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Article **Hematology**

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High-level β-globin expression and preferred intragenic integration after lentiviral transduction of human cord blood stem cells

Suzan Imren,[1] Mary E. Fabry,[2] Karen A. Westerman,[3] Robert Pawliuk,[3] Patrick Tang,[1] Patricia M. Rosten,[1] Ronald L. Nagel,[2,4] Philippe Leboulch,[3,5] Connie J. Eaves,[1,6] and R. Keith Humphries[1,7]

[1]Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, British Columbia, Canada. [2]Department of Medicine (Division of Hematology) and Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York, USA. [3]Genetics Division, Department of Medicine and Harvard Medical School, Brigham and Women's Hospital, Harvard New Research Building, Boston, Massachusetts, USA. [4]Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York, USA. [5]Department of Medicine and Harvard Medical School, Brigham and Women's Hospital, Boston, Massachusetts, USA. [6]Departments of Medical Genetics and [7]Medicine, University of British Columbia, Vancouver, British Columbia, Canada.

Transplantation of genetically corrected autologous hematopoietic stem cells is an attractive approach for the cure of sickle-cell disease and β-thalassemia. Here, we infected human cord blood cells with a self-inactivating lentiviral vector encoding an anti-sickling $\beta^{A\text{-}T87Q}$-globin transgene and analyzed the transduced progeny produced over a 6-month period after transplantation of the infected cells directly into sublethally irradiated NOD/LtSz-*scid/scid* mice. Approximately half of the human erythroid and myeloid progenitors regenerated in the mice containing the transgene, and erythroid cells derived in vitro from these in vivo–regenerated cells produced high levels of $\beta^{A\text{-}T87Q}$-globin protein. Linker-mediated PCR analysis identified multiple transgene-positive clones in all mice analyzed with 2.1 ± 0.1 integrated proviral copies per cell. Genomic sequencing of vector-containing fragments showed that 86% of the proviral inserts had occurred within genes, including several genes implicated in human leukemia. These findings indicate effective transduction of very primitive human cord blood cells with a candidate therapeutic lentiviral vector resulting in the long-term and robust, erythroid-specific production of therapeutically relevant levels of β-globin protein. However, the frequency of proviral integration within genes that regulate hematopoiesis points to a need for additional safety modifications.

Introduction

Sickle-cell disease (SCD) and β-thalassemia are life-threatening inherited disorders of red cell malfunction of major worldwide importance. Both are caused by alterations to the β-globin gene, which is required for normal hemoglobin production in adults. Currently, allogeneic bone marrow transplantation is the only therapy that can produce permanent cures in patients with these disorders (1, 2). However, this approach is limited by lack of suitably matched donors and the significant morbidity and mortality associated with the treatment.

Transplantation of genetically corrected autologous hematopoietic stem cells (HSCs) could circumvent both of these drawbacks, and the development of this approach has therefore been a goal of basic and preclinical studies for many years. These investigations have led to a number of important advances including the identification of the locus control region (LCR) of the β-globin gene cluster (3–6) and the demonstration of the importance of specific elements within the LCR in promoting high-level, erythroid-specific, and position-independent expression of the β-globin gene (7–10).

Efforts to incorporate these LCR elements into oncoretroviral vectors have yielded some promising results in both mouse (11–14) and human targets (15, 16). However, the generation of high titers of intact oncoretroviral vectors encoding sufficient portions of the LCR to achieve maximal β-globin transgene expression in primary cells has proven difficult (11, 15–20). In addition, obtaining clinically relevant yields of transduced HSCs remains a challenge with these vectors. This is due to the decline in HSC activity that occurs during the several days of growth factor stimulation in culture required for their efficient transduction (21–23).

Lentiviral vectors can also infect primitive primary human hematopoietic cells at high efficiencies (50% and more) but with much shorter in vitro–transduction protocols (24–32). Also, the rev-responsive element in the vector facilitates the export of unspliced transcripts into the cytoplasm, thereby promoting higher protein expression (33). Lentiviral vectors may also be less prone to silencing (34, 35). Thus, a number of groups have been evaluating the potential of lentiviral vectors as potentially superior vehicles for globin gene transfer applications (36–41). We have focused on the development of a lentiviral vector containing an anti-sickling human $\beta^{A\text{-}T87Q}$-globin expression cassette that includes a 2.7-kb region of the LCR (comprising the DNase I–hypersensitive 2, 3, and 4 elements) (37, 38). Evaluations of the therapeutic utility of this vector in murine models of SCD (37) and β-thalassemia (38) have, in both cases, demonstrated pancellular expression of

Nonstandard abbreviations used: BFU-E, burst forming unit–erythroid; Epo, erythropoietin; FL, Flt-3 ligand; HSC, hematopoietic stem cell; LCR, locus control region; LM-PCR, linker-mediated PCR; LTR, long terminal repeat; NOD/SCID, NOD/LtSz-*scid/scid*; NOD/SCID-*nu/nu*, NOD/LtSz-*scid/scid nu/nu*; SCD, sickle-cell disease; SF, Steel factor; TAE, Tris-acetate EDTA; Tpo, thrombopoietin.

Conflict of interest: C.J. Eaves and R.K. Humphries are paid consultants of StemCell Technologies Inc., suppliers of some of the culture reagents used in this study; R. Pawliuk, K.A. Westerman, and P. Leboulch are officers of Genetix Pharmaceutical Inc.

