vectors at transducing engrafting HSC, we used CB CD34$^+$ cells, which engraft more efficiently in NOD/SCID mice than CD34$^+$ cells isolated from BM or peripheral blood [30]. To maximally preserve SRC activity, we decided to use a short 4-day protocol of ex vivo culture including LV or RV transduction. Cyropreserved CB CD34$^+$ cells were thawed and cultured in QBSF-60 containing KFT overnight, and then transduced with concentrated LV or RV supernatants. Since the titer of VSV-G-pseudotyped GIN-MU3 LV was two- to threefold higher than that of MGIN RV, concentrated GIN-MU3 LV supernatants were diluted in each experiment to the same titer as the MGIN RV. The ratio of vector particles to cells (MOI) ranged from 30 to 80, similar to what we had used in RV transduction in previous studies [1, 3]. To develop a clinically applicable protocol, the time of vector exposure to cells

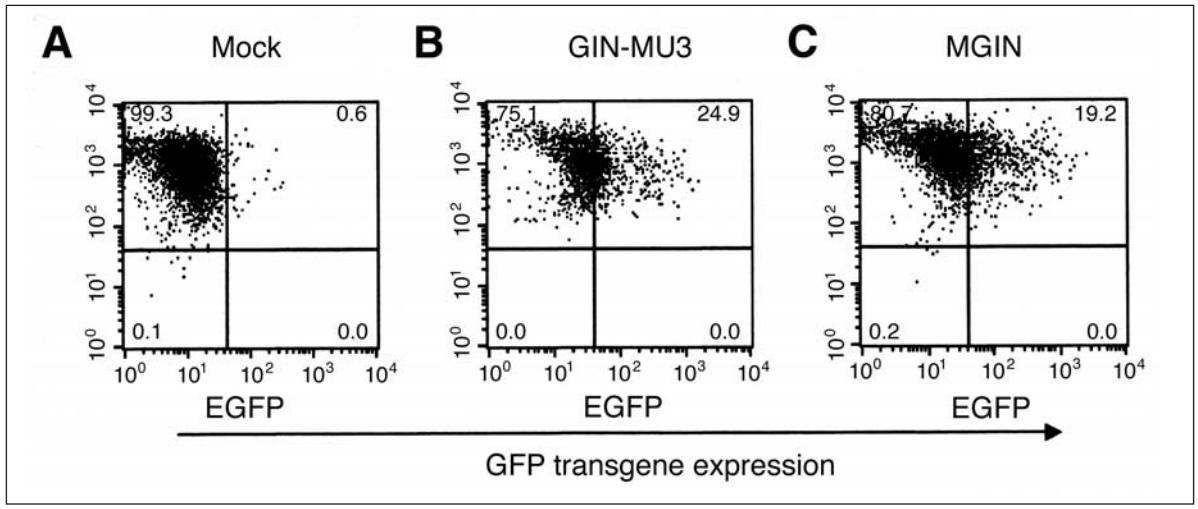

Figure 3. Representative FACS analysis of CD34 and GFP expression in transduced CD34⁺ cells immediately after LV (B, middle plot) and RV (C, right plot) transduction. *At day 4 (2 days after transduction), transduced CB CD34⁺ cells were stained with an anti-CD34 antibody and analyzed by FACS for CD34 and GFP transgene expression. Note that nearly 100% of cells expressed CD34. The percentage of cells in each quadrant is also indicated.*

was limited to 4 hours, and two transduction procedures (spinoculation) were performed on days 1 and 2. Cells were cultured in fresh QBSF-60 medium containing KFT for 2 additional days (to allow cell recovery and GFP expression). On day 4, total cell numbers had increased by ~2 fold and a minor reduction of cell viability after transduction was observed due to the exposure to the VSV-G-pseudotyped viruses (data not shown). Aliquots of cultured cells were analyzed by FACS for CD34 and GFP expression (Fig. 3). Under the conditions used, nearly all the cultured cells were CD34⁺. Similar percentages (19%-25%) of GFP⁺ cells were detected in LV- and RV-transduced cells by FACS (Fig. 3). We observed that percentages of EGFP⁺ cells increased after an additional 2-4 days in culture under the same condition (data not shown).

