Lentiviral vector transduction of NOD/SCID repopulating cells results in multiple vector integrations per transduced cell: risk of insertional mutagenesis

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Efficient vector transduction of hematopoietic stem cells is a requirement for successful gene therapy of hematologic disorders. We asked whether human umbilical cord blood CD34+CD38lo nonobese diabetic/severe combined immunodeficiency (NOD/SCID) repopulating cells (SRCs) could be efficiently transduced using lentiviral vectors, with a particular focus on the average number of vector copies integrating into these primitive progenitor cells. Mouse bone marrow was analyzed by fluorescence-activated cell-sorter scanner and by semiquantitative polymerase chain reaction (PCR) to determine the transduction efficiency into

SRCs. Lentiviral vector transduction resulted in an average of 22% (range, 3%-90%) of the human cells expressing green fluorescent protein (GFP), however, multiple vector copies were present in human hematopoietic cells, with an average of 5.6 ± 3.3 (n = 12) copies per transduced cell. To confirm the ability of lentiviral vectors to integrate multiple vector copies into SRCs, linear amplification mediated (LAM)–PCR was used to analyze the integration site profile of a selected mouse showing low-level engraftment and virtually all human cells expressing GFP. Individually picked granulocyte macrophage colony-forming unit colonies derived from

the bone marrow of this mouse were analyzed and shown to have the same 5 vector integrants within each colony. Interestingly, one integration site of the 5 that were sequenced in this mouse was located in a known tumor-suppressor gene, BRCA1. Therefore, these findings demonstrate the ability of lentiviral vectors to transduce multiple copies into a subset of NOD/SCID repopulating cells. While this is efficient in terms of transduction and transgene expression, it may increase the risk of insertional mutagenesis. (Blood. 2003;101:1284-1289)

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Introduction

The hematopoietic stem cell (HSC) is an ideal target cell for vector-mediated gene therapy of both inherited and acquired hematological diseases, as both the quantity and distribution of vector containing progeny cells from a transduced HSC should be sufficient for a therapeutic response. Thus, vectors capable of stable integration into a target cell's genome, such as the murine oncoretroviral-based vectors and lentiviral-based vectors, have been tested for their efficiency of transduction in primitive human hematopoietic cells from peripheral blood, bone marrow, and umbilical cord blood in both in vitro and in vivo assays.[1-9] The true HSC, defined by its capacity for long-term hematopoietic reconstitution by virtue of its high proliferative potential and its ability to self-renew, is, paradoxically, quiescent at most points in time. It is this property of the stem cell that has impeded vector-mediated gene therapy–based treatment of disease. However, lentiviral-based vectors have been shown to transduce primitive nondividing hematopoietic cells[5,10] including nonobese diabetic/severe combined immunodeficiency (NOD/

SCID) repopulating cells (SRCs) with minimal stimulatory conditions.[6-9]

Using lentiviral vectors, it has been demonstrated that in order to achieve a maximum transduction efficiency into primitive hematopoietic cells, a high multiplicity of infection (MOI) is required.[11,12] However, even at an MOI as high as 1000 transducing units (TU)/cell and viral concentrations exceeding 10^7 TU/mL, transduction of all primitive hematopoietic cells without stimulation remains an elusive goal.[9] Moreover, the consequence of the high concentration of virus on the fraction of cells permissive to transduction has not been addressed with regards to the integrated vector copy number per transduced cell. Lentiviral vector transduction and transgene expression analyses in hematopoietic cells have assumed single (or very few) vector copies per cell, based on single oncoretroviral vector copies found in murine hematopoietic clones derived from beige/nude/xid mice.[13] However, a study performed in a murine embryonic stem cell line demonstrated that the lentiviral vector copy number per transduced clone could be

increased to as many as 12 copies per cell by increasing the MOI used during infection.[14]

In order to assess the transduction efficiency in human hematopoietic stem cells and their progeny, we transplanted lentivirally transduced primitive human $CD34^+CD38^{lo}$ cells into NOD/SCID mice. Surprisingly, an average of 5.6 ± 3.3 (n = 12) copies per transduced hematopoietic cell were detected in the transduced progeny of the SRCs. The capability of lentiviral vectors to transduce multiple vector copies into SRCs was further verified using a highly sensitive linear amplification mediated (LAM)–polymerase chain reaction (PCR) technique[15,16] to track the individual vector integration sites in the genome of the transduced cell. Therefore, we propose that the subset of SRCs permissive to lentiviral vector transduction is susceptible to multiple vector copy integration into genomes of each cell. While this is efficient in terms of gene transfer into human NOD/SCID repopulating cells, multiple copy integrations of lentiviral vectors in hematopoietic cells may increase the risk for insertional mutagenesis.