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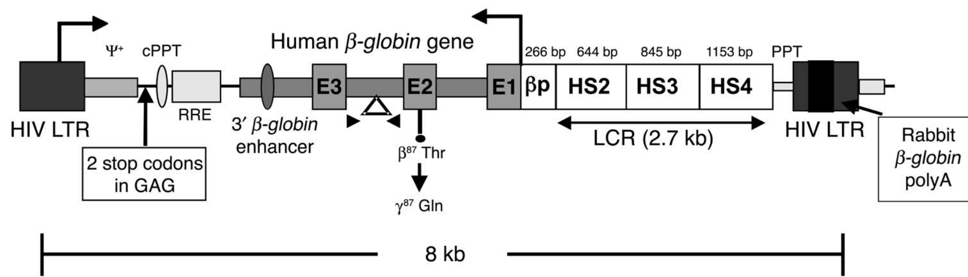

Figure 1
Schematic diagram of the self-inactivating $\beta^{A\text{-}T87Q}$-globin lentiviral vector used in this study. Features include the HIV LTR; the packaging signal (ψ^+); the central polypurine tract (cPPT); the rev-responsive element (RRE); the β-*globin* promoter, from *Sna*BI to the cap site (βp); the 3′ β-*globin* enhancer (to the downstream *Avr*II site); the 372-bp IVS2 deletion (indicated by the triangle); the $\beta^{A\text{-}T87Q}$ mutation (ACA Thr [β^{87} Thr] to CAG Gln [γ^{87} Gln]); and the DNase I–hypersensitive sites, *Sma*I to *Xba*I (HS2), *Sac*I to *Pvu*II (HS3), and *Stu*I to *Spe*I (HS4) of the β-*globin* LCR; 2 stop codons in the ψ^+ packaging signal; the 400-bp deletion in U3 of the right-hand HIV LTR; and the rabbit β-*globin* polyA signal. The locations of the $\beta^{A\text{-}T87Q}$-globin transgene-specific primers in exons 2 and 3 (E2, E3) are indicated by arrowheads.

the transduced β-globin gene with nearly complete correction of the disease phenotype in recipients of transduced HSCs containing an average of 3 proviral integrations per cell.

The present study was undertaken to test the ability of this vector to transduce HSCs of human origin and to measure the level of β-globin transgene expression achieved in their erythroid progeny. The results show efficient transduction of HSCs in normal cord blood as detected by their ability to repopulate sublethally irradiated immunodeficient (NOD/LtSz-*scid/scid*, or NOD/SCID) mice for at least 6 months. In addition, production of clinically relevant levels of β-globin protein in the erythroid progeny of the transduced HSCs has been demonstrated. Additional evidence of the preferred intragenic integration of such vectors has also been obtained including insertions into genes implicated in human leukemia.

Results

Efficient lentiviral transfer of a $\beta^{A\text{-}T87Q}$-globin transgene into primitive human cord blood cells. As an initial test of the ability of our safety-modified $\beta^{A\text{-}T87Q}$-globin lentivirus (Figure 1) to transduce primitive human hematopoietic cells, the proportion of vector-containing hematopoietic colonies was determined on cells plated immediately after a 16-hour exposure of a $CD34^+$ cell–enriched cord blood cell suspension to virus following a prior 48-hour period of stimulation in vitro with the following growth factors alone: Flt-3 ligand (FL), Steel factor (SF), thrombopoietin (Tpo), and hyper–IL-6 (hIL-6). PCR analysis of individual colonies (both granulopoietic and erythroid) using primer pairs specific for the $\beta^{A\text{-}T87Q}$-globin transgene and for GAPDH showed that the DNA from 15 of 40 $GAPDH^+$ colonies analyzed (38%) also contained the $\beta^{A\text{-}T87Q}$-globin transgene. To determine the efficiency of transducing more primitive cells with multiple-lineage in vivo–repopulating ability, 26 NOD/SCID and 3 NOD/LtSz-*scid/scid nu/nu* (NOD/SCID-*nu/nu*) mice were transplanted with infected cord blood cells. In these experiments, $CD34^+$ cell–enriched suspensions (n = 4; including experiments 1 and 2) or $CD34^+CD38^-$ cells isolated by FACS (n = 1) were first stimulated with growth factors for 18 hours, exposed to $\beta^{A\text{-}T87Q}$-globin lentivirus for the next 6 hours, and then injected into the mice. FACS analysis of serial femoral marrow aspirates obtained from these mice confirmed that the transplants had regenerated persistent populations of hematopoietic ($CD45/71^+$) cells. Figure 2A shows representative data from two experiments. The variation in the number of human cells regenerated in these two experiments

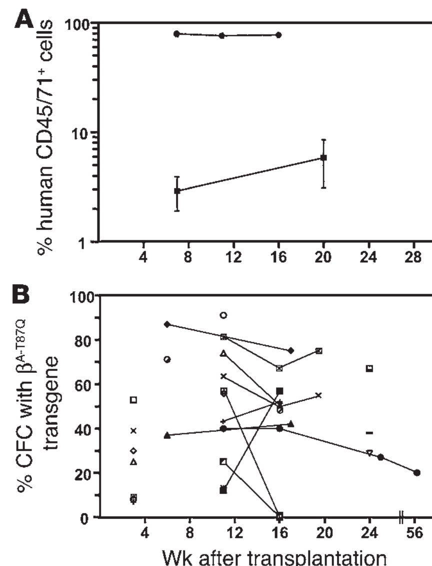

Figure 2
Assessment of 16 NOD/SCID mice and 3 NOD/SCID-*nu/nu* mice transplanted with $\beta^{A\text{-}T87Q}$-globin lentivirus-infected cord blood cells. (**A**) Time course studies of the total number of human hematopoietic ($CD45/71^+$) cells generated in two representative experiments. Values are the mean ± SEM for 8 mice in experiment 1 (filled squares) and 11 mice in experiment 2 (filled circles). Of all cells present in the marrow in experiment 1, 20 weeks after transplant, 4% ± 2% were B-lymphoid ($CD19/20^+$) cells and 0.7% ± 0.4% were mature granulopoietic ($CD15^+$) cells; of all cells present in the marrow in experiment 2, 16 weeks after transplant, 63% ± 4% were B-lymphoid cells and 7% ± 1% were mature granulopoietic cells. (**B**) PCR analysis to detect provirus-positive hematopoietic colonies produced by human CFCs isolated from the marrow of individual mice (n = 18) at different time points after transplant in a total of five experiments. Values determined at 3 weeks after transplant are not connected to the values determined at later time points because the cells present at these two different times are thought to be derived from different types of repopulating cells (43). The overall proportion of colonies with the $\beta^{A\text{-}T87Q}$-globin transgene in all 17 recipients analyzed 11–24 weeks after transplant was 45% ± 6% (range 12%–91%).

