**In Vivo Transduction of Hematopoietic Stem Cells after Neonatal Intravenous Injection of an Amphotropic Retroviral Vector in Mice**

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Hematopoietic stem cells (HSC) are important targets for gene therapy. Most protocols involve *ex vivo* modification, in which HSC are transduced *in vitro* and injected into the recipient. An *in vivo* delivery method might simplify HSC gene therapy. We previously demonstrated that iv injection of an amphotropic retroviral vector (RV) into newborn mice resulted in long-term expression from hepatocytes. The goal of this study was to determine if HSC were also transduced. After neonatal administration of \( \frac{1}{10} \times 10^{10} \) transducing units/kg of RV, peripheral blood cells had \( \sim 0.1 \) copy of RV per cell for up to 22 months. At 18 months, RV sequences were detected in T, B, and myeloid cells from bone marrow (BM). Unfractionated BM was transplanted into naive recipients after total body irradiation. Recipients maintained similar levels of the RV in their blood cells for 10 months, at which time RV sequences were present at the same integration site in all lineages of cells from BM. We conclude that neonatal iv injection of RV results in transduction of HSC in mice, which might be used for BM-directed gene therapy. Transduction of blood cells after liver-directed neonatal gene therapy might have adverse effects in patients, although no leukemias developed here.

**Key Words:** amphotropic, retroviral vector, hematopoietic stem cell, neonatal gene therapy, LAM-PCR, clonal marking

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**INTRODUCTION**

Hematopoietic stem cells (HSC) are an attractive target for gene therapy, as their modification might result in long-term correction of blood diseases [1–3]. Indeed, patients with severe combined immunodeficiency due to deficiencies of the common \( \gamma \) chain for cytokine receptors [4] or adenosine deaminase [5] have achieved improvement in immune function after gene therapy with a retroviral vector (RV) derived from the Moloney murine leukemia virus (MLV). Most studies on gene therapy for HSC have involved *ex vivo* modification, in which HSC are obtained from blood or bone marrow (BM), transduced *in vitro*, and then reintroduced into the same or a congenic animal or patient. There are two potential disadvantages to this approach. First, this involves isolation and culture of HSC, which is laborious and carries some risk of contamination with infectious agents. Second, *in vitro* culture of HSC can reduce their ability to reconstitute blood cells long term, which may involve a decrease in self-renewing capacity or homing efficiency [6–15].

A method for efficient *in vivo* delivery of RV to HSC might avoid some of the adverse effects of *ex vivo* gene therapy. Indeed, it was recently demonstrated that direct intrafemoral injection of packaging cells into adult rabbits [16] or intrafemoral injection of RV into adult mice [17,18] or neonatal sheep [19] resulted in modification of HSC. Similarly, intraperitoneal injection into fetal sheep resulted in transduction of HSC [20], while *in utero* intraperitoneal or intrahepatic injection of an amphotropic MLV-based RV resulted in transduction of blood cells from rhesus monkeys, although a VSV-G-pseudotyped RV of the lentiviral class was about threefold more...
efficient [21]. Finally, iv injection of an RV derived from a lentivirus transduced blood cells in the short term, although long-term contribution to hematopoiesis was not examined [22].

We have recently demonstrated that neonatal administration of an amphotropic MLV-based RV resulted in efficient transduction of hepatocytes [23,24], which results in long-term expression in the liver. We demonstrate here that this gene therapy approach also results in transduction of pluripotent HSC in mice. This simple approach might therefore be used to transduce blood cells for the purpose of gene therapy. On the other hand, transduction of blood cells is a potential adverse effect of this neonatal liver-directed gene therapy approach that should be considered for clinical trials.