Materials and methods

Lentiviral vector production

The 3-plasmid expression system used to generate the lentiviral vectors has been described.[17] The construction of the transfer vector plasmids, pHR′ PGK GFP, pLOX-EΔW-GFP (EF-1α GFP SIN), pHR′ CMV GFP SIN, has previously been documented.[7,12,17-21] The pHR′ GFP MESVltr plasmid containing the murine embryonal stem cell virus (MESV) long terminal repeat (LTR) promoter/enhancer within the lentiviral vector LTR was constructed as follows. First the HIV was cloned into the pSP73 plasmid. The self-inactivating (SIN) deletion was then created by removing the fragment between the EcoRV and PvuII sites. The MESV LTR promoter was then cloned into the SIN-deleted LTR. The GFP gene from Clontech (Palo Alto, CA) was then inserted upstream of the hybrid LTR along with the 5′ LTR and truncated gag portion of the phosphoglycerate kinase (PGK) GPF vector generating the final pHR′ GFP MESVltr plasmid. The pHR′ GFP MESVltr vector virus could be generated via a stable packaging cell line as described by Klages et al.[22] Concentration and titration of all vectors supernatants was performed as previously described.[7]

Purification and transduction of $CD34^+CD38^{lo}$ cells

Umbilical cord blood samples were collected and $CD34^+$ cells isolated as previously described.[7] On the day of transduction, $CD34^+$ cells were thawed and stained using anti-CD34 and anti-CD38 antibodies (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA). $CD34^+CD38^{lo}$ cells (lowest 6% $CD38^{lo}$) were sorted using the FACS Vantage turbo sorter (BDIS). Lentiviral transduction of $CD34^+CD38^{lo}$ cells was performed as previously described[7] with the following exceptions: transduction was performed in a 96-well plate to approximate a similar cell density as the $CD34^+$ cell transductions (ie, $5-20 \times 10^4$ cells/cm^2), using an MOI of 100 TU/cell or a minimum viral concentration of 5×10^6 TU/mL.

NOD/SCID transplant recipients

The NOD/SCID mice were bred, maintained, and irradiated as previously described.[7] Within 24 hours of the irradiation procedure, transplantation of 7000-15 000 transduced or mock-transduced $CD34^+CD38^{lo}$ cells (or 35 000 $CD34^+$ for selected PGK mouse 7.23, see Woods et al[7] for experimental design) with 1 million irradiated (1500 cGy) CD34-depleted, mononuclear cells in 0.5 mL volume of phosphate-buffered saline supplemented with 1% bovine serum albumin was performed via tail-vein injection.

FACS analysis of NOD/SCID bone marrow

NOD/SCID bone marrow cells were then stained with monoclonal antibodies and cells analyzed by fluorescence-activated cell-sorter scanner (FACS)

as previously described.[7] Lineage marker antibodies anti-huCD33, CD15, and CD19 were used to verify positive engrafting mice as having both lymphoid and myeloid cell reconstitution.

PCR analysis of colony-forming units granulocyte–macrophage (CFU-GM) colonies from NOD/SCID mice

Colony plating and picking were performed as previously described,[7] however, PCR was performed using the GFP/lentiviral LTR primer pair (GFP 625F: 5′-CCT GAG CAA AGA CCC CAA CGA GAA-3′, and SIN/ALU1: 5′-GGG TCT GAG GGA TCT CTA GTT ACC A -3′), with a primer annealing temperature of 55°C for 1 minute, an Mg^{+2} concentration of 1.5 mM (Invitrogen AB, Lidingö, Sweden) and 33 cycles run (Peltier Thermal Cycler 200; MJ Research, Watertown, MA).