Figure 3

Lineage-specific expression of human $\beta^{A\text{-}T87Q}$ -globin protein in human erythroid cells generated in vitro. (**A**) Photomicrograph of Wright-Giemsa–stained slide preparation of cells from erythroid suspension cultures initiated 14 days previously with cells obtained immediately after exposure to lentivirus. (**B**) FACS analysis of the cells shown in **A** after staining with antibodies specific to human glycophorin-A. (**C**) Representative HPLC profile of a cell lysate obtained from a 14-day culture of cord blood cells set up immediately after infection. Peaks representing differently eluting β-globin proteins are indicated. (**D**) Isolation by FACS of human $CD45/71^+$ cells from bone marrow (BM) aspirates of transplanted mice to initiate CFC or suspension cultures that support terminal human erythroid cell differentiation. (**E**) Representative HPLC profile of a cell lysate from a 14-day suspension culture of human erythroid cells initiated with cells obtained from a mouse that received a transplant of transduced cord blood cells 11 weeks previously. (**F**) RT-PCR analysis of RNA extracted from cells shown in **A** (right panels) and human $CD19/20^+$ B-lymphoid cells isolated by FACS (left panels) from a marrow sample of a repopulated mouse 19 weeks after transplant. The $\beta^{A\text{-}T87Q}$-globin transcript was detected in erythroblasts even when diluted 32 times (top right panel), whereas no signal was seen in the extract of B-lymphoid cells (top left panel). Triangles indicate decreasing concentration.

is probably due to differences in the number of $CD34^+$ cells transplanted per mouse (1.65×10^5 versus 4.0×10^5) and also to differences in frequency of repopulating cells in the two pools of cord blood used. As expected (42), by 16–20 weeks after the transplant, the regenerated human cells were predominantly B-lymphoid ($CD19/20^+$), with a minority of mature granulopoietic ($CD15^+$) elements, in almost all (15/19) of the mice. Overall, from five experiments, 19 of 29 recipients were monitored for a minimum of 18 and up to 24 weeks, and 75% of these maintained significant levels of human lymphoid and myeloid cells (35% ± 9% total human cells; 23% ± 6% human B-lymphoid cells, and 7% ± 3% mature human granulopoietic cells).

Figure 2B shows the results of the PCR analysis of the regenerated human CFCs isolated from 15 NOD/SCID and 3 NOD/SCID-*nu/nu* mice at different time points after the transplant (5–40 colonies per mouse per time point indicated). In all but 1 of the 15

Table 1
Expression of β^{A-T87Q}-globin protein in human erythroid cells produced ex vivo from the in vivo progeny of transduced cord blood cells transplanted into NOD/SCID mice

	% β^{A-T87Q}/% β^{A-T87Q} + % β^{A}		% β^{A-T87Q}/% β-like	
Wk after transplant	Ery susp cult	BFU-E	Ery susp cult	BFU-E
8	34	55	12	35
11	54		33	
11	47		31	
11	46		28	
11	21		13	
11	14		7	
11	ND	38	ND	25
16	33	41	17	18
16	29		18	
24	10	14	6	10
24	59		29	
Mean	35 ± 5	37 ± 9	19 ± 4	22 ± 5

A total of 1–10 pooled BFU-E–derived colonies per sample was used. Ery susp cult, erythroid suspension culture; ND, not done.

positive NOD/SCID mice evaluated between 3 and 6 months after the transplant and also in the single positive NOD/SCID-*nu/nu* recipient followed for evaluation until 13 months after transplant, more than 20% of the CFCs were found to contain the transgene. In 2 mice, as many as 80–90% of the regenerated human CFCs were positive. Based on the data for all of the CFCs analyzed (obtained at least 11 weeks after the transplant from 17 mice in five experiments), the overall average transduction efficiency of long-term NOD/SCID mouse–repopulating cells was 45%.

High levels of β^{A-T87Q}-globin protein are produced in the erythroid progeny of transduced human cord blood cells. To investigate the levels of β^{A-T87Q}-globin protein produced by erythroid cells derived from transduced human cord blood cells, we transferred aliquots of cord blood cells immediately after virus exposure to culture conditions designed to promote erythroblast generation and differentiation over a period of 14 days (see Methods). In the two such experiments performed, more than 85% of the cells at the time of harvest had phenotypic features of maturing glycophorin-A^+ erythroblasts (Figure 3, A and B). HPLC analysis of lysates of these cells showed that β^{A-T87Q}-globin protein composed 48% and 59% of all β-globin chains (and 24% and 41% of all β-like globin chains) in the two experiments (Figure 3C).

The ability of transduced HSCs to generate erythroid progeny that would also produce this level of β^{A-T87Q}-globin protein was then investigated. Direct assessment of in vivo–generated human erythroblasts is, unfortunately, not possible in NOD/SCID mice repopulated with human hematopoietic cells because of the failure of these hosts to support terminal human erythropoiesis beyond 3–4 weeks after irradiation (43). Therefore, we used the FACS to isolate the human ($CD45/71^+$) cells present in marrow samples obtained from engrafted mice and then stimulated these to differentiate into erythroid cells in culture, either in CFC assays or in suspension cultures (Figure 3D). HPLC analysis of lysates from the cells harvested from the suspension cultures showed β^{A-T87Q}-globin protein to represent 35% of all β-globin chains and 19% of all β-like globin chains, with values as high as 59% and 31%, respectively (Table 1). Figure 3E shows a representative HPLC profile resulting from a culture initiated with cells present in a mouse receiving a transplant of transduced cord blood cells 11 weeks before. Similar levels of β^{A-T87Q}-globin protein (37%) were also detected in lysates of single or 5–10 pooled erythroid colonies produced from regenerated burst forming unit–erythroids (BFU-Es; Table 1).