RESULTS

DNA Copy Number in Blood Cells from Hemophilia B Mice

We previously reported that iv injection of $1 \times 10^{10}$ transducing units (TU)/kg of an RV expressing canine factor IX (cFIX) resulted in efficient transduction of liver cells from hemophilia B mice and stable expression of cFIX at 7 μg/ml (140% of normal) [24]. However, it was unclear if blood cells were transduced with this approach. We therefore isolated DNA from peripheral blood cells at 6 months after transduction and tested it by TaqMan real-time PCR using primers specific for the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) of the RV, with normalization for genomic DNA levels using primers specific for mouse β-actin. All mice had detectable copies of RV, which varied from 0.05 to 0.2 copy per cell, with an average of 0.10 ± 0.01 [standard error of the mean (SEM)] copy/cell, as shown in Fig. 1. RV DNA sequences were undetectable in DNA from blood cells of two nontransduced hemophilia B mice that was isolated at the same time as the other samples (data not shown). DNA from blood cells of some of these animals was also analyzed at 22 months after transduction, at which time the DNA copy number was similar at 0.11 ± 0.02 copy per cell. We conclude that neonatal iv injection of RV results in moderate copies of RV in the blood cells, which were stable for almost 2 years.

Secondary Transplant

One criterion for transduction of HSC is achieving long-term engraftment with transduced cells in a naïve recipient after bone marrow transplant (BMT). We therefore performed neonatal transduction in phenotypically normal mice from the mucopolysaccharidosis (MPS) VII colony, which can be transplanted into MPS VII [α-glucuronidase (GUSB)-deficient] mice of the colony. The degree of engraftment can then be assessed by determining the percentage of blood cells that express GUSB in a histochemical stain. Five normal C57BL/6 mice were injected with hAAT-cFIX-WPRE shortly after birth. They achieved a stable level of cFIX in blood, which was 7.7 μg/ml (154%) at 15 months. Blood cells had 0.10 ± 0.04 copy of RV DNA per cell, which was similar to that observed in hemophilia B mice. We isolated BM cells at 18 months after birth from these mice and compared the RV DNA copy number in total BM with that in liver and spleen, as shown in Fig. 2. For all donors, the highest copy number was observed in liver, which ranged from 0.4 to 5.8 copies/cell and averaged 2 ± 1 (SEM) copies/cell. This was 17- to 22-fold higher than the copy number in spleen (0.12 ± 0.03 copy/cell) and total BM (0.092 ± 0.034 copy/cell). We conclude that the liver is the organ that is most efficiently transduced with this approach, but spleen and BM have substantial copies as well.

We injected BM cells from these RV-transduced mice into irradiated MPS VII mice of the same colony. GUSB staining of peripheral blood cells and BM cells of recipients at 6 and 10 months after BMT, respectively, demonstrated that all recipients achieved >90% engraftment (data not shown). We analyzed blood cells from the BM recipients for the RV DNA copy number by real-time PCR at several times after BMT, and these values were compared with the copy number in blood cells of the donor, as shown in Fig. 3. Donor 1 (D1) had 0.2 copy per cell in

![Blood Cells of Hemophilia B Mice](Image)
blood cells at 15 months after transduction. His recipients (R1A and R1B) had somewhat lower numbers of copies per cell at 0.02 to 0.04, but these were maintained for 10 months after BMT in one animal (the other animal died at 6 months from skin problems). Donor 2 (D2) had a similar copy number of 0.23 copy of RV DNA per cell in blood cells at 15 months after birth. All three of his recipients (R2A, R2B, and R2C) maintained high copies of the DNA in blood cells for 10 months after BMT. Similarly, transplantation of cells from D3, D4, or D5 resulted in the appearance of RV sequences in blood cells of the six recipients altogether that were evaluated. Maintenance of RV DNA sequences in blood cells for a total of 28 months after transduction is consistent with the hypothesis that HSC were transduced after the iv injection of RV in the newborn period.

Assessment of the RV Marking Efficiency in Cells of Different Lineages

Another criterion for transduction of a pluripotent HSC is that it can generate cells of different lineages. We therefore fractionated some of the cells isolated from BM of the donors at 18 months after transduction according to their lineage. Similarly, we fractionated BM obtained from the recipients of BMT at 10 months after transplantation. Examples of the fluorescence-activated cell sorting (FACS) for the recipient R2A are shown in Figs. 4A, 4C, and 4E. The average percentage of cells of each lineage in all recipients was 62.1 ± 1.7% (SEM) for myeloid cells (Gr-1+/Mac1+), 10.1 ± 0.8% for B cells (B220+), and 8.1 ± 0.7% for T cells (CD3+). We reanalyzed sorted cells from each lineage by FACS, as shown for R2A in Figs. 4B, 4D, and 4F. On average, 96.9 ± 0.6, 95.6 ± 0.2, and 92.4 ± 0.5% of cells that were collected after sorting for myeloid, B, or T cells, respectively, were of the correct lineage.