Semiquantitative PCR analysis of bone marrow from NOD/SCID mice

DNA from NOD/SCID mouse total bone marrow was extracted using Gentra Puregene DNA Isolation Kit (Minneapolis, MN). PCRs for all samples were performed using the following protocol. Platinum Taq DNA Polymerase PCR kit (Invitrogen) was used with a final concentration of $MgCl_2$ of 1.5 mM, 0.4 mM deoxynucleoside triphosphates, and 0.4 μM for each primer. The primers used for scoring human genomic content were based on human β-actin gene sequence, huAktII F 5′ CCC CAG TGT GAC ATG GTG CAT 3′ and R 5′ CGA AGT CCA GGG CGA CGT A 3′ and were specific for human DNA amplification at primer annealing temperature of 65°C. The primers used for scoring vector copy number were based on GFP sequence GFP108 F 5′ GAT GCC ACC TAC GGC AAG CTG AC and GFP629 R 5′ CGC TTC TCG TTG GGG TCT TTG CT and were specific for vector amplification at 62°C. The total DNA per reaction sample was 15-30 ng for the β-actin PCR and 40 to 80 ng for the GFP PCR, both in a total reaction volume of 50 μL. The β-actin reaction cycle number was 32, while the GFP reaction cycle number was 35. The amplified product was electrophoresed on a 1% agarose gel containing ethidium bromide at 0.5 μg/mL. Quantity One version 4.2.1 computer software and digital imaging system from Bio-Rad Laboratories (Hercules, CA) were used to analyze the DNA quantities. The standard curve for amount of human genomic DNA per sample was generated by mixing mouse and human mononuclear cells (MNCs) at varying dilutions followed by DNA extraction. The vector copy standard curve was generated by the serial dilution of DNA from a clonally expanded HeLa cell containing a single elongation factor-1α (EF-1α) SIN vector integrant. The vector copy number per human cell was calculated by dividing the copy number per total genomes (calculated from the standard curve for copy number) by the number of human genomes per total genomes (calculated from the standard curve for quantity of human DNA).

LAM-PCR analysis of mouse 7.23 bone marrow and CFU-GM colonies

DNA from total mouse bone marrow and individually picked CFU-GM colonies from mouse 7.23 was analyzed for vector copy number and integration site location using the high sensitivity linear amplification mediated (LAM)–PCR method.[16] The mouse was selected for LAM-PCR analysis on the basis that it was transplanted in a cell-dose–limiting dilution experiment because it was one of a few mice that engrafted positively, and virtually all the human cells expressed GFP, suggesting repopulation by a single transduced SCID repopulating cell.[7] Due to the variable nature of the efficiency of the LAM-PCR band amplification for each of the distinct integration bands, not all bands were visible in all of the colonies tested. In cases where a particular band size could not be seen in one colony but was present in another colony that shared other common band sizes, a PCR tracking analysis was performed. For this, primers generated from the known integration site sequenced from other colony DNA combined with the previously used LTR primers could be used to screen for the presence of the integration site in the colony by nested or seminested PCR on the DNA from each colony. To prevent the possibility of false bands generated from contaminating cells from an adjacent colony, colonies were picked from
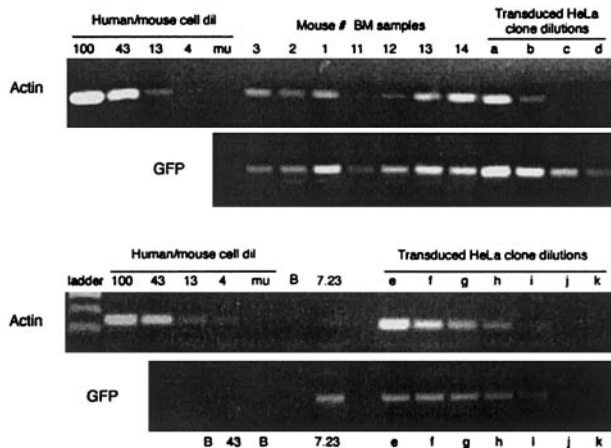
Figure 1. Semiquantitative PCR analysis of NOD/SCID bone marrow cells reveals multiple vector copies per transduced cell. Figure shows agarose gel used to quantify the amount of human DNA (human β-actin) and vector copy number (GFP) per total bone marrow sample. The standard curve for amount of human genomic DNA per sample was generated by mixing mouse and human MNCs at varying dilutions followed by DNA extraction. The well labeled 100 contains 100% human cells followed by 43% human cells and so on. The vector copy standard curve was generated by dilution of DNA from a clonally expanded HeLa cell containing a single EF-1α SIN vector integrant (wells a-d correspond to dilutions containing 300, 100, 30, and 10 pg/μL, respectively, and wells e-k correspond to dilutions of 1500, 900, 300, 100, 30, 10, and 3.3 pg/μL, respectively. The upper panel shows PCR for human β-actin and GFP on mouse bone marrow from cells transduced using the PGK lentiviral vector (mouse nos. 1-3) and EF-1α SIN lentiviral vector (mouse nos. 11-14). The lower panel shows the analysis of bone marrow sample from control mouse 7.23. The copy number values determined from the semiquantitative PCR analysis are shown in Table 1.

