Lentivirus Gene Transfer in Murine Hematopoietic Progenitor Cells Is Compromised by a Delay in Proviral Integration and Results in Transduction Mosaicism and Heterogeneous Gene Expression in Progeny Cells

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Human immunodeficiency virus type 1-based lentivirus vectors containing the green fluorescent protein (GFP) gene were used to transduce murine Lin− c-kit+ Sca1+ primitive hematopoietic progenitor cells. Following transduction, the cells were plated into hematopoietic progenitor cell assays in methylcellulose and the colonies were scored for GFP positivity. After incubation for 20 h, lentivirus vectors transduced 27.3% ± 6.7% of the colonies derived from unstimulated target cells, but transduction was more efficient when the cells were supported with stem cell factor (SCF) alone (42.0% ± 5.5%) or SCF, interleukin-3 (IL-3), and IL-6 (53.3 ± 1.8%) during transduction. The vesicular stomatitis virus glycoprotein-pseudotyped MGIN oncoretrovirus control vector required IL-3, IL-6, and SCF for significant transduction (39.3 ± 9.4%). Interestingly, only a portion of the progeny cells within the lentivirus-transduced methylcellulose colonies expressed GFP, in contrast to the homogeneous expression in oncoretrovirus-transduced colonies. Secondary plating of the primary GFP+ lentivirus vector-transduced colonies revealed vector PCR+ GFP+ (42%), vector PCR+ GFP− (46%), and vector PCR+ GFP− (13%) secondary colonies, indicating true genetic mosaicism with respect to the viral genome in the progeny cells. The degree of vector mosaicism in individual colonies could be reduced by extending the culture time after transduction and before plating into the clonal progenitor cell assay, indicating a delay in the lentiviral integration process. Furthermore, supplementation with exogenous deoxynucleoside triphosphates during transduction decreased mosaicism within the colonies. Although cytokine stimulation during transduction correlates with higher transduction efficiency, rapid cell division after transduction may result in loss of the viral genome in the progeny cells. Therefore, optimal transduction may require activation without promoting intense cell proliferation prior to vector integration.

Hematopoietic stem cells are an attractive target for gene therapy, as they can both self-renew and differentiate into all blood lineages, thus supporting hematopoiesis throughout the lifetime. Gene transfer into hematopoietic stem cells can potentially provide a cure for many inherited and acquired diseases of the hematopoietic and immune systems (25). So far the success of gene therapy in the hematopoietic system has been limited by inefficient gene transfer. Due to the quiescent nature of human hematopoietic stem cells, they are fairly poor targets for conventional oncoretrovirus vectors, which require cell division for integration (22, 26). Lentivirus proteins have nuclear localization signals which facilitate entry of the preintegration complex into the nuclei of nondividing cells (4, 22, 42). This enables lentivirus vectors to transduce nondividing cells, and they therefore represent a promising tool for gene therapy of hematopoietic stem cells (27, 30, 31, 36, 38, 40). Lentivirus vector transfer vectors have been shown to transduce both dividing and nondividing cells from various species, including cell lines and primary cells such as neurons (2, 10, 12, 19, 44), myocytes (18), and hepatocytes (18, 34); retinal (28), corneal (41), cochlear (16), and, pancreatic islet (15) cells; and various populations of hematopoietic cells (1, 5, 11, 13, 30, 39, 40). Continuous vector development has focused on the generation of vectors that are both efficient and safe (32). The tropism of the vector is widened by pseudotyping the virus by vesicular stomatitis virus glycoprotein (VSV-G) envelope (23), which also provides high stability for the virus and facilitates concentration for high titers. For safety reasons the accessory genes vif, vpr, vpu, and nef, involved in the pathogenesis of wild-type human immunodeficiency virus (HIV), have been deleted. The probability of generation of replication-competent recombinants (RCRs) is minimized by segregating the cis- and trans-acting elements in three different plasmids, as well as developing self-inactivating lentiviruses with deletions in the U3 region of the 3′ long terminal repeat (LTR) (29,45). Lentivirus vectors do not transduce any viral genes into the target cells, minimizing the likelihood of immune reactions. The α vivo transduction used for hematopoietic cells omits the need to expose the patient to the virus systemically, reducing the risk of toxicity potentially associated with high quantities of the vector, as shown with in vivo transduction of murine hepatocytes (34).