Gene Transduction into Colony-Forming Progenitor Cells

Since transgene expression immediately following transduction may not reflect the level of stable viral integration [31-32], CFC assays were performed to test the efficiency of stable gene transfer into clonogenic erythroid, myeloid or mixed progenitor cells. Table 2 shows the results from two representative experiments. GFP gene expression was observed in the majority of all three types of progenitor cells (BFU-E, CFU-GM, and CFU-mix). Similar types and numbers of GFP⁺ colonies were observed in GIN-MU3 LV and MGIN RV-transduced cells. The intensities of GFP expression from GIN-MU3 were similar to those from MGIN reported previously [25].

Gene Transfer into NOD/SCID Repopulating Cells

Several studies have demonstrated that in vitro assays do not predict the in vivo engraftment activity of human HSC. We decided therefore to use the SRC assay (with NOD/SCID recipient mice) as a surrogate measure of gene transfer into engrafting human HSC. We performed a total of five independent experiments, testing the GIN-MU3 LV and MGIN

Table 2. Gene transfer into CFC progenitor cells by the GIN-MU3 LV or the MGIN RV

Expt.	Vector type	% of GFP⁺ CFC			
		BFU-E	CFU-GM	CFU-mixed	Total CFC
A	LV	61.1 ± 22.0	74.5 ± 7.9	87.5 ± 12.5	74.5 ± 17.6
	RV	60.9 ± 19.3	82.1 ± 9.0	81.1 ± 8.1	74.6 ± 15.4
B	LV	97.0 ± 5.2	94.9 ± 2.4	93.7 ± 5.5	95.1 ± 4.2
	RV	55.9 ± 16.9	73.9 ± 5.2	77.3 ± 19.6	68.4 ±16.8

Total and GFP⁺ colonies of each type formed in methylcellulose were numerated after 14 days in culture. Percentages of GFP⁺ colonies (mean and standard derivation) were calculated from three triplicates. To compare with the data shown in Table 4, the percentages of GFP⁺ colonies of all the three types were recalculated (using the same data), and are shown in the last column.