methylcellulose plates (35 mm) where mouse bone marrow was plated so that, on average, only 1 CFU-GM colony was present per plate. Furthermore, in order to exclude the possibility of adjacent colony cell contamination, DNA extracted from larger colonies was serially diluted. LAM-PCR analysis revealed the presence of all known vector integration sites in all dilutions provided that the internal control band was still detectable. LAM-PCR also was used to analyze several additional colonies from mice other than 7.23. The colonies derived from these mice were generated from single cell plating of human CD45^{+} CD34^{+} GFP^{+} FACS-sorted cells on TC microwell plates (Nunc, Rochester, NY). The DNA sequence analysis of bands from these colonies was performed as described above.

Results

Experimental design

In this study we asked how many vector copies could be detected following lentiviral vector transduction of NOD/SCID repopulating cells. CD34^{+}CD38^{lo} umbilical cord blood cells were transduced as described in "Materials and methods" and transplanted into NOD/SCID mice. Bone marrow from the mice was analyzed 6 weeks later for proviral vector copy number, and the integration sites were characterized in whole bone marrow and hematopoietic colonies as described below.

Semiquantitative PCR reveals multiple vector copy numbers per transduced NOD/SCID repopulating cell

Using semiquantitative PCR analysis on DNA extracted from the total bone marrow of 12 transplanted NOD/SCID mice, quantities of human DNA and vector number were determined. Analysis was performed on mice transplanted with the PGK, EF-1α SIN, MESVltr, and CMV SIN lentiviral vector-transduced cells. In addition, a mouse whose engraftment and GFP levels had previously been published was selected as a control mouse for analysis based on its unique engraftment profile. This mouse was transplanted in a cell-dose–limiting dilution experiment, and virtually all the human cells expressed GFP, suggesting that it was repopulated by a single transduced SCID repopulating cell.[7] The average vector copy number per GFP-expressing cell for the mice (excluding the selected control mouse 7.23) was 5.6 ± 3.3 vector copies/GFP[+] cell. The analysis of the selected control mouse 7.23 revealed 6.5 vector copies/GFP[+] cell (Figure 1; Table 1). The average for the PGK-transplanted mice was found to be 3.7 ± 1.4 copies/GFP[+] cell, 3.9 ± 1.5 copies/GFP[+] cell for the EF-1α SIN vector, 5.7 ± 4.0 for the MESVltr, and 10.6 and 11.6 copies/GFP[+] cell for the 2 mice transplanted with CMV SIN vector-transduced cells. In order to ensure that the high copy number was the result of multiple copy integrations into a subset of SRCs and not single copies into most cells, standard PCR for the presence of the vector in individually picked CFU-GM colonies derived from the bone

Table 1. Average vector copy number per transduced cell in the bone marrow of NOD/SCID mice as determined by semiquantitative PCR analysis

Mouse no.	hu genomes per 100 total genomes	Vector copies per 100 genomes	Vector copies per human cell	TE FACS (%)	Vector copy no. per GFP+ human cell
PGK vector					
1	18.6	6.17	0.33	16.0	2.1
2	13.5	1.70	0.13	3.0	4.2
3	17.8	1.37	0.08	1.6	4.8
(selected mouse 7.23)	7.1	46.1	6.47	91	6.5
EF-1α SIN vector					
11	8.5	1.09	0.13	4.0	3.2
12	11.3	2.82	0.25	7.7	3.3
13	20.0	6.01	0.30	4.9	6.1
14	26.0	4.32	0.17	5.3	3.1
MESVltr vector					
36	0.4	0.88	2.00	85	2.4
37	2.1	9.96	4.68	90	5.2
38	2.1	4.47	2.16	21	10.3
CMV SIN vector					
47	75.5	129.09	1.71	15.0	11.6
48	3.4	4.35	1.28	12.0	10.6