Several studies have demonstrated the superiority of HIV type 1 (HIV-1)-based lentivirus vectors to oncoretrovirus vectors in transducing human hematopoietic progenitor cells and human candidate stem cells. This includes CD34+ cells from different sources including bone marrow, cord blood, and mo-
bilized peripheral blood progenitor cells, as well as purified cells with the CD34+/CD38− immunophenotype (1, 5, 11, 13, 30, 39, 40) that are known to support long-term hematopoiesis. High transduction efficiency has been demonstrated in in vitro assays as well as in vivo in xenograft models, for example, in immunodeficient NOD/SCID mice which act as hosts for the transplantation of human hematopoietic cells (30). Although the NOD/SCID mouse assay is the most commonly used assay so far for the study of human candidate stem cells, it is limited by the short life span of the recipients as well as by the inability to support differentiation to all hematopoietic lineages.

The development of lentiviral gene therapy into clinical use will require preclinical trials in animal models. This will be invaluable for in vivo testing of new lentiviruses, e.g., to achieve optimal expression levels in differentiated progeny cells or to provide lineage-specific or regulatable expression of the transgene. The animal disease models are also crucial for testing the effects of lentivirus gene transfer in vivo, as well as assessing the safety of the vector system in immunocompetent hosts.

The aim of this work was to study the efficiency of lentivirus gene transfer into murine hematopoietic stem cells under quiescent and proliferating conditions. In this study we have demonstrated that purified Lin−c-kit+ Sca1− primitive murine hematopoietic progenitor cells can be transduced by lentivirus vectors driven by an internal promoter, EF-1α (a gypsy from Sterne-Osikin) conditions and that high transgene expression levels can be achieved using the elongation factor 1α (EF-1α) promoter. However, the transduction efficiency is consistently higher if the target cells are transduced with cytokine support. Furthermore, the final gene transfer efficiency in the daughter cells is compromised by a latency of lentivirus vector integration, and optimal gene transfer of primitive murine hematopoietic progenitor cells depends on adjustment of the cytokine stimulation and proliferation kinetics of the target cell during and after transduction.

MATERIALS AND METHODS

Lentivirus vector constructs. Lentivirus vectors were generated by transient transfection in 293T cells using the three-plasmid system as previously described (31). The packaging plasmid pCMV R8.91 provides the Gag, Pol, Tat, and Rev proteins to package the viral particle in 293T cells. The envelope coding plasmid pMD.G provides the vector with a VSV-G envelope, which broadens the host range and stabilizes the viral particle. The transfer vector plasmid, pHR'EF-1α, contains the enhanced green fluorescent protein (GFP) marker gene (6, 7, 8, 35) driven by an internal promoter, EF-1α (a gypsy from Sterne-Osikin). Children's Hospital, Boston, Mass.), as described earlier with other internal promoters (31).

Transduction of murine hematopoietic progenitor cells. Sorted cells were washed, and all lentivirus transductions and liquid cultures were performed in serum-free X-Vivo 15 medium (BioWhittaker, Walkersville, Md.) with 1% bovine serum albumin (Stem Cell Technologies, Vancouver, British Columbia, Canada), β-ME, l-glutamine, and penicillin-streptomycin, with and without cytokine supplementation. The transductions were performed either in 96-well plates (non-tissue culture treated; Falcon; BD Biosciences, San Jose, Calif.) coated with fibronectin (Retronectin; Takara Shuzo, Otsu, Japan) or on terasaki plates (Falcon). In 96-well plates, 2,000 to 10,000 cells were transduced in each well in a volume of 100 μl, whereas in terasaki plates, 500 to 1,000 cells were transduced in each well in a volume of 20 μl. Viral supernatants were supplemented with 10% calf serum (IL-6/20% murine IL-1b/10%, or an equal mixture of IL-6/20% murine IL-1b/10% murine IL-1a/10%) and 10% fetal bovine serum (FBS) at 108 to 5 × 1010 transducing units (TU)/ml and nonconcentrated serum-containing supernatants (titers, 1 × 109 to 5 × 1010 TU/ml) were used.