RT-PCR analysis of RNA from erythroblasts generated in vitro from transduced cord blood cells confirmed the presence of β^{A-T87Q}-globin transcripts in these cells. A strong signal could be detected when the cDNA prepared from these extracts was diluted up to 32 times (Figure 3F, top right panel). In contrast, similar analysis of RNA extracts prepared from human B-lymphoid cells isolated by FACS from 2 mice repopulated with transduced cells showed no evidence of β^{A-T87Q}-globin transcripts in the undiluted cDNA (Figure 3F, top left panel). This demonstrates the erythroid specificity of expression of the β^{A-T87Q}-globin transgene achieved in human cells transduced with this vector, as previously observed in murine cells (37).

Polyclonal reconstitution of NOD/SCID mice transplanted with β^{A-T87Q}-globin lentiviral vector–infected cord blood cells. To investigate the number and stability of transduced clones obtained in NOD/SCID mice transplanted with β^{A-T87Q}-globin lentiviral vector–infected cord blood cells, and to obtain an estimate of the number of copies present in each repopulating cell, we used "bubble" linker-mediated PCR (LM-PCR). A total of 121 human CFC–derived colonies from 11 mice that had been confirmed to contain the β^{A-T87Q}-globin transgene were analyzed individually. These included CFCs from 2 mice that were assessed at different time points after receiving transplants. Agarose gel electrophoresis of the LM-PCR products showed that the cells in each colony contained, on average (± SEM), 2.1 ± 0.1 copies of provirus. Multiple unique integration patterns were frequently detected in the different CFCs harvested from a single mouse, indicating the presence of multiple clones transduced by the β^{A-T87Q}-globin lentivirus contributing to the regenerated human cell population present (Figure 4, A–C). Analysis of colonies generated from sequential marrow aspirates indicated dynamic changes in clonal prevalence during the first 6 months after the transplant in both mice followed in this way. Representative data from one of these mice is shown in Figure 4, D and E.

Preferential intragenic integration of β^{A-T87Q}-globin lentiviral vector into human HSCs. The products of the LM-PCR include genomic sequences flanking the integrated provirus, thus allowing the site of proviral integration to be ascertained by DNA sequencing. Using this approach, we analyzed the sequences of 48 gel-purified fragments that contained the expected vector-related long terminal repeat (LTR) sequence linked to a flanking genomic sequence. Of these 48 fragments, 35 were unique and were obtained from 33 individual colonies generated in vitro from human CFCs produced in the mice. Of 48 fragments, 13 (27%) were repeats; that is, the same sequence was observed in at least 2 colonies generated from the same mouse. For 7 of the 35 inserts (20%), no match was found in the human genome database. Of the remaining 28 inserts, 24 mapped within identified genes (86%) and 4 were repetitive elements that could not be mapped. The chromosomal distribution and names of the mapped genes are shown in Figure 5 and in Table 2, respectively. Notably, several integrations occurred in known oncogenes (e.g., *MLL* and *NUP214*, both implicated in acute myeloid leukemias), genes involved in signal transduction pathways (e.g., *SCAP2*), or genes with tumor suppressor activity (e.g., *CGBP*). Interestingly, the majority of intragenic integration events occurred in introns (21/24 or 88%).