The results reveal multiple vector copies present per transduced cell, suggesting multiple lentiviral vector copy integration into NOD/SCID repopulating cells (from Figure 1). To calculate the copy number per transduced cell, the vector copy number per human cell was divided by the proportion of GFP^{+} cells as determined by FACS. The average vector copy number per transduced cell and the SD are shown for each vector group. TE indicates transduction efficiency. The average for the PGK vector (excluding selected mouse 7.23) was 3.7 ± 1.4; for the EF-1α SIN vector, 3.9 ± 1.5; for the MESVltr vector, 5.7 ± 4.0; for all groups, 5.6 ± 3.3.
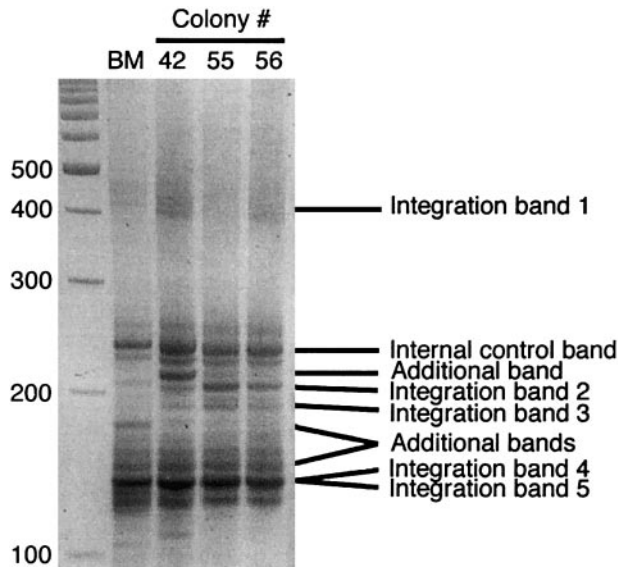
Figure 2. Linear amplification mediated (LAM)-PCR analysis reveals the presence of 5 vector integration sites in individually picked hematopoietic clones from mouse 7.23. Multiple integration sites were detected in each of CFU-GM colonies analyzed, demonstrating the ability of lentiviral vectors to transduce multiple copies of the vector into human repopulating cells. Figure shows spredex gel band analysis following LAM-PCR on DNA from bone marrow and individually picked CFU-GM colonies from mouse 7.23. Similar band profiles could be seen in both the total bone marrow and colony analyses. Bone marrow and colony numbers 42, 55, and 56 are shown. All 5 integration bands are labeled, along with the internal control band, which is a byproduct of the reaction. Due to the sensitivity of the LAM-PCR technique, additional bands were occasionally amplified. Sequencing revealed that these additional bands contained identical sequences to the known integration sequences. However, their difference in size compared to the true integration site band was due to DNA fragment ligation prior to amplification, from the linker cassette ligation procedure. (Integration bands containing either an extra linker cassette or an extra linker cassette along with an additional fragment of human genomic DNA were seen.) All true integration bands contain the 116 base pair (bp) of vector sequence upstream of the vector/human genome junction. Bands < 116 bp were demonstrated to be the result of a recombination between the HIV LTR and the linker cassette and found not to contain human genomic DNA. These results conclusively demonstrate the ability of lentiviral vectors to transduce multiple vector copies into human repopulating cells.

marrow of these mice was performed. These analyses revealed that the transduction efficiency as measured by percentage of PCR-positive colonies was similar to the transduction efficiency as determined by FACS, suggesting that most colonies were negative for transduction, and the subset of SRCs permissive to transduction had multiple vector integrants (data not shown). These results show the ability of lentiviral vectors to transduce human hematopoietic repopulating cells with multiple vector copies integrated into the genomes of repopulating hematopoietic progenitor cells.