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Preparation of high-titer virus vectors. Lentivirus vectors were generated by transient transfection in 293T cells using the three-plasmid system as described earlier (31). Briefly, the transfection was performed by CaPO4 precipitation in Dulbecco's modified Eagle medium (DMEM)—10% fetal bovine serum (Gibco BRL, Cleveland, Ohio)—100 IU of penicillin/ml—100 μg of streptomycin (Gibco BRL)/ml, followed by a medium change after 18 h. Viral supernatants were harvested 24 and 48 h later and concentrated by ultracentrifugation. The viral supernatants were titrated on HeLa cells by serial dilutions and analyzed 96 h after transduction.

After scoring for GFP expression, individual colonies were picked for PCR analysis. The cells within each colony were cultured for 2 weeks, and each secondary colony was analyzed. PCR from hematopoietic colonies. After scoring for GFP expression, individual methylcellulose colonies were picked for PCR analysis. The cells within each colony were cultured for 2 weeks, and each secondary colony was analyzed by microscopy, FACS, and PCR.
96°C for 15 min. The PCR to detect the presence of the vector gene was performed by primers within the GFP gene (GFP56F; 5' GAG CTG GAC GGC GAC GTA AAC G, and GFP629R, 5' CGC TTC TCG TTG GGG TCT TTG CT). Amplification was performed in 1.5 mM MgCl2 with 30 cycles of 95, 60, and 72°C, and the PCR products were analyzed on ethidium bromide-agarose gels. The integrity of DNA samples was controlled by PCR for mouse actin.

RESULTS

Optimal lentivirus vector design for expression in murine hematopoietic cells. To select an optimal expression cassette for high expression of lentivirus vectors in murine hematopoietic cells, 10 different expression vectors were tested in murine hematopoietic cells (Fig. 1). The vectors EF-1α, EF-1α–WPRE, PGK, PGK-SIN, PGK-WPRE-SIN, CMV, CMV-WPRE, CMV-SIN, CAG, and CAG-WPRE were named according to the internal promoter and whether the WPRE or the SIN deletion was present. The purified hematopoietic cells were transduced, grown in liquid culture in the presence of IL-3, IL-6, and SCF for 3 days, and then analyzed by FACS. The vector containing the EF-1α promoter generated the highest mean fluorescence intensity (MFI) among the GFP cells by FACS (EF-1α, 179 ± 8; PGK, 58 ± 3; CMV, 39 ± 2; CAG, 40 ± 0.5; n = 3 experiments) and was comparable to the MFI (162 ± 2) generated by the oncoretrovirus, vector MGIN (Fig. 3). Addition of the WPRE did not provide an improvement in murine hematopoietic cells, in contrast to the human cell lines HeLa and 293T, where two- to threefold improvement was seen (data not shown). Inclusion of the SIN deletion did not have a clear effect on the expression levels in murine hematopoietic cells (data not shown). Therefore, we chose the EF-1α vector without addition of the WPRE element and without the SIN deletion for all further experiments presented below.

Gene transfer efficiencies of lentivirus vectors in Lin− c-kit+ Sca1+ murine hematopoietic progenitor cells. Lentivirus gene transfer efficiency in Lin− c-kit+ Sca1+ clonogenic progenitors was analyzed in methylcellulose colony assays. The lentivirus vector transduction efficiency, as scored by the percentage of GFP+ colonies in methylcellulose, was high under all 20-h transduction conditions tested. The scoring was initially performed by microscopy and confirmed by FACS analysis of the individual GFP+ colonies. If the transduction was performed without cytokines or serum, the transduction efficiency with the lentivirus vector was 27.3 ± 6.7%, whereas there was no significant transduction with the oncoretrovirus controls (1.26 ± 0.8% with the concentrated supernatant under serum-free conditions, or 1.7 ± 0.3% with the unconcentrated supernatant with a final serum concentration of 3%) (Fig. 4). The lentivirus transduction efficiency within the clonogenic progenitors was higher if cytokine support was used. When SCF was added, lentivirus transduction generated 42.0 ± 5.5% GFP+ colonies, in contrast to the concentrated and unconcentrated oncoretrovirus MGIN controls, which resulted in 3.3 ± 1.8 and 9.7 ± 1.8% GFP+ colonies, respectively. When transduction was performed with SCF, IL-6, and IL-3, 53.3 ± 1.8% of the lentivirus-transduced colonies were positive for GFP, in comparison to 9.3 ± 1.2% (serum free) and 39.3 ± 9.4% (with serum) of the oncoretrovirus controls.