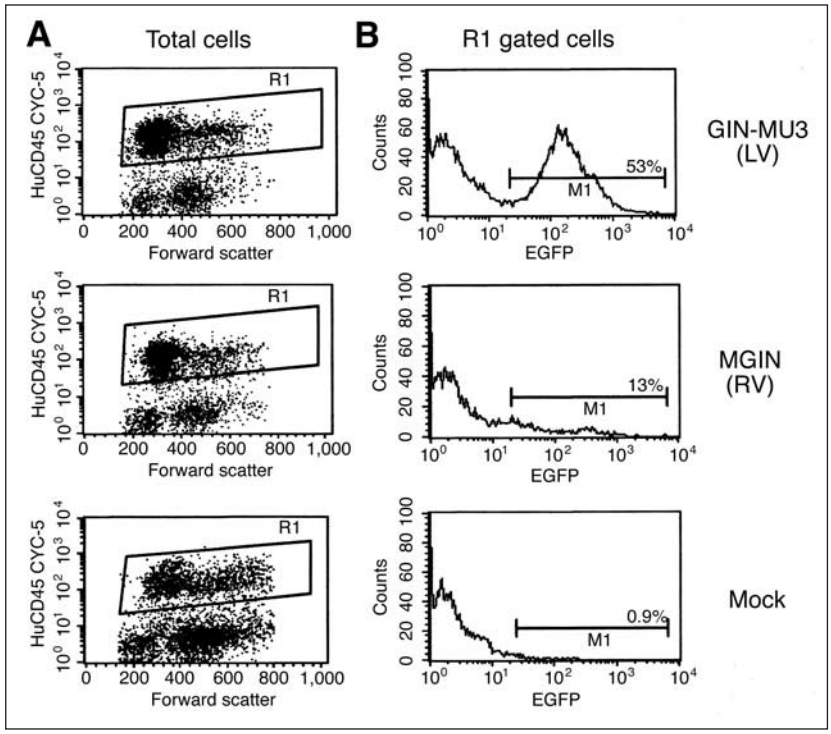

Figure 4. A representative FACS analysis of engrafted human cells at 6 weeks post-transplantation. *GIN-MU3-, MGIN-, or mock-transduced CD34⁺ cells were transplanted into recipient mice. After 6 weeks, mouse BM cells were harvested and analyzed first to determine the presence of human cells using a specific antibody against human CD45 (common leukocyte) antigen (A). The CD45⁺ human cells were gated (in R1) and analyzed for GFP expression (B). The data were from experimental animal #111 (GIN-MU3 transduced), #121 (MGIN-transduced), and #101 (mock-transduced), respectively, as shown in Table 3.*

RV vectors in parallel. In each experiment, 6-10 × 10^5 expanded CB CD34⁺ cells (after LV or RV transduction during a 48-hour period and culturing for a further 48 hours in serum-free culture) were transplanted into NOD/SCID mice. Six to 15 weeks later, the presence of engrafted human cells (expressing the human CD45 antigen) within mouse BM was analyzed. GFP transgene expression in either the total or a subset of engrafted human (CD45⁺) cells was also analyzed by three-color FACS analysis.

In the first experiment (Expt. 1), transplanted NOD/SCID mice with transduced CD34⁺ cells were terminated at 6 weeks post-transplantation (Fig. 4). We found that LV- and RV-transduced cells engrafted at similar levels (Fig. 4A). GFP-expressing human (CD45⁺) cells after 6-week engraftment were observed by either vector (Fig. 4B). Engraftment and gene transduction results are summarized in Table 3. GFP⁺ transduced cells were detected in human CD13⁺ (myeloid), CD19⁺ (B lymphoid), and CD34⁺ cell subsets in each transplanted animal with transduced cells by either vector. However, the percentages of GFP⁺ human cells with the MGIN RV were lower than in each subset as well as in total engrafted human (CD45⁺) cells. Despite a lower percentage in GFP⁺ human cells by the MGIN LV, the intensities of GFP transgene expression were similar in transduced human cells by either vector (Fig. 4B). Notably, the intensity of transgene expression was much higher than previously reported using an LV vector containing the CMV IE promoter

Table 3. Short-term engraftment and GFP expression in engrafted human cells after transduction[a]

Transduction vector	Mouse ID	% human CD45⁺ cells	% of GFP⁺ cells among indicated human cells[b]			
			CD45⁺	CD19⁺	CD13⁺	CD34⁺
LV	111	77.0	54.0	46.3	48.6	45.7
	112	21.4	29.7	64.5	33.3	57.0
	113	17.5	20.4	46.1	29.2	54.7
	Mean ± SE	29.4 ± 14.2	34.7 ± 14.2	46.6 ± 14.4	34.4 ± 4.7	51.5 ± 6.0
RV	121	70.2	13.2	18.6	14.4	4.5
	122	14.2	12.3	13.0	26.2	10.4
	123	36.4	5.9	12.0	16.5	21.3
	Mean ± SE	30.9 ± 12.0	10.5 ± 3.2	15.7 ± 4.6	27.4 ± 9.5	12.3 ± 6.7
Mock	101	40.3	(0.9)	(0.4)	(0.5)	(0.3)

[a] 3 × 10^5 cryopreserved human CB CD34⁺ cells were transduced during a 48-hour period and transplanted after a further 48 hours in culture. Engrafted cells within mouse BM were analyzed 6 weeks post-transplantation. FACS analysis on mouse #111, #121, or #101 is shown in Figure 4.