LAM-PCR reveals that mouse 7.23 was repopulated by a single SRC that contained 5 integrated lentiviral vector copies

To verify the ability of the lentiviral vector to transduce multiple vector copies into human hematopoietic repopulating cells, high-sensitivity LAM-PCR was used to analyze the mouse 7.23 for all vector integration events. As both the number of vector integration events and the sequence of the vector-human genome junctions could be determined using low quantities of DNA, both the mouse total bone marrow and the CFU-GM colonies could be analyzed using this method. In the total bone marrow, 5 vector integration sites could be distinguished as determined by bands on the gel (Figure 2). A CFU-GM colony derived from the bone marrow of a NOD/SCID mouse describes a clonal situation where all cells in the CFU-GM colony would originate from a single SRC. Colony numbers 42, 55, and 56 demonstrated the presence

of all 5 vector integration sites, demonstrating that the lentiviral vector was capable of transducing multiple vector copies into candidate hematopoietic stem cells. Further analysis of 12 additional colonies from the mouse reveal that 10 of 12 colonies contained all 5 vector integration sites and all 12 colonies contained at least 2 of the known sites (Table 2). These results demonstrate that at the time of harvest, the hematopoietic system of this mouse was reconstituted by a single SRC that contained 5 integrated lentiviral vector copies, confirming the ability of the lentiviral vector to transduce multiple copies into the genomes of SRCs. To further verify the ability of lentiviral vectors to integrate multiple vector copies into the genomes of cells, several additional colonies from mice other than from mouse 7.23 were analyzed by LAM-PCR. Colonies were derived from 3 additional mice, and these were generated from single cell plating of human CD45^{+} CD34^{+} GFP^{+}-sorted cells on terasaki plates. The DNA sequence analysis of all bands from the 3 colonies further demonstrate multiple vector copy integration into SRCs in 2 of these 3 mice with 6, 1, and 3 vector integrants present, respectively.

Integration into a tumor suppressor gene

DNA sequencing of the vector integration bands reveals the precise locations of the vector integrants in the target cell's genome. Interestingly, in mouse 7.23, integration site number 1 is located in a known tumor suppressor gene, breast cancer 1 (*BRCA1*) (Table 3), in which mutations therein are known to be involved in impaired double-stranded DNA repair and associated with an increased proclivity to multiple solid tumor malignancies.[23-25] The proviral vector was found to be integrated into the 17th intron of the *BRCA1* gene.

Table 2. Individually picked CFU-GM colonies analyzed by LAM-PCR reveal this mouse was repopulated by a single repopulating cell that possessed 5 individual vector integrants

Mouse 7.23 Colony number	Integration site no.					hu β-actin
	1	2	3	4	5	
7.23-44	+	+	+	+	+	+
7.23-47	+	+	+	+	+	−
7.23-63	+	+	+	+	(+)	+
7.23-68	+	+	+	+	+	+
7.23-69	+	+	+	+	+	(+)
7.23-85	(+)	+	+	+	−	+
7.23-87	+	+	+	+	+	−
7.23-88	+	+	+	+	+	+
7.23-90	+	+	+	+	+	+
7.23-91	+	+	+	+	+	+
7.23-92	−	−	+	+	−	−
7.23-93	+	+	+	+	+	+
7.23 total bone marrow	+	+	+	+	+	+
Nontransplanted BM	−	−	−	−	−	−
MOCK-transduced colony	−	−	−	−	−	+

The 5 vector/human genome junction bands shown in Figure 2 could be seen in virtually all individually picked CFU-GM colonies derived from the NOD/SCID mouse. The same 5 bands could be detected in the total bone marrow of this mouse. The results of this analysis reveal that this mouse was repopulated by a single SRC that gave rise to virtually all the hematopoietic cells in this mouse. In some cases when a particular band was barely visible, colony DNA was analyzed by PCR tracking, where the known sequences of the vector and specific site in the human genome (Table 3) can be used to generate primers and allow for more sensitive detection of the presence of the band.[16] PCR for human β-actin is used as a control. (+) denotes weak presence of amplified product from PCR tracking analysis.