Heterogeneity of GFP expression within GFP+ colonies. Although lentivirus transduction resulted in a high percentage of GFP+ colonies, microscopy and FACS analysis of individual GFP+ colonies revealed that only a portion of the cells within each colony expressed the GFP gene (Fig. 5). The ratio of...
GFP+ cells within each colony was lowest when no cytokines were used during transduction (19.2 ± 5.0%), in comparison with 34.2 ± 6.0% when SCF was used alone and 45 ± 11% when SCF, IL-6, and IL-3 were used during transduction. In contrast, 87.8 ± 5.2% of the cells in GFP+ colonies transduced in the presence of IL-3, IL-6, and SCF with the MGIN oncoretrovirus, control vector were GFP+ (Fig. 5, bottom). Mosaicism of GFP expression was observed in transduced colonies from all lentivirus-vectors tested, irrespective of the nature of the internal promoter (data not shown).

Analysis of secondary hematopoietic colonies for GFP expression and presence of the vector genome. To study whether the heterogeneity of GFP expression in the methylcellulose colonies was due to transcriptional silencing or true genetic mosaicism with respect to the presence of the proviral vector genome, individual GFP+ colonies were plated further for secondary colony assays. Purified hematopoietic progenitors were transduced in the presence of IL-3, IL-6, and SCF, then plated into methylcellulose cultures, and GFP+ hematopoietic colonies of various types or morphologies (colony-forming units mix [CFU-mix], colony-forming units granulocyte-macrophage [CFU-GM], etc.) were picked at days 5 to 6 and plated into secondary methylcellulose cultures. Individual secondary colonies were then scored for GFP expression by FACS and for the presence of vector genome in the daughter cells by PCR. Within the 30 secondary colonies that derived from 7 different primary GFP+ colonies, only 42% were GFP positive by both FACS and PCR (Table 1). None of the colonies were positive by FACS without being PCR positive, demonstrating the high sensitivity of the PCR assay. Approximately half of the colonies (46%) were negative both by FACS and by PCR. Some of the colonies (13%) were positive by PCR without demonstrating any expression of GFP by FACS, representing either integrated copies where the expression from the EF-1α promoter was silenced or cases where the GFP gene was amplified by PCR from a nonintegrated vector. The presence of GFP+ PCR− daughter colonies in the progeny of primary GFP+ colonies indicates that the lentivirus gene transfer is not fully completed between the time of transduction and the time when the transduced progenitor divides. Therefore, the vector genome seems to integrate after proliferation in the methylcellulose culture starts, and as a consequence, vector integration is seen in only a portion of each progenitor’s progeny cells. In contrast, all secondary colonies derived from the oncoretrovirus-transduced colonies expressed GFP and contained the proviral DNA, as detected by PCR. The difference between the number of secondary colonies containing the lentiviral and oncoretroviral DNA, respectively, is highly significant by the chi-square test (P < 0.01).

Effect of time and proliferation kinetics after transduction on heterogeneity of GFP expression and final gene transfer efficiency. To study the time course needed for completion of lentivirus gene transfer in mouse hematopoietic progenitor cells, the transduced cells were cultured for an extended period (an additional 24 to 72 h) before plating into methylcellulose clonal assays. By delaying the start of a clonal assay after transduction in SCF, the degree of mosaicism was reduced. The percentage of GFP+ cells within the GFP+ colonies increased from 34 ± 8% at day 0 to 55 ± 6% when the cells were plated at day 1 posttransduction and to 61 ± 9 and 68 ± 11% at days 2 and 3 posttransduction, respectively (Fig. 6A). The difference between day 0 and day 3 was significant (P < 0.02), as was the difference between day 0 and day 2 (P < 0.05), by Student’s t test. The difference between day 0 and day 1 was not statistically significant (P = 0.11).