[b] Specific GFP signals were detected only in transduced human cells. The background green fluorescence signals (≤1.0%) in human cells are listed in parenthesis.

[10]. These data confirmed our previous results that the MGIN RV can transduce SRC and direct transgene expression in multiple lineages of progenies derived from transduced SRC at 4-7 weeks post-transplantation [1, 3-4]. We also found that the GIN-MU3 LV can direct transgene expression equally efficiently in engrafted human cells of multiple lineages as MGIN RV. In addition, it appears that the GIN-MU3 LV transduced SRC more efficiently than the MGIN RV.

We further examined gene transduction into long-term engrafted human cells by terminating transplanted NOD/SCID mice at 10-12 weeks (Expt. 2-4) and at 15 weeks (Expt. 5) post-transplantation. In addition, the presence of transduced human CFC progenitor cells within the BM of transplanted NOD/SCID mice was also assayed subsequently. The FACS analyses of long-term engraftment and gene transduction at 15 weeks are shown in Fig. 5, and summarized results of Expt. 2-5 are shown in Table 4.

RV-transduced human cells had similar levels of long-term engraftment in NOD/SCID mice as mock- or GIN-MU3-transduced cells (Fig. 5A). However, percentages of detected GFP^+ human ($CD45^+$) cells transduced by the MGIN RV were much lower than those by the GIN-MU3 LV (Fig. 5A), or those by the same vector after short-term (6 weeks) engraftment (Fig. 4B). Similarly, low numbers of cells that expressed low levels of GFP signals were found in human $CD19^+$, $CD13^+$, or $CD34^+$ cell subsets in RV-transduced cells after transplantation as were GFP^+ human colonies (Table 4). In contrast, higher percentages of GFP^+ human cells were detected in the GIN-MU3-transduced human cells, including $CD19^+$, $CD13^+$, and $CD34^+$ cell subsets at 15 weeks post-transplantation (Fig. 5B). Notably, the intensity of GFP transgene expression in these transduced cells after a 15-week engraftment remained high. Combined with animals used in Expt. 2-5 (Table 4), approximately 37% (n = 8) of human cells that engrafted in the BM of recipient NOD/SCID mice expressed the GFP gene introduced by the GIN-MU3 LV at 10-15 weeks post-transplantation. The fact that every animal that was transplanted with GIN-MU3-transduced cells contained GFP^+ cells of multiple lineages (erythroid, myeloid, and B lymphoid progenies) suggests that multipotential HSC were likely transduced by the GIN-MU3 LV.

Discussion

Here we reported gene transfer studies of $CD34^+$ human hematopoietic progenitor cells involving a novel LV, GIN-MU3, which contains the U3 region of the MSCV LTR in place of the HIV-1 LTR U3 region. Like the RV MGIN, which we and others have previously used, the expression of the GFP reporter gene is controlled by the MSCV U3 promoter. These two vectors, which differ only in the vector backbone (LV versus RV), provided a unique opportunity to directly compare the efficiency of LV- versus RV-mediated gene transduction of SRC. Using VSV-G-pseudotyped RV and LV supernatants produced in parallel, we transduced CB $CD34^+$ cells involving a short period of ex vivo culture in serum-free medium in order to better preserve and transduce maximal numbers of long-term engrafting HSC. In addition, we attempted to reduce the potential toxicity of VSV-G-pseudotyped viruses by reducing the time that vector particles were incubated with cells to 8 hours, and the concentration of viruses to an MOI of ≤80. During the 4-day ex vivo culture and transduction (48-hour prestimulation and transduction followed by a 48-hour culture period post-transduction), there was a twofold increase in cell number, and essentially all the cells continued to express CD34. Under these conditions, both vectors transduced CFC at a similarly high level (68%-95%). The GIN-MU3 LV mediated a high level of sustained transgene expression in multiple lineages of human cells engrafted in the transplanted animals at both early (6 weeks) and late (up to 15 weeks) time points post-transplantation. However, the counterpart MGIN RV vector resulted in a low percentage of GFP^+ human cells, particularly after long-term engraftment (10-15 weeks).