We tested DNA from these fractionated BM cells for RV DNA sequences by real-time PCR, as shown in Figs. 4G to 4J. Donor 1 had 0.13, 0.14, 0.28, and 0.17 copy per cell in total BM, myeloid, B, and T cells, respectively. These values are consistent with the DNA copy number of 0.20 copy per cell in his peripheral blood. DNA was detectable in cells of all lineages from BM for one of the recipients of Donor 1 (R1B), although the levels were only 10 to 50% of the levels found in cells of the same lineage from D1. This may represent relatively inefficient engraft-
ment of the transduced clone(s) in the recipient of D1. We did not analyze BM cells from the second recipient, R1A. Similarly, D2 had detectable copies of DNA in total BM, myeloid, and B cells, although there was insufficient DNA for analysis of T cells. DNA was detectable in cells of all lineages in all three of the recipients of Donor 2. Similarly, D3 and both of his recipients (R3A and R3B) had detectable DNA sequences in cells of all lineages. The recipients of D5 differed in that RV DNA sequences were undetectable in myeloid cells of both mice, although DNA sequences were detectable in total BM, B, and T cells. This may represent a failure to transduce a pluripotent stem cell in this donor, the failure of the transduced pluripotent HSC to engraft and contribute to hematopoiesis in these recipients, or the relatively low sensitivity of the real-time PCR assay. An insufficient amount of DNA was available from several of the samples from D4 and his recipients, so these data are not shown. We conclude that cells from all lineages contain RV DNA sequences for recipients of three different donors at 28 months after the initial transduction of the donor.

Identification of Integration Sites for D2 and His Recipients

A final criterion for transduction of a pluripotent stem cell is that cells of different lineages contain the same RV integration site. To identify integration sites, we subjected DNA obtained from the BM of the donor with the highest RV DNA copy number (D2) and the blood of his recipients (R2A, R2B, and R2C) to ligation amplification-mediated PCR (LAM-PCR) and analyzed the samples on a polyacrylamide gel (Fig. 5). DNA from D2 resulted in a major band at 227 bp, which is the size expected for a sequence derived from the 3′ V LTR and adjacent upstream sequence of the RV. The failure to identify any other major bands may be due to the fact that DNA from the BM of D2 contained many different integration sites, none of which were dominant. LAM-PCR products of DNA from the recipients contained the same 227-nt band from the 3′ V LTR as well as minor bands. To identify the integration sites, we cloned DNA from the LAM-PCR. The fragments with letters in Fig. 5 represent LAM-PCR fragments that derived from amplification of the 5′ LTR and mouse genomic sequence. Integration A represents insertion into an intergenic region on chromosome 3 that is 30 kb away from the guanylate cyclase 1 soluble α3 gene (100% identical with nt 2743366 to 2743075 of Mm3_78449_32). Integration B represents insertion into an intergenic sequence on chromosome 3 that is 25 kb away from the guanylate cyclase 1 soluble gene (100% identical with nt 2743366 to 2743075 of Mm3_78449_32). Integration C represents insertion 0.5 kb upstream of the peptidylprolyl isomerase A (100% identical to nt 758916 to 758981 of Mm6_39399_32). Clone C from R2B was identical to clone C from R2C but was truncated for unclear reasons.