In an effort to analyze the effect of cytokine stimulation and proliferation kinetics after transduction on the final gene transfer efficiency as judged by the total percentage of GFP+ cells in
The difference between the oncoretroviral and lentiviral samples was highly significant (P < 0.001). In an attempt to further improve the gene transfer process in murine hematopoietic progenitors and stem cells, deoxynucleotides were added during transduction. Addition of 50 μM dNTPs was shown to increase the total ratio of GFP+ cells under all conditions tested, from 6.5 ± 1.0% to 14.4 ± 2% with SCF alone (statistically significant; P < 0.03). These results together suggest that the second exposure to the lentivirus vector may facilitate entry of more viral particles into the target cell population, as well as give more time to complete the integration process before plating for clonal assays, thus increasing the percentage of GFP+ cells in the progeny.

Effect of exogenous dNTP supplementation on gene transfer efficiency. In an attempt to further improve the gene transfer process in murine hematopoietic progenitors and stem cells, deoxynucleotides were added during transduction. Addition of 50 μM dNTPs was shown to increase the total ratio of GFP+ cells under all conditions tested, from 6.5 ± 1.0% to 14.4 ± 2% with SCF alone (statistically significant; P < 0.03). These results together suggest that the second exposure to the lentivirus vector may facilitate entry of more viral particles into the target cell population, as well as give more time to complete the integration process before plating for clonal assays, thus increasing the percentage of GFP+ cells in the progeny.

Table 1. Lentivirus transduced GFP+ primary hematopoietic colonies generate GFP+ secondary colonies lacking the provirus

<table>
<thead>
<tr>
<th>Primary colony</th>
<th>No. (%) of secondary colonies with the following phenotype:</th>
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<tbody>
<tr>
<td></td>
<td>FACS+ PCR+</td>
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<tr>
<td>Lentivirus transduction</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>3 (50%)</td>
</tr>
<tr>
<td>B</td>
<td>2 (25%)</td>
</tr>
<tr>
<td>C</td>
<td>5 (39%)</td>
</tr>
<tr>
<td>D</td>
<td>1 (33%)</td>
</tr>
<tr>
<td>E</td>
<td>6 (66%)</td>
</tr>
<tr>
<td>F</td>
<td>2 (40%)</td>
</tr>
<tr>
<td>G</td>
<td>1 (25%)</td>
</tr>
<tr>
<td>Total</td>
<td>20 (42%)</td>
</tr>
<tr>
<td>Oncoretrovirus transduction</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>I</td>
<td>4 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td>9 (100%)</td>
</tr>
</tbody>
</table>

* Cells were transduced as described in Materials and Methods in the presence of IL-3, IL-6, and IL-3. These were then plated into methylcellulose cultures. Primary GFP+ colonies (A through I) were picked and replated into secondary methylcellulose cultures. The secondary colonies were picked individually and analyzed by FACS, and the presence of the proviral DNA was detected by PCR. The difference between the oncoretroviral and lentiviral samples was highly significant by the chi-square test (P < 0.01).
lentivirus vectors to transduce and express genes in primitive hematopoietic progenitor cells and their progeny, in an effort to create a mouse model for lentivirus gene transfer for future studies involving testing of new expression cassettes and therapeutic genes in a true in vivo model. Furthermore, the aim was to gain insight into the prerequisites for optimal gene transfer of hematopoietic cells by lentivirus vectors, by studying the gene transfer efficiency into quiescent as well as cytokine-stimulated primitive murine hematopoietic progenitor cells. Lin− c−kit+ Sca1− cells were obtained from the bone marrow by depleting the cells expressing lineage-specific markers, and further sorting for c−kit+ Sca1+ cells, resulting in a 1,000-fold enrichment for primitive hematopoietic progenitor and stem cells. In an effort to achieve high transgene expression levels in murine hematopoietic cells, different lentivirus vectors were tested in these target cells and their progeny. Our results show that high expression levels of the GFP marker gene can be obtained by using the EF-1α promoter in the lentivirus vector. The expression level from the EF-1α lentivirus vector was similar to the level from the oncoretrovirus LTR in the MGIN vector, which expresses very well in hematopoietic cells (8).

