Plenary paper

A novel murine model of Cooley anemia and its rescue by lentiviral-mediated human β-globin gene transfer

Stefano Rivella, Chad May, Amy Chadburn, Isabelle Rivière, and Michel Sadelain

Patients affected by β-thalassemia major require lifelong transfusions because of insufficient or absent production of the β chain of hemoglobin (Hb). A minority of patients are cured by allogeneic bone marrow transplantation. In the most severe of the hitherto available mouse models of β-thalassemia, a model for human β-thalassemia intermedia, we previously demonstrated that globin gene transfer in bone marrow cells is curative, stably raising Hb levels from 8.0-8.5 to 11.0-12.0 g/dL in long-term chimeras. To fully assess the therapeutic potential of gene therapy in the context of a lethal anemia, we now have created an adult model of β^0-thalassemia major. In this novel model, mice engrafted with β-globin–null (Hbb$^{th3/th3}$) fetal liver cells succumb to ineffective erythropoiesis within 60 days. These mice rapidly develop severe anemia (2-4 g/dL), massive splenomegaly, extramedullary hematopoiesis (EMH), and hepatic iron overload. Remarkably, most mice (11 of 13) treated by lentivirus-mediated globin gene transfer were rescued. Long-term chimeras with an average 1.0-2.4 copies of the TNS9 vector in their hematopoietic and blood cells stably produced up to 12 g/dL chimeric Hb consisting of muα_2:huβ_2 tetramers. Pathologic analyses indicated that erythroid maturation was restored, while EMH and iron overload dramatically decreased. Thus, we have established an adult animal model for the most severe of the hemoglobinopathies, Cooley anemia, which should prove useful to investigate both genetic and pharmacologic treatments. Our findings demonstrate the remarkable efficacy of lentivirus-mediated globin gene transfer in treating a fulminant blood disorder and strongly support the efficacy of gene therapy in the severe hemoglobinopathies. (Blood. 2003;101: 2932-2939)

© 2003 by The American Society of Hematology

Introduction

The β-thalassemias are caused by more than 200 mutations that lead to decreased or absent production of the β chain of hemoglobin (Hb).[1-3] Most of the mutations are point mutations in the β-globin gene that either decrease the level of transcription or mRNA stability or lead to nonfunctional mRNA, as is the case in nonsense and frameshift mutations. Other mutations include deletions in the β-globin gene, as well as rare deletions located distally to the β-globin gene itself.[1,3,4] There is a large spectrum in the severity of the disease found in homozygotes and compound heterozygotes. Patients affected by β-thalassemia intermedia do not require chronic transfusion therapy in the first years of life, but they often worsen over time, eventually developing hypersplenism, osteopenia, extramedullary hematopoiesis (EMH), and iron overload. Some patients eventually require splenectomy and blood transfusions. In patients with β-thalassemia major, or Cooley anemia, the absent or extremely reduced production of the β chain of Hb causes severe ineffective erythropoiesis, massive erythroid hyperplasia in the bone marrow and extramedullary sites, and hemolysis. The ensuing iron overload can lead to endocrine deficiencies, cirrhosis, and cardiac failure. In the absence of lifelong transfusions, the disease is lethal.[5] Current disease management consists of prenatal diagnosis, transfusion therapy, or allogeneic bone marrow transplantation.[1,3,4] Only the latter is curative, but this option is limited to a minority of patients for whom a histocompatible donor can be identified.

New approaches aimed at ameliorating the condition of patients with severe β-thalassemia are greatly needed. Genetic approaches based on the transfer of a regulated human β-globin gene in autologous hematopoietic stem cells (HSCs) represent an attractive potential treatment.[6] Their implementation has been hampered by the difficulty of appropriately regulating expression of globin genes in the progeny of virally transduced HSCs. The therapeutic globin gene must indeed be expressed in a tissue-specific fashion and at a high level.[7,8] We recently demonstrated that complex lentiviral vectors harboring the human β-globin gene and an optimized combination of proximal and distal transcriptional control elements direct erythroid-specific and sustained globin gene expression in murine bone marrow chimeras.[9] In mice with β-thalassemia intermedia, the TNS9 lentiviral vector durably ameliorated anemia.[9,10] A similar vector encoding a variant globin gene reduced sickle cell formation in 2 murine models of sickle cell disease (SCD).[11] Obtained in hitherto available models of severe hemoglobinopathies, these encouraging findings do not address whether stem cell–based gene therapy is potentially curative for a deficiency as profound as that found in Cooley anemia.