We and others have previously reported that MGIN or a similar RV can transduce SRC assessed approximately 4-8 weeks after transplantation [1, 3-6]. The current study differs from previous studies in several key aspects. First, VSV-G-pseudotyped viruses were used here while amphotropic or GALV-pseudotyped MGIN viruses were used in previous studies. VSV-G is generally considered to be a more effective envelope because it appears to recognize a ubiquitous cellular receptor that does not need to be upregulated. There are few reported studies using VSV-G-pseudotyped RV for transducing human $CD34^+$ cells [7, 33]. The best result shown was ~25% gene marking (with an MOI of 130 in transduction) in engrafted CFC 6-8 weeks post-engraftment [33]. However, the data presented here and shown in [7] indicated that VSV-G-pseudotyped RV is not more efficient to transduce CB $CD34^+$ cells. It remains to be established whether VSV-G is superior or inferior to envelopes from other viruses such as GALV (for RV only) and feline endogenous virus RD114 for both RV and LV [7].

Second, the preactivation period for SRC transduction by RV and LV was only 18 to 40 hours, which is believed to be suboptimal for RV. Interestingly, under the conditions employed, the transduction efficiencies of CFC by RV were high and comparable to those achieved by the LV. However, it is widely held that a high efficiency of CFC transduction does not necessarily correlate with efficient transduction of more primitive hematopoietic progenitors. Third, the duration of post-transplantation reconstitution in this study ranged from 6-15 weeks, greater than that (4-7 weeks) in previous studies [1, 4-6]. After RV transduction and transplantation,