Using the EF-1α lentivirus vector, we studied how mouse hematopoietic progenitor cells can be transduced most efficiently. Our results demonstrate that the VSV-G-pseudotyped lentivirus vectors can transduce a high percentage of murine Lin− c−kit− Sca1− hematopoietic stem cells with or without cytokine stimulation, as judged by the ratio of GFP-positive colonies in clonogenic progenitor assays. However, the transduction efficiency was consistently higher when the transduction was performed with cytokine support. Supplementation with SCF during transduction increased the transduction efficiency, although SCF alone will not induce rapid cell division. The transduction efficiency was increased further by using SCF, IL-6, and IL-3, a combination that effectively stimulates proliferation of hematopoietic progenitor and stem cells. The oncoretrovirus control vector did not show significant transduction unless the cells were stimulated with SCF, IL-6, and IL-3. It is known that optimal oncoretrovirus transduction requires longer cytokine stimulation than the 20-h transduction protocol used here allows (8). These results demonstrate the superiority of lentivirus gene transfer to oncoretrovirus transduction when the cells to be transduced have undergone little or no activation. However, the efficiency of lentivirus transduction of murine hematopoietic progenitors was increased for cytokine-stimulated cells. This is consistent with the results of Sutton et al. (39), which showed that the efficiency of transduction of human CD34+ hematopoietic cells is higher when they are in the G1 or G2/S phase of the cell cycle than when they are in G0. Cell cycle activity is probably not required for lentivirus vector transduction of hematopoietic progenitors, in contrast to murine hepatocytes, which need to be actively dividing in order to be efficiently transduced in vivo (34).

Interestingly, when the daughter cells within individual GFP+ colonies in methylcellulose were analyzed for GFP expression by flow cytometry, only a portion of the cells were found to express GFP. In contrast, GFP expression was more homogeneous in colonies transduced with the oncoretrovirus control vector. The heterogeneity of GFP expression in the lentivirus transduced colonies was most evident if the transduction was performed without cytokine stimulation. The lack of GFP expression in a portion of cells within the progeny of a single transduced progenitor cell raised the question of whether the GFP mosaicism observed is true genetic mosaicism with respect to the presence of the integrated proviral vector genome or is due to transcriptional silencing of the internal promoter in the vector. To address this question, the...
progeny cells from individual GFP-positive colonies were plated into secondary methylcellulose culture, and the secondary colonies were analyzed for the presence of the viral genome and GFP expression. Interestingly, both GFP<sup>+</sup> PC<sup>+</sup> as well as GFP<sup>-</sup> PC<sup>-</sup> secondary colonies originating from the primary GFP<sup>+</sup> colonies were found. Some of the colonies were positive by PCR without showing any detectable GFP expression by FACS. These colonies may represent colonies that contain the proviral genome but are transcriptionally inactive. The presence of both GFP<sup>+</sup> PC<sup>+</sup> and GFP<sup>-</sup> PC<sup>-</sup> secondary colonies in the progeny of a single cell demonstrates true genetic mosaicism with respect to the vector genome in the progeny cells, suggesting a latency of viral integration using these vectors, target cells, and transduction conditions. It is also possible that the vector has integrated initially but has subsequently been lost from the target cell. This possibility is not likely.

To study whether vector mosaicism in the progeny cells was the result of delayed integration, we tested whether the degree of GFP heterogeneity within the colonies could be reduced by extending the time available for integration before the transduced cells were plated for clonal assays. The results show that the degree of GFP mosaicism could be reduced if the colony assay was started at later time points, suggesting that lentivirus integration cannot always be completed during the time from the beginning of transduction until progenitor proliferation starts. If the extended culture was performed under low proliferative conditions, with SCF alone, the number of positive colonies was maintained, and the final ratio of GFP<sup>+</sup> cells in their progeny could be increased. In contrast, when the cells were cultured after transduction under high proliferative conditions, with SCF, IL-6, and IL-3, the total percentage of GFP<sup>+</sup> cells was not increased by extended liquid culture. These results, taken together, show that although murine Lin<sup>−</sup> c-kit<sup>−</sup> Sca1<sup>+</sup> hematopoietic progenitor cells can be more readily transduced with cytokine stimulation than without, rapid cell division prior to integration may result in loss of the unintegrated vector genome from some of the progeny cells, lowering the total gene transfer efficiency. In support of the concept of delayed integration of lentivirus vectors is evidence that wild-type HIV-1 proviral DNA can persist as long as 3 weeks extrachromosomally in quiescent T cells prior to integrating upon T-cell activation (37).