Three mouse models of β-thalassemia have been generated to date. These mouse models are affected by different degrees of β-thalassemia intermedia. The *th1* model results from the deletion of the β^major gene; Hbb^th1/th1 homozygotes exhibit a moderate form of thalassemia.[12] A second model (*th2*) was generated by insertional disruption of the β^major gene, causing lethality in Hbb^th2/th2 homozygotes but only a very mild phenotype in the heterozygotes.[13] The third model, *th3,* was generated by deletion of both the β^major and β^minor genes.[14] Mice homozygous for this deletion die late in gestation, while heterozygotes are viable and thalassemic. Adult Hbb^th1/th1 and Hbb^th3/+ mice exhibit anemia (slightly more severe in the latter model), abnormal red cell morphology, and splenomegaly, and develop spontaneous hepatic iron deposition similar to that found in humans with β-thalassemia intermedia. The lack of an animal model for Cooley anemia has limited the full investigation of the pathophysiology underlying this disease and hampered the evaluation of both pharmacologic and genetic treatments.

Here we describe a severe anemia model in which mice engrafted with β-globin–null (Hbb^th3/th3) fetal liver cells succumb to ineffective erythropoiesis within 60 days. This novel model enabled us to investigate whether globin gene transfer could rescue a massive protein deficit. We show that treatment with the TNS9 vector durably rescues most treated chimeras. In hematopoietic chimeras with a mean vector copy number of 1.0 to 2.4 in hematopoietic and blood cells, stable production of chimeric Hb (Hbb^hu, consisting of chimeric murine β_2:human β_2 tetramers) was achieved, reaching levels up to 12 g/dL and effectively treating the anemia. These findings strongly support the feasibility of a genetic treatment for β-thalassemia and have general implications for devising stem cell–based treatments for severe blood disorders.

Materials and methods

Hematopoietic chimeras

Donor fetal liver cells (FLCs) were harvested from embryonic day (ED) 14.5 embryos obtained by intercrossing Hbb^th3/+ mice (Jackson Laboratories, Bar Harbor, ME). Embryo genotypes were screened by analyzing Hb by cellulose acetate electrophoresis (Figure 1A). FLCs were resuspended in X-VIVO-15 serum-free medium and supplemented with 10 ng/mL IL-1α, 100 U/mL IL-3, 150 U/mL IL-6, 10 ng/mL Kit ligand obtained from Genzyme (Cambridge, MA), 0.5 mM β-mercaptoethanol obtained from Sigma (St Louis, MO), 200 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. FLCs were then pelleted and resuspended in serum-free medium containing concentrated lentiviral supernatant and supplemented with 4 mg/mL polybrene obtained from Sigma, 200 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and cytokines as above, and cultured for 8 hours. Transduced FLCs (2×10^6) were then intravenously injected into each of the irradiated female recipients to establish bone marrow chimeras. Recipient mice (11- to 14-week-old C57/BL6 mice) were irradiated with 12 Gy (split dose 2×6 Gy) on the day of transplantation. Vector stocks were generated by triple transfection into 293T cells, as previously described.[9] Concentrated viral stocks with titers of $1-2 \times 10^8$ infectious particles/mL were used to infect 2×10^6 FLCs in a final volume of 1 mL (multiplicity of infection = 50). The lentiviral vectors TNS9 and pHR′eGFP (enhanced green fluorescent protein), derived from pHR′LacZ,[15] have been previously described.[9]

Hematologic studies

Blood samples were obtained by retro-orbital puncture under ether anesthesia. Total hemoglobin levels, red cell counts, hematocrit levels, neutrophil counts, platelet counts, and reticulocyte counts were measured on a blood cell count analyzer (H System, Bayer, Leverkusen, Germany). Plasma erythropoietin (EPO) concentrations were measured using an enzyme-
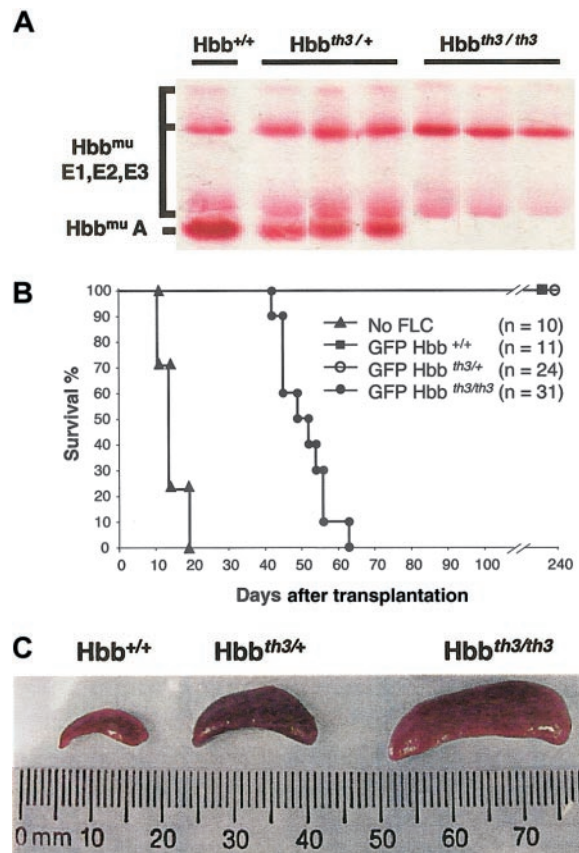