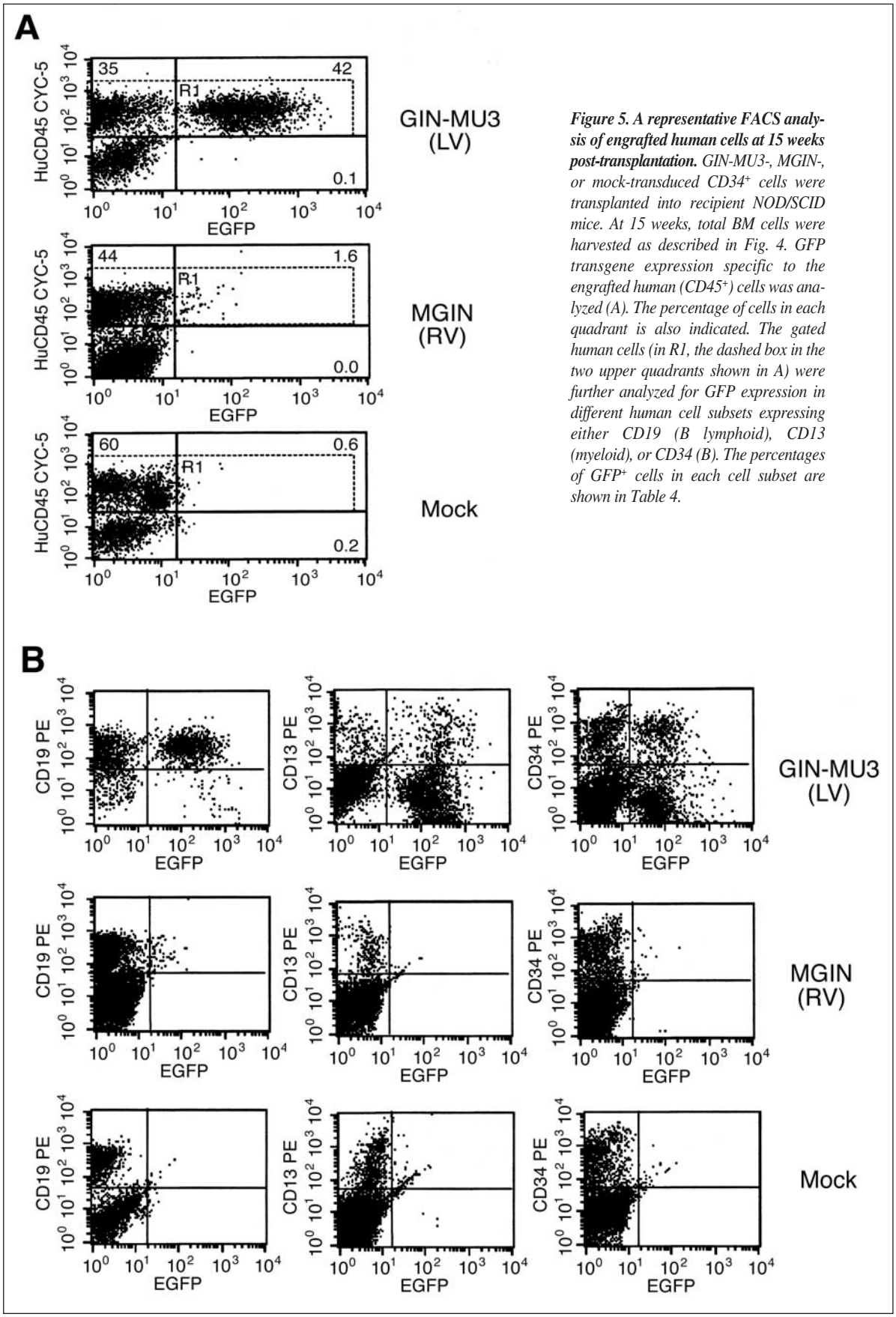

Figure 5. A representative FACS analysis of engrafted human cells at 15 weeks post-transplantation. *GIN-MU3-, MGIN-, or mock-transduced CD34⁺ cells were transplanted into recipient NOD/SCID mice. At 15 weeks, total BM cells were harvested as described in Fig. 4. GFP transgene expression specific to the engrafted human (CD45⁺) cells was analyzed (A). The percentage of cells in each quadrant is also indicated. The gated human cells (in R1, the dashed box in the two upper quadrants shown in A) were further analyzed for GFP expression in different human cell subsets expressing either CD19 (B lymphoid), CD13 (myeloid), or CD34 (B). The percentages of GFP⁺ cells in each cell subset are shown in Table 4.*

Table 4. Long-term engraftment and GFP expression in transduced human cells at 10-15 weeks post-transplantation[a]

Expt #	Post-transplant	Transduction vector	Mouse ID	% human CD45+ cells	% of GFP+ cells among indicated human cells[b]				
					CD45+	CD19+	CD13+	CD34+	CFC[c]
2, 3, 4	10-12 weeks	LV	211	49.0	40.4	50.1	54.2	41.2	45.3
			212	47.9	38.5	54.2	50.1	61.1	26.0
			311	77.1	14.5	18.3	3.1	6.1	52.5
			312	60.7	34.0	40.3	32.4	48.9	35.0
			411	9.4	45.5	49.5	36.0	42.5	N/A
			412	9.0	46.7	52.1	33.7	27.4	N/A
		RV	221	66.2	2.1	4.3	4.9	4.4	14.7
			222	64.9	1.8	2.6	4.3	11.5	N/A
			421	31.0	2.6	1.5	1.5	3.6	N/A
			422	41.5	1.8	1.0	1.0	7.0	N/A
		Mock	201	40.1	(0.3)	(0.9)	(0.4)	(0.3)	0
			301	51.2	(0.2)	(0.2)	(0.2)	(0.6)	0
			401	30.3	(0.9)	(0.2)	(0.2)	(0.3)	N/A
5	15 weeks	LV	511	77.0	54.5	44.1	52.3	35.6	19.7
			512	10.9	24.7	31.4	34.4	22.3	22.3
		RV	521	45.6	1.6	1.9	0.8	1.5	0.4
			522	3.6	0.8	1.0	0.9	0.9	0
		Mock	501	60.6	(0.6)	(0.2)	(0.3)	(0.3)	0

[a] 3×10^5 to 5×10^5 cyropreserved human CB CD34+ cells were transduced during a 48-hour period and transplanted after a further 48 hours in culture.