The results with the lentivirus transduction of mouse hematopoietic stem cells and progenitor cells point out that although viral entry with the VSVG envelope in these target cells appears to be efficient, as shown by the high ratio of GFP<sup>+</sup> colonies in the methylcellulose assay, there may be other bottlenecks that compromise the final gene transfer efficiency in the progeny. In addition to entry into the target cells, optimal gene transfer requires efficient reverse transcription of the viral RNA, transport into the nucleus, and integration in the genome of the target cell. Our aim was to see whether we could affect any of these processes and increase overall transduction efficiency as well as decrease the GFP mosaicism within the progeny cells. In an effort to maximize vector entry into the target cells, the cells were exposed for a second hit of vector together with prolongation of the transduction time by an additional 20 h. Indeed, a second exposure to the vector increased the final gene transfer efficiency two-fold, as determined by the ratio of GFP<sup>+</sup> cells in the progeny, with a modest increase in the ratio of GFP<sup>+</sup> colonies. When the overall transduction efficiency was high, e.g., over 40% GFP<sup>+</sup> cells, an increase in the MFI was also observed. These results show that the double transduction may be useful if high gene transfer efficiency is required. The beneficial effect may in part depend on entry of more viral copies into the target population, and in part on the extended time available to complete gene transfer before rapid proliferation of the target cells ensues. It is of interest in this context that a recent report demonstrates that the 99-nucleotide central DNA flap that creates a DNA triplex in HIV-1 will increase the nuclear import of HIV-1 and HIV-1-based vectors (42). It is possible that the presence of this sequence, which is lacking in our vectors, would increase gene transfer efficiency and reduce the mosaicism in the progeny of the target cells.

The transduction efficiency of hematopoietic progenitors was lowest, and the mosaicism within the colonies was highest, when the cells were transduced without any cytokine support. Studies with wild-type HIV and other retroviruses have shown that reverse transcription of the virus cannot be efficiently completed in quiescent lymphocytes, or the mutation rate in the reverse transcripts may be higher (14, 17, 20, 21). One factor hindering reverse transcription may be nucleotide imbalances (24) in quiescent cells. Attempts have been made to improve retrovirus transduction efficiency by supplementing with nucleotides before transduction (43); however, no significant improvement due to dNTPs was seen in transducing rat neurons with lentivirus vectors (2). Interestingly, in hematopoietic progenitor cells, supplementation of the transduction mixture with 50 μM dNTPs increased the ratio of GFP<sup>+</sup> cells in the progeny roughly two-fold. The effect was seen most clearly in the unstimulated cells. The number of transduced colonies was not changed, although the overall ratio of the GFP<sup>+</sup> cells within the colonies doubled, indicating that the mosaicism of GFP expression in the colonies could be reduced by providing exogenous nucleotides during transduction. This raises the possibility that reverse transcription might be a rate-limiting step for the rapid establishment of the lentiviral provirus in mouse hematopoietic progenitor cells.

In conclusion, we have found that mouse Lin<sup>−</sup> c-kit<sup>-</sup> Sca1<sup>+</sup> hematopoietic stem and progenitor cells are sensitive targets for lentivirus gene transfer, and high expression levels can be achieved by using EF-1α as an internal promoter. However, the final gene transfer efficiency with the vector type used depends on the growth conditions during and after transduction. Cytokine stimulation or, alternatively, supplementation with nucleotides during transduction was associated with higher gene transfer efficiency, whereas rapid proliferation after transduction resulted in a lower ratio of GFP-expressing progeny cells. These findings may help in understanding the mechanisms affecting lentivirus gene transfer efficiency in hematopoietic progenitor and stem cells, and in developing optimal transduction protocols for these target cells.

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