Table 3. DNA sequence of vector/human genome junctions in the repopulating cell from mouse 7.23

Integrant site no.	PCR fragment size	Genomic size	Genomic sequence	BLAST search
1	391 bp	279 bp	AAGAGCGAGATTCTTGTCTCAAAAAAAAAA...	human BRCA1 (chr 17)
2	205 bp	93 bp	AATAAGTGTATATATACACAA...	human chr 2
3	188 bp	76 bp	AAAGGGCACAAGAGCTAACAT...	human chr 3
4	139 bp	27 bp	GACAGTCCATTTATACTCTTCCAAATT	human chr 16
5	138 bp	26 bp	CAGAGCATCATCTGTGAGTTCTAATT	human chr 22

Sequencing reactions were performed on the 5 vector integration site bands extracted from gel in Figure 2 using a primer from within the vector extending outward into the genomic DNA. Integration site sequences were then compared to known genomic sequence databases by Basic Local Alignment Search Tool (BLAST) search to determine the precise location of the vector integrant in the human genome. Interestingly, one vector sequence showed a vector had integrated into intron 17 of a known tumor-suppressor gene, BRCA1.

Discussion

The goal of this study was to assess the ability of lentiviral vectors to transduce primitive human repopulating cells. For this, we lentivirally transduced human CD34+CD38lo cells and transplanted these into NOD/SCID mice. For the first time, we show that lentiviral vectors are capable and regularly transduce multiple vector copies into the genomes of NOD/SCID repopulating cells. Semiquantitative PCR analysis on mouse bone marrow revealed that the average lentiviral vector copy number per GFP-expressing cell in the progeny of SRCs is approximately 5.6 ± 3.3 (range, 2.1-11.6) copies/GFP+ cell. Multiple vector copies could be detected in the progeny of CD34+CD38lo SRCs, suggesting that the occurrence of multiple-vector integration was not a rare event, given the high MOI and low cytokine stimulatory conditions used during the transduction. This finding was further verified and confirmed using the highly sensitive LAM-PCR technique, which determined the precise vector integration sites in hematopoietic clones from the bone marrow of NOD/SCID mouse 7.23 from a previous cell-dose–limiting dilution experiment.[7] This mouse, which showed GFP expression in virtually all human cells, revealed upon LAM-PCR analysis that the single SRC contributing to hematopoiesis at the time of harvest had 5 separate vector integration sites, proven by the fact that all vector integrants were seen in virtually all the hematopoietic clones analyzed. The ability of lentiviral vectors to transduce a subpopulation of CD34+CD38lo SRCs (ie, 22% of the total transplanted SRCs) demonstrates that most of the SRCs are impervious to transduction despite the high viral particle numbers used during the transduction. Factors that may block the transduction of the remaining SRCs may be at the level of receptor binding and viral uptake, reverse transcription, or integration, which may be related to the inability of lentiviral vectors to transduce cells in the G_0 stage of the cell cycle as opposed to cells in G_1.

The novel finding that the lentiviral vectors transduce multiple vector copies into SRCs may have implications for the safety of lentiviral vectors for use in gene therapy applications as the risk for insertional mutagenesis is increased. A recent article provided proof of principle that in some rare occasions one random integration event by an oncoretroviral vector can lead to an insertional mutagenesis event characterized by a severe myeloid leukemia causing abnormal hematopoiesis in all secondary transplant recipient mice and lethality in all tertiary recipients.[26]

Because the theoretical chances of activation of a proto-oncogene are small (10^{-7}),[27] it is a striking coincidence that the only mouse analyzed by the highly sensitive integration site-specific technique in this present study demonstrates that on one occasion the vector had integrated into the 17th intron of a known tumor-suppressor gene, BRCA1, most notably known to be involved in breast and ovarian cancers[25] and DNA damage repair.[23] Although no unusual phenotype was apparent at the time the mice were humanely killed, most leukemias require a minimum time for a leukemic phenotype to develop, well in of excess of the 6 weeks as performed here. In addition, in order for its oncogenic properties to be manifested, BRCA1 must also be accompanied by at least one other mutation.[25] The recent finding that integration of the HIV-1 virus preferentially occurs in active regions of the genome further potentiates the risk for insertional mutagenesis if one assumes that lentiviral vectors integrate into similar sites as the wild-type HIV-1.[28] It has been previously shown that lentiviral vectors are capable of multiple vector copy integration in embryonic stem cells when transduced at high MOI and that the number of vector copies per cell can be reduced by reducing the MOI[14,29] and suggests that a reduction in vector copy in lentivirally transduced hematopoietic cells may be possible using lower viral titers during the transduction. Our findings show that lentiviral vectors are capable of integrating multiple vector copies into the genomes of primitive human repopulating cells, and this warrants development of transduction methods that yield efficient transduction with fewer copies per cell.

Acknowledgments

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