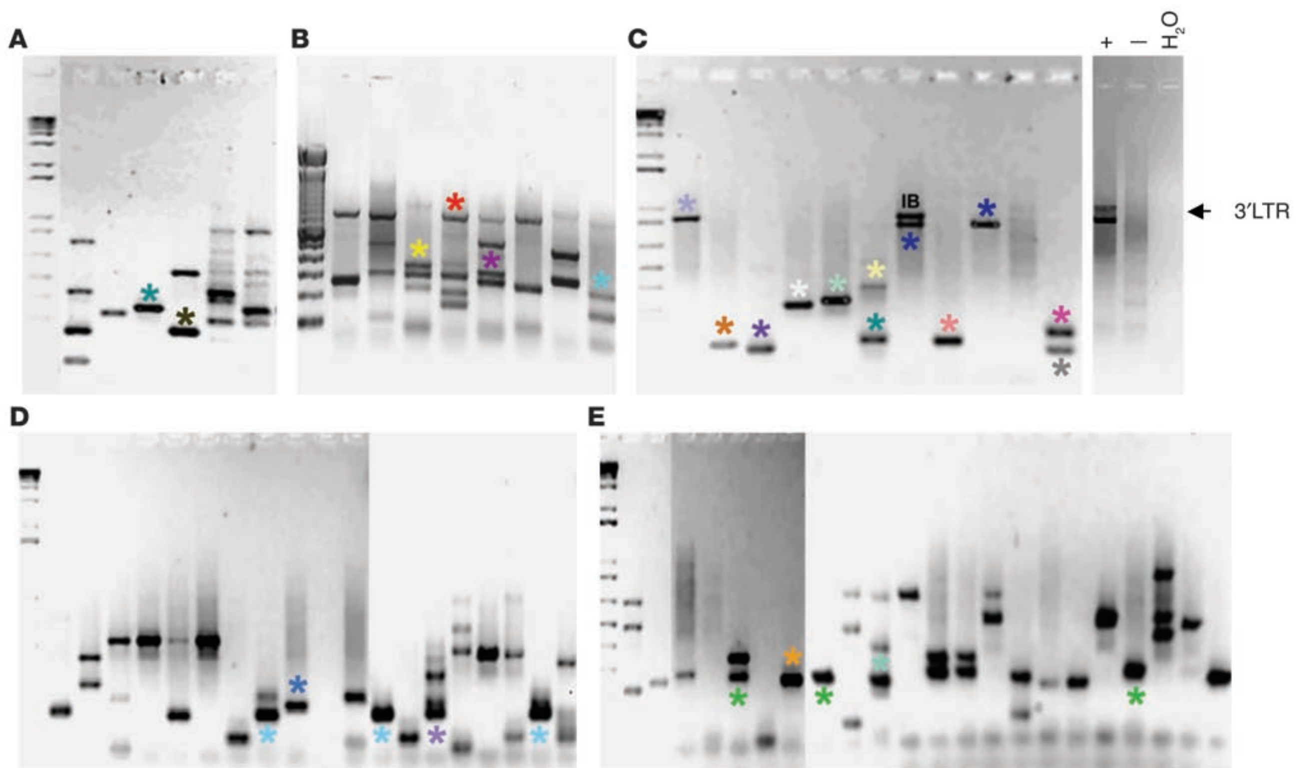

Figure 4
Bubble LM-PCR analysis of the number and dynamics of β^{A-T87Q} -globin lentivirus-positive clones in mice transplanted with transduced cord blood cells. (**A**–**C**) Analysis of individual CFC-derived colonies from 3 recipients (**A**, 1 recipient from experiment 4 analyzed 24 weeks after transplant; **B** and **C**, 2 mice from experiment 2, described in Figure 2A, analyzed 19 weeks after transplant). DNA from a transgene-negative colony was used as a negative control for the PCR, and DNA from a mouse spleen colony with a single integration confirmed by Southern blotting was used as a positive control. Note that the expected 750-bp 3′ LTR–related fragment (IB) could not be detected in all of the colonies. The presence of identical faint bands in the analyses of several colonies is probably caused by cross contamination that occurred while plucking these colonies from the methylcellulose cultures. (**D** and **E**) Analysis of CFC-derived colonies from a third mouse from experiment 2 described in Figure 2A analyzed 11 and 19 weeks after transplant. Bands with asterisks were gel purified and sequenced. Asterisks with the same color indicate colonies that showed the same integration site upon sequencing.

Discussion

Historically, the development of gene therapy approaches to SCD and β-thalassemia has been plagued by problems inherent in the design of stable vectors encoding human β-globin cassettes sufficient to direct the permanently very high levels of expression needed for the correction of these diseases. More recently, the risks of insertional mutagenesis have highlighted the need to achieve these goals with minimal copies of proviral integrants per cell (44). Here, we present the first evidence that a recently described self-inactivating lentiviral vector can be used to efficiently engineer the sustained production of anticipated therapeutic levels of an anti-sickling β^{A-T87Q}-globin protein in the erythroid progeny of primary human hematopoietic cells with in vivo–repopulating ability. We focused on normal cord blood as a target population for these initial studies because of the high frequency of NOD/SCID mouse–repopulating cells in cord blood (45, 46) and their reported ease of transduction after brief in vitro exposure to virus (25, 28, 29, 32, 47–49). The demonstrated levels of anti-sickling β^{A-T87Q}-globin protein in these cells constitute a significant advance and now underscore the need to extend these studies to adult HSCs.

Many groups have now shown that human HSCs with NOD/SCID mouse–repopulating activity can be efficiently transduced after very brief exposure to lentiviral vectors in vitro without affecting their subsequent hematopoietic potential or ability to express the acquired transgene (25, 31, 32, 48, 50–52). However, most of these findings have been limited to vectors carrying a ubiquitously expressed reporter or drug resistance gene. It remained to be demonstrated that such models could be extended effectively to vectors encoding a therapeutic gene and any additional sequences required for regulating their level and cell-specific expression. The findings presented here are the first to document this achievement with a vector carrying a β-globin gene. At least 20% of the CFCs regenerated in mice that had been repopulated with multiple clones of human cord blood cells exposed to concentrated preparations of this virus (0.5×10^9 to 1×10^9 infectious units/ml) had the vector transgene, and values of more than 50% were noted in several mice. These findings indicate that the β^{A-T87Q}-globin lentivirus can be used to transduce human cord blood cells with long-term NOD/SCID mouse–repopulating activity with the same efficiency as has been reported for simpler constructs (25, 27, 28, 31, 32, 48, 53).

Because we had found that prior overnight exposure to growth factors can increase the proportion of NOD/SCID mouse–repopulating cells that are ultimately transduced (data not shown), we adopted this approach. No perturbation of the normal pattern

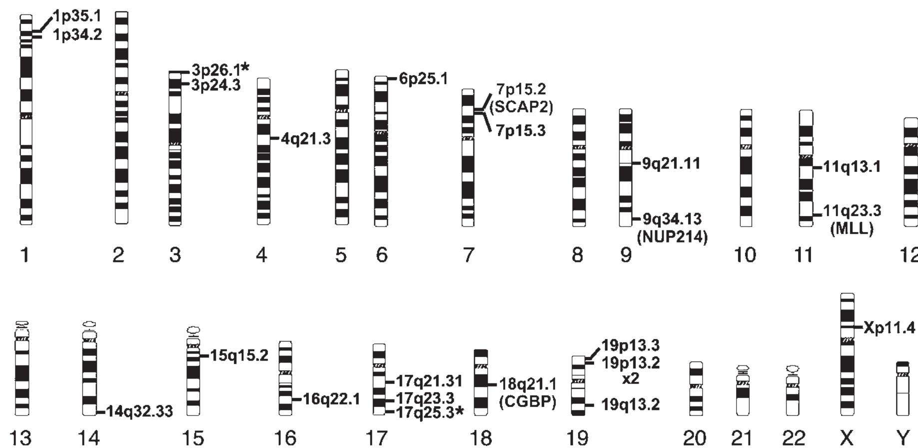

Figure 5
Chromosomal distribution of the sites of $\beta^{A\text{-}T87Q}$-globin lentiviral vector integration. Asterisks indicate sites of integration within predicted genes.

of differentiation was observed in mice repopulated with the transduced cells. Also, multiple-lineage hematopoiesis was stable for as long as 4–6 months after transplant in the NOD/SCID hosts and for at least 13 months after transplant in the one NOD/SCID-*nu/nu* mouse that could be followed. Taken together, these findings indicate that the transduction protocol used had no significant or consistent biological effect on cord blood HSCs.