[b] Specific GFP signals were detected only in transduced human cells. The background green fluorescence signals (≤1.0%) in human cells are listed in parenthesis.

[c] Human CFC assays post-transplantation are described in **Materials and Methods** and Table 2. A and B in Table 2 were the corresponding CFC assays (prior to transplantation) in Expt. #5 and #2, respectively. Human cell colonies were absent due to either the lack of human CFC or technical failure. N/A = not applicable.

high percentages of GFP+ human cells were detected when transplanted NOD/SCID mice were terminated after 6 weeks in this study (Expt. 1, Table 3), slightly lower than or similar to what was observed previously by *Novelli et al*. (4 weeks) [1], *Dorrell et al*. (6-7 weeks) [4], or *Hennemann et al*. (6-8 weeks) [5-6]. By comparison, we found that a low percentage of engrafted human cells expressed the GFP transgene mediated by the MGIN RV vector after 10-15 weeks post-transplantation (Expt. 2-5). In contrast, the GIN-MU3 LV mediated a high level of transgene expression in engrafted human cells even after 15 weeks post-transplantation.

Since the GIN-MU3 LV contained the same promoter (and reporter gene cassette) as the MGIN RV, the reduced, low level of transgene expression by the MGIN RV is presumably not primarily due to the "silencing" of the MSCV U3 promoter per se [34]. Rather, the RV backbone is probably the primary genetic determinant for the lack of transgene expression. Transgene silencing due to other vector sequences is certainly a possibility. However, an alternative possibility is that the MGIN RV, under the condition employed, did not transduce the subset of human SRC that is responsible for long-term engraftment. Clearly, human CD34+ cells are heterogeneous, and the concept would be consistent with published results of HSC subset heterogeneity from long-term mouse syngeneic transplantation studies [35-36] and the sheep in utero transplant model for human CD34+ HSC [37]. This interpretation is consistent with the conclusion from a recent study using viral integration to track the fate of multiple SRC clones, which was published during the preparation of this manuscript [38]. It would also provide a reason why long-term engrafting human cells have rarely been transduced by RV in early clinical trials, and would explain the data obtained using a nonhuman primate model where percentages of transgene-expressing myeloid cells were diminished over time [8]. Long-term and systemic studies to distinguish between the two hypotheses (which are not necessarily mutually exclusive) are currently under way.

Irrespective of the underlying mechanisms responsible, our data suggest that modified LV may be advantageous for transfer of functional transgenes into HSC. First, LV-mediated gene transduction allows a reduction in the prestimulation and ex vivo culture periods required by RV. This should help to better preserve engrafting HSC while reducing the manipulations required prior to transplantation. Second, LV containing permissive promoters such as the MSCV LTR can provide a sustained and high level of transgene expression in vivo after long-term engraftment. Very recently, several groups have reported the development of newer generations

of LV containing internal viral and cellular promoters for enhanced transgene expression in hematopoietic cells [39-42]. In particular, during the preparation of this manuscript, *Woods et al*. reported that sustained transgene expression was achieved in transduced human cells engrafted in primary and second NOD/SCID mice using an improved LV and a short transduction protocol similar to ours [42]. Collectively, these studies provide proof-of-principle that LV vectors have the potential to become important delivery vehicles for HSC-based gene therapy. All these studies used CB cells and NOD/SCID mice as a surrogate assay. It remains to be determined whether the observation can be translated to adult BM and blood $CD34^+$ cells, and in clinical settings.

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