FIG. 4. Evaluation of the DNA copy number in BM cells of different lineages in the donors and their recipients. (A–F) Examples of FACS to obtain cells of different lineages. The left side (A, C, and E) shows the cells that were collected by FACS from recipient R2A during sorting using antibodies that recognize Gr-1 and Mac-1 (A, myeloid cells), B220 (C, B cells), and CD3 (E, T cells). The right side (B, D, and F) shows the analysis of the sorted fractions from R2A to demonstrate their purity. The box shows the regions that were collected or found to be positive for the postsort samples. The numbers represent the percentages of cells whose values fell within the box. (G–J) Comparison of DNA copy number in BM cells of different lineages between donors and recipients. Cells from donors were sorted in a fashion similar to that shown for the recipients except an antibody directed against the T cell receptor was used to sort for T cells. DNA from the sorted populations was used to determine the RV DNA copy number. Results from each donor and its recipient(s) are shown in separate graphs. (G) Donor 1 (D1). DNA copy numbers in D1 and one recipient (R1B) are shown for the indicated cell types. (H) Donor 2 (D2). DNA copy numbers in D2 and three recipients are shown. There was insufficient DNA for analysis of the DNA copy number in T cells of D2. (I) Donor 3 (D3). DNA copy numbers in D3 and two recipients are shown. (J) Donor 5 (D5). DNA copy numbers in D5 and two recipients are shown. Both RSA and RSB had undetectable copies per cell in myeloid cells, as indicated by a bar up to 0.0015 copies per cell. There was insufficient DNA available for analysis of the T cells from D3, which appears without a bar. For D4, several samples had insufficient DNA for analysis, and none of the values are shown.
We used the insertion sites to design primers that would specifically amplify DNA from a particular integration site. DNA from total BM of R2B was used to assess the sensitivity of each PCR, as shown in Fig. 5C. For integrations A, B, and C, 3, 100, and 30 ng of DNA from total BM, respectively, was required to see a signal. We then performed PCR on samples from blood or BM of D2 and his recipients, R2A, R2B, and R2C, as shown in Fig. 5D. To demonstrate that the PCR was specific for the integration site, we tested all primer sets with DNA from blood or total BM of a different donor (D1) and a recipient from still another donor (R3B). No PCR products were identified in these negative controls for any of the primer sets, demonstrating that the PCR is specific for that particular integration site.

Integration A was present in DNA from cells of all lineages for all recipients of D2, although the copy number was relatively low for the myeloid cells from R2C. This suggests that all three of the recipients received the same pluripotent stem cell, which contributed to cells of all lineages. A caveat here is that the PCR for integration A was quite sensitive (Fig. 5C) and some sorted fractions were only 92% pure. This makes it possible that a signal in the sorted populations was due to the low level of contamination with other cell types. Similarly, we detected integration site C in cells of all lineages from R2A and R2B, although this integration site was not detected in myeloid cells of R2C. This suggests that a pluripotent HSC with integration C was transplanted into all recipients, although the HSC may have stopped contributing to hematopoiesis in R2C, or the clone may be present in insufficient copies in myeloid cells for detection. We detected integration B in cells of all lineages that we evaluated for D2, although there was insufficient DNA for evaluation of T cells in D2. Integration B was also present in B cells of all recipients and in T cells of R2A and R2B. However, it was not detected in T cells of R2C or in myeloid cells of any of the recipients. This is consistent with the hypothesis that this clone ceased to contribute to pluripotent hematopoiesis in the recipients. We conclude that cells of all lineages can contain the same integration site, which suggests that this method of RV delivery results in transduction of pluripotent HSC.

Although all three of the integration sites that were analyzed were present in all three recipients of BM from D2 as assessed by PCR using primers specific for the integration site, LAM-PCR identified the sequence in only one of the three recipients for two of the clones and in two of the three recipients for the third clone. Since the LAM-PCR does not reliably identify all the clones in our hands, it is likely that additional integration sites that were not identified with LAM-PCR were present.