We did not attempt to document any changes in NOD/SCID mouse–repopulating cell activity that may have been incurred by the use of an overnight infection protocol, nor did we try to determine how these might have affected an ultimate net gain or loss in the absolute yield of transduced HSCs (32, 54). Clearly such data would be important to design a clinical protocol. The latter would also require re-examination of the timing and growth factor treatment needed to optimize the transduction of NOD/SCID mouse–repopulating cells in adult sources of human HSCs, since these are present at much lower frequencies than in cord blood (45, 55). Adult sources of HSCs are also known to be qualitatively as well as quantitatively different from cord blood HSCs in terms of the rate at which they can be activated into G_1 and the rate at which HSC function may be lost in vitro (16, 42, 56–59).

We took advantage of the high gene transfer to human HSCs obtained in many experiments and the resultant high degree of repopulation of some mice with transduced human cells to investigate the clonal dynamics of this process. To identify different clones by the detection of unique proviral integration events, we analyzed hematopoietic colonies produced in vitro from in vivo–regenerated progenitors using a bubble LM-PCR method. Bubble LM-PCR was chosen because it is simpler to implement than other methods for detecting proviral inserts in small numbers of cells (fewer than 10^3). However, we do not yet know the sensitivity of this protocol, particularly where multiple integrations may be encountered and hence the number of proviral copies per cell may be underestimated. Nevertheless, this analysis allowed multiple clones (CFCs with different inserts) to be identified in individual animals, providing strong evidence of their polyclonal reconstitution by transduced human HSCs. CFCs with different patterns of integration were also seen in a single repopulated mouse examined over a 2-month interval (i.e., 3 and 5 months after transplant). This is similar to what has been observed in large animals (60).

Table 2
Identity of intragenic $\beta^{A\text{-}T87Q}$-globin lentiviral transgene integration sites

Accession no.	Chromosomal band	Ref Seq gene name	Biological process
NM_144621	1p35.1	*BOZF1*	Unknown
NM_006367	1p34.2	*CAP1*	Signal transduction
NT_022517.90	3p26.1	Predicted gene	–
NM-014744	3p24.3	*TBC1D5*	Signal transduction
BC017432	4q21.3	*MLLT2*	Cell growth and maintenance
AF285118	6p25.1	*CGI-203 like gene*	Unknown
NM_003930	7p15.2	*SCAP2*	Signal transduction and metabolism
NM_018846	7p15.3	*SBBI26*	Unknown
NM_001163	9q21.11	*APBA1*	Development and cell adhesion and metabolism
NM_005085	9q34.13	*NUP214*	Cell growth and maintenance
NM_006795	11q13.1	*EHD1*	Unknown
NM_005933/ NM_004397	11q23.3	*MLL* and *DDX6*[A]	Transcription and cell growth and maintenance
NG_001019	14q32.33	*IGHG3*	Immune response
NM_174916	15q15.2	*UBR1*	Metabolism
NM_182619	16q22.1	*LOC348174*	Unknown
NM_001002909	17q21.31	*KIAA0553*	Unknown
NM_001433	17q23.3	*ERN1*	Metabolism
NT_024871.61	17q25.3	Predicted gene	–
NM_014593	18q21. 1	*CGBP*	Transcription
NM_052847	19p13.3	*GNG7*	Signal transduction
NM_080665	19p13.2[B]	*MGC19604*	Unknown
NM_153358	19p13.2[B]	*FLJ90396*	Transcription
NM_003370	19q13.2–q13.3	*VASP*	Cell motility
NM_003688	Xp11.4	*CASK*	Metabolism

[A]The integration is in the second intron of the *DDX6* gene, which is located in the fifth intron of the *MLL* gene. [B]Genomic locus hit twice in two independent experiments.

The present studies are also important in their demonstration of the high lineage-specific levels of β^{A-T87Q}-globin produced in the erythroblast progeny of transduced HSCs. In many examples, these levels were above the estimated values of 20% of total β-globin per erythroid cell required to achieve a therapeutic effect in SCD and β-thalassemia (41, 61), even when all the β-like globin chains present in the cells were taken into consideration. Although the transgene copy number was estimated as 2 copies per cell, no meaningful calculation of the amount of β^{A-T87Q}-globin protein produced per transgene copy was possible because the extracts analyzed were obtained from a highly heterogeneous mixture of erythroblasts at different stages of differentiation. Nevertheless, it is clear that the results obtained here represent a significant improvement over the levels of β-globin transgene–derived transcripts and protein recently documented in erythroblasts derived from human cord blood cells transduced with an oncoretroviral vector containing a similar β-globin cassette with a smaller LCR fragment (16).

We found that the majority (24/28, 86%) of the proviral integrations occurred within genes identified in RefSeq, and these included a number of genes that possess cell signaling or regulatory functions or that have been associated with leukemia. This proportion is significantly higher than would be expected ($P < 0.0001$) from the current estimates that genes occupy approximately 35% of the genome (62, 63). Thus our findings support the concept of preferred intragenic integration of lentiviral vectors into human DNA, as noted by others who reported 67% and 58% intragenic HIV-1 integrations in human lymphoid (64) and HeLa cells (65), respectively. We also observed preferential integration into introns (21/24 = 88% of inserts characterized), as reported by others (64), perhaps reflecting the greater relative size of introns as compared with exons (62, 63). Taken together, these findings refocus attention on the possibility that such integration could result in activation or ablation of the expression of endogenous genes following the transduction of human HSCs, with functional consequences. As such, they point to the need for improvements in vector design aimed at both insulating integration sites from the effects of the provirus and minimizing the integrated proviral copy number required for therapeutic levels of expression.

Methods

Lentiviral vector design and production. The β^{A-T87Q}-globin lentiviral vector used (Figure 1) was based on a vector previously described in detail (37) that was then modified to include the following additional safety features: a 400-bp deletion in U3 plus replacement of U5 in the right LTR with the polyadenylation/termination sequence of the rabbit β-globin gene and mutation of two potential ATG initiation codons in the 3′ portion of the packaging signal (details available upon request). High-titer recombinant virus pseudotyped with vesicular stomatitis virus glycoprotein-G was initially produced by using a standard 4-plasmid packaging system and later by using a recently developed "super-split" 7-plasmid packaging system (66). Recombinant virus made using the safety-modified vector and super-split 7-plasmid packaging system was used in the last two experiments (one of which is experiment 2 presented in Figure 2A), whereas in the first three experiments (including experiment 1 in Figure 2A), the original vector (37) was used. Harvested virus-containing supernatants were concentrated by two rounds of ultracentrifugation approximately 1,000- to 2,000-fold to achieve titers of 0.5×10^9 to 1×10^9 infectious units/ml. Viral titers were determined using quantitative Southern blot analysis to compare vector DNA levels in cells containing a known number of integrated proviral copies with those in NIH 3T3 cells infected with test virus preparations. Absence of replication-competent virus in the viral supernatants was verified by a mobilization assay analogous to an assay previously employed for oncoretroviral vectors (67).

Isolation and transduction of human cord blood cells. Cord blood was obtained from consenting mothers undergoing cesarean delivery of healthy, full-term infants, and low-density (less than 1.077 g/ml) cells were isolated by centrifugation on Ficoll-Hypaque (Pfizer). CD34$^+$ cell–enriched populations (65–98% CD34$^+$ cells) were obtained by one of the following methods: (a) removal of lineage marker-positive cells using a column (n = 2); (b) sorting by FACSVantage SE (n = 1; BD); (c) positive (EasySep) selection using magnetic beads (n = 1; StemCell Technologies Inc.). In one experiment, CD34$^+$CD38$^-$ cells were isolated by FACS as previously described (46). Cells were stimulated overnight for in vivo experiments and for 48 hours for in vitro experiments at densities less than or equal to 2×10^5 cells/ml in Iscove's medium supplemented with 1% BSA, 10 μg/ml bovine pancreatic insulin, and 200 μg/ml human transferrin (BIT; StemCell Technologies Inc.), 10^{-4} mol 2-mercaptoethanol, 2 mM glutamine, 100 ng/ml FL (Immunex Corp.), 100 ng/ml SF, 50 ng/ml Tpo (Genentech Inc.), and 100 ng/ml hIL-6 (provided by S. Rose-John, Christian-Albrechts University, Kiel, Germany). The following day, the cells were pelleted, resuspended in fresh growth factor–supplemented medium with 5 μg/ml protamine sulfate and 0.5×10^8 to 5×10^8 infectious units/ml (MOI = 9–140; 140 in experiment 1, 9 and 90 in experiment 2), placed in a 24-well plate coated with 2 μg/cm^2 Retronectin (Takara Shuzo Co.) or with 5 μg/cm^2 fibronectin (Sigma-Aldrich), and then incubated at 37°C for 6 hours. In experiment 2, cells were plated in a Retronectin-coated well, which was then preloaded with virus for 2 hours at 4°C.

In vitro CFC assays. Appropriate aliquots of human cells were plated in 1 ml of methylcellulose-containing medium (Methocult H4230; StemCell Technologies Inc.) supplemented with 50 ng/ml human SF (purified from human SF cDNA-transfected Cos cell supernatants) and 20 ng/ml each of human IL-3 (Novartis), IL-6 (Cangene), GM-CSF (Novartis), granulocyte-CSF (G-CSF; Novartis), and 3 U/ml erythropoietin (Epo; StemCell Technologies Inc.) (68). The cultures were then incubated for 2 weeks at 37°C, and colonies of terminally differentiating erythroid, myeloid, and mixed erythroid-myeloid cells (from BFU-E, GM-CFU, and granulocyte-erythroid-megakaryocyte-macrophage–CFU [GEMM-CFU], respectively) were identified.

Animals. NOD/SCID mice and NOD/SCID-*nu/nu* mice were bred and maintained in microisolators at our center. Original breeding pairs of NOD/SCID-*nu/nu* mice were provided by J. Nolta (University of Washington, St. Louis, Missouri, USA), who obtained these mice by serially backcrossing the *nude* gene onto the NOD/SCID background for seven generations to give a host that supports human hematopoiesis in a fashion that is similar to the NOD/SCID mouse but without the development of endogenous thymomas and the resultant reduced lifespan of the NOD/SCID mouse (69).

At 8–10 weeks of age, mice were irradiated with 350 cGy of ^{137}Cs γ-rays the day prior to being intravenously injected with virus-infected human cells. (These cells were the progeny of an initial aliquot containing 1.7×10^5 to 4×10^5 CD34$^+$ or 7×10^4 CD34$^+$CD38$^-$ cord blood cells per mouse.) Thereafter, mice were given acidified water containing ciprofloxacin (100 mg/l; Bayer AG). Marrow cells were aspirated (70) 3, 7, 11, and 16 weeks after transplantation; the mice were killed 4–8 weeks later, and all cells from both tibiae and femurs were harvested for analysis. For phenotyping studies, cells were stained with human-specific monoclonal antibodies for total hematopoietic cells (CD45 and CD71), progenitors (CD34), B-lymphoid cells (CD19 and CD20), and granulopoietic cells (CD15), as previously described (71). Human multiple-lineage engraftment was defined as the detection of five or more CD19/20$^+$CD34$^-$ and five or more CD15$^+$ events per 2×10^4 live (propidium iodide–negative) events analyzed. In some instances, human CD45/71$^+$ cells

were isolated using a 3-laser FACSVantage SE (BD), as previously described (46), and then plated in methylcellulose cultures to generate hematopoietic colonies or placed in erythroid differentiation cultures.

PCR analysis. Cell lysates were prepared by incubating the cells from individually plucked colonies at 42°C for 30 minutes in the presence of proteinase K (1 mg/ml). PCR was performed on 2 μl (1/25th) of these lysates using primers specific for human GAPDH (5′-ACCGTCAAGG CTGAGA-AACGG-3′ and 3′-ACGTACTCAGCGCCAGCATC-5′) and for the β^{A-T87Q}-globin transgene (5′-GGGCACCTTTGCCCAG-3′ and 3′-TGGTCACGTCC-GACGGATA-5′) to amplify the expected 100-bp and 600-bp fragments, respectively. After 40 cycles of denaturation (30 seconds at 94°C), annealing (30 seconds at 63.5°C), and extension (60 seconds at 72°C), PCR products were separated on a 1.5% Tris-acetate EDTA (TAE) agarose gel.