**DISCUSSION**

We demonstrate here that a simple iv injection of an amphotropic MLV-based RV into newborn mice results in a DNA copy number of ~0.10, which is maintained for up to 22 months. Further, transplantation of BM cells obtained at 18 months after transduction into congenic recipients resulted in similar copies of RV in blood cells for 10 months, which represents a total of
28 months during which blood cells remained positive. Finally, the same integration site was present in cells of all lineages in BM cells from the recipients of BMT for some of the integration sites that were analyzed. We conclude that this gene therapy approach results in transduction of pluripotent HSC in mice. Our results are consistent with the fact that neonatal administration of a VSV-G-pseudotyped MLV-based RV [25] or a lentiviral RV [26] resulted in transduction of spleen cells for at least 9 months. This may have been due to the contribution of BM-derived cells, although BM and blood cells were not specifically examined in these studies. Intravenous injection of an RV of the lentiviral class into adults may also result in transduction of HSC, as BM cells contained ~0.2 copies per cell when analyzed 40 days later [22]. However, since long-term evaluation was not performed, it is not yet clear if this represents transduction of HSC or of progenitor cells.

The efficiency of transduction of blood cells in this study was somewhat surprising, as in vivo delivery systems do not allow prolonged contact of RV with cells. Furthermore, levels of the amphotropic receptor are low in cultured HSC, and MLV-based RV with the amphotropic envelope are less efficient at transducing murine HSC in vitro than are MLV-based vectors with other envelope proteins [27,28]. It is possible that the transduction would have been even higher with an RV containing...
other envelope proteins or that the process of isolation and culture of HSC alters the expression of the amphotropic receptor. It is possible that the relatively high transduction efficiency relates to the fact that hematopoiesis occurs in the liver at birth in mice and the RV has good access to HSC due to the high rate of blood flow to the liver.

Recipients were also tested for expression of the RV. Although the internal human α1-anti-trypsin promoter drives expression in the liver, the LTR of the RV can direct expression in nonhepatic cells (see Fig. 5A). However, the cF IX protein was undetectable in plasma (<1 ng/ml) at 2 and 6 months after BMT (data not shown), which suggests that the RV was not expressed efficiently from blood cells at 20 and 24 months, respectively, after the initial transduction. Consistent with this result, RV RNA levels in BM were low at 10 months after BMT (28 months after transduction) in the recipients, at 0.4% of that in liver, as assessed by reverse transcriptase real-time PCR (data not shown). We conclude that the RV did not express well from the blood cells at this very late time after transduction, which is consistent with the fact that the MLV LTR can shut off in hematopoietic cells in mice [29,30]. However, we recently analyzed BM at 6 weeks after iv injection of a related RV (hAAT-cGUS-WPRE [34]) into newborn MPS VII mice. We found that RNA levels in BM were 2.5-fold the levels found in liver, suggesting that expression in BM cells is quite high in the early period after transduction. This suggests that this gene therapy approach might result in sufficient expression of a therapeutic protein in BM-derived cells if a promoter that does not shut off over time is used.

An in vivo delivery method of an RV for gene therapy has potential advantages. One is that it would be simple to deliver, as it would avoid the need for HSC harvesting, in vitro culture, and reinfusion. In addition, this might obviate the fact that ex vivo culture can reduce the ability of HSC to engraft and result in production of cells of all lineages. Our results in mice are consistent with the finding that iv injection of an RV results in stable transduction of blood cells at a somewhat lower copy number (~0.02 copies per cell) for over 3 years in dogs (T. O’Malley and M. Haskins, unpublished data). Thus, it is possible that an iv injection in the neonatal period might be used for BM-directed gene therapy. However, further studies will need to address the efficiency of this approach in other species and ultimately in humans, which might differ due to difficulties in scaling up to larger animals, differences in stem cell cycling status, or the amount of hematopoiesis in liver vs bone marrow at birth. In addition, the efficiency of this approach will need to be addressed in adults, which have little hematopoiesis in the liver.

This demonstration that iv injection of an RV results in stable transduction of HSC has potential disadvantages. In particular, HSC transduction would not be necessary for most liver-directed gene therapy approaches and might increase the risk of an adverse effect. Indeed, it was recently demonstrated that insertional mutagenesis contributed to the leukemias that developed in two patients with severe combined immunodeficiency due to mutation of the common γ chain who received gene therapy with an MLV-based RV [31]. Although none of the animals that were evaluated here had evidence of leukemia (data not shown), this potential risk will need to be considered if a neonatal iv administration of an RV is being considered for gene therapy.
REFERENCES