RT-PCR analysis. Total RNA was extracted using a commercial kit (Trizol; Gibco BRL, Invitrogen Corp.) and reverse transcribed by random priming using 1 μg of total RNA and SuperScript II Reverse Transcriptase (Invitrogen Corp.) at 42°C for 30 minutes, followed by denaturation at 72°C for 10 minutes and snap cooling to 4°C for 5 minutes. A PCR was then performed on undiluted and diluted (1/2–1/4–1/8–1/16–1/32–1/64) cDNA using the same primer sets as those used for PCR to amplify the expected 100-bp fragments for the β^{A-T87Q}-globin and GAPDH transcripts. After 40 cycles of denaturation (30 seconds at 94°C), annealing (30 seconds at 63.5°C), and extension (60 seconds at 72°C), the PCR products were separated on a 1.5% TAE agarose gel.

Erythroid differentiation cultures and analysis of globin protein. Cells were cultured for the first 9 days in α-medium plus 15% FCS plus 1 U/ml Epo, 100 ng/ml SF, 40 ng/ml IGF-1 (R & D Systems), 10^{-6} mol freshly dissolved hydrocortisone (Sigma-Aldrich), 10^{-6} mol 17β-estradiol (Sigma-Aldrich), 1.28 μg/ml iron-saturated transferring (StemCell Technologies Inc.), and 10^{-4} mol 2-mercaptoethanol. The cells were then transferred to fresh α-medium plus 15% FCS, 1 U/ml Epo, 1 μg/ml insulin (Sigma-Aldrich), 1.28 μg/ml iron-saturated transferrin, and 10^{-4} mol 2-mercaptoethanol for another 3–5 days; finally, cells were harvested and lysed using acid-acetone. Globins were analyzed by HPLC using a Vydac large-pore (300 Å) C_4 column and a modified acetonitrile/H_2O/trifluoroacetic acid gradient as previously described (72). The amount of β^{A-T87Q}-globin protein was then calculated, both as a percentage of the total β-globin present (β^{A-T87Q}-globin/β^{A-T87Q}-globin + β^{A}-globin × 100%) and as a percentage of all β-like proteins present (β^{A-T87Q}-globin/β^{A-T87Q}-globin + β^{A}-globin + γ^{G}-globin + γ^{A}-globin × 100%).

LM-PCR. Integrated LTR and flanking genomic sequences were amplified and then isolated using a modification of the bubble LM-PCR strategy (73). Aliquots of the cell lysates from transgene-positive colonies (5–10 μl; one-fifth to one-tenth) were digested with *Hin*fI (New England Biolabs), and the fragments were then ligated overnight at room temperature to a double-stranded bubble linker (5′-CTCTCCCTTCTCGAA TCGTAACCGTTCG-TACGAGAATCGCTGTCCTCTCCTTG-3′ and 5′-ANTCAAGGAGAGGAC-GCTGTCTGTCGAAGGTAAGGAACGGACGAGAGAAGGGA GAG-3′) prior to performing a first PCR (PCR-A) on 10 μl (one-tenth) of the ligation product using a linker-specific Vectorette primer (5′-CGAATCGTAACC-GTTCGTACGAGAATCGCT-3′) and an LTR-specific primer (LTR-A: 5′-CAACACACACATTGAAGCACTCAAGGCAAG-3′) and under the following conditions: one cycle of 94°C for 2 minutes, 20 cycles of 94°C for 30 seconds and 65°C for 1 minute, and one cycle of 72°C for 2 minutes. The bubble linker contains a 30-nucleotide nonhomologous sequence in the middle region that prevents binding of the linker primer in the absence of minus strand generated by the LTR-specific primer. A 1-μl aliquot of the PCR-A reaction (one-fifteenth) was then used as a template for a second nested PCR (PCR-B) using an internal LTR-specific primer (LTR-B: 5′-GAGA-GCTCCCAG GCTCAGATCTGGTCTAAC-3′) and the same linker-specific Vectorette primer as was used in PCR-A with the following conditions: one cycle of 94°C for 2 minutes, 30 cycles of 94°C for 60 seconds and 72°C for 1 minute, and one cycle of 72°C for 2 minutes. Ten microliters (one-half) of the final PCR-B product were electrophoresed using 2% agarose TAE gel. Individual bands were excised and purified using the Qiaex II Gel Extraction Kit (QIAGEN) for sequencing. Two types of marked fragments were obtained. One was always 750 bp, representing the vector sequence bound by the primer specific to the 3′ LTR and the next *Hin*fI restriction site. The other was of variable length (158 bp or more) depending on the distance to the next *Hin*fI site in the host genome adjacent to the 5′ LTR.

Sequence analysis. Gel-purified DNA bands were sequenced directly from the LTR-B primer with the ABI PRISM Model 377 according to the manufacturer's instructions. Only sequences containing both complete 5′ LTR and intact *Hin*fI-linked Vectorette ends were analyzed. Sequencing of fragments with identical lengths sometimes yielded different integration sites. The sensitivity of determining the number of proviral integrations per colony could be increased by further cloning of the gel-isolated fragments. BLAST searches were performed using the University of California Santa Cruz (UCSC) genome project website (http://genome.ucsc.edu) to identify the genomic location of the flanking sequences. Chromosomal localizations of the mapped genes were determined using the Ensembl map viewer (http://www.ensembl.org).

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Address correspondence to: R. Keith Humphries, Terry Fox Laboratory, 601 West 10th Avenue, Vancouver, British Columbia V5Z 1L3, Canada. Phone: (604) 877-6070, ext. 3095; Fax: (604) 877-0712; E-mail: khumphri@bccrc.ca.

Connie J. Eaves and R. Keith Humphries contributed equally to this work.

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