Figure 1. Establishment of an adult model of β⁰-thalassemia. (A) Cellulose acetate analysis showing the hemoglobin patterns in ED 14.5 Hbb^+/+, Hbb^th3/+, and Hbb^th3/th3 embryos. E1, E2, and E3 indicate the 3 embryonic hemoglobins. (B) Mice engrafted with Hbb^th3/th3 hematopoietic FLCs die 6 to 9 weeks after receiving transplants. Donor FLCs harvested from ED 14.5 embryos were injected in irradiated C57BL/6 recipient mice. Mice engrafted with Hbb^th3/th3 FLCs died or had to be killed 7 to 9 weeks after receiving transplants. Recipients of Hbb^+/+ and Hbb^th3/+ FLCs survived for at least 8 months (duration of follow-up). (C) Spleen sizes in mice engrafted with Hbb^+/+, Hbb^th3/+, and Hbb^th3/th3 FLCs at 6 to 8 weeks after receiving transplants.

linked immunosorbent assay (ELISA) kit for human EPO (R&D Systems, Oxon, United Kingdom).

Protein analyses

Red cell lysates from freshly collected peripheral blood were analyzed by cellulose acetate electrophoresis obtained from Helena Laboratories (Beaumont, TX). Hemoglobin bands were visualized by Ponceau S staining and quantitated by densitometry, as previously described.[9]

Southern blot analysis

For quantification of vector copy number in HSC chimeras, genomic DNA from tissues was isolated, digested with *Bam*HI, and studied by Southern blot analysis using a [^{32}P]dCTP-labeled *Nco*I/*Bam*HI fragment of the human β-globin gene as probe. TNS9-transduced single-copy murine erythroleukemia (MEL) clone controls also were digested with *Bam*HI. Radioactive bands were quantitated by phosphorimager analysis (Molecular Dynamics).

Gene expression analysis

Total RNA was extracted from bone marrow (BM), spleen, and blood using TRIzol (Gibco-BRL). Quantitative primer extension assays were performed as previously described,[9] using the Primer Extension System-AMV Reverse Transcriptase kit (Promega) with [^{32}P]dATP end-labeled primers specific

for lentiviral-encoded human β-globin (5′-CAGTAACGGCAGACTTCTC-CTC-3′), mouse β-globin (5′-TGATGTCTGTTTCTGGGGTTGTG-3′), and mouse β-globin (5′-CCTTGATGTTGCTTTTGTCTTC-3′) with predicted extension products of 90 bp, 53 bp, and 69 bp, respectively. Radioactive bands were quantitated by phosphorimager analysis (Molecular Dynamics). All measurements were standardized to RNA samples obtained from YAC transgenic mice harboring one copy of the human β-globin gene (line A85.68,[16]) as previously described.[9]

Tissue pathology

Four-micron tissue sections were stained with hematoxylin and eosin or with a Gomori iron stain and examined under light microscopy, as previously described.[10] Slides of control and treated mice were assessed in a blind manner.

Statistical analysis

For statistical analyses, we used the permutation rank sum statistic to determine whether hematologic parameters differed between treated and mock-treated groups. A low P value is evidence that the 2 populations are different. The Wilcoxon rank sum statistic was used to compare survival curves between groups.

Results

Establishment of an adult mouse model of β-thalassemia major

Homozygote mice lacking adult β-globin genes ($Hbb^{th3/th3}$) die late in gestation.[14] We therefore attempted to adoptively transfer the hematologic deficit to adult mice by transplanting FLCs harvested from screened ED 14.5 embryos (Figure 1A). Congenic C57BL/6 recipient mice were irradiated (12 Gy) before intravenous infusion of 2×10^6 FLCs. Mice engrafted with $Hbb^{+/+}$ or $Hbb^{th3/+}$ FLCs survived for at least 8 months, corresponding to the duration of this study (n = 11 and 24, respectively; Figure 1B). In contrast, recipients of $Hbb^{th3/th3}$ cells showed a progressive decrease of body mass and activity, eventually requiring termination 7 to 9 weeks after transplantation (T_{50} = 50 days, n = 31). These mice died significantly later than radiation controls (T_{50} = 15 days, n = 10, $P < .01$), suggesting that the cause of death was neither failure to engraft nor pancytopenia.

$Hbb^{th3/th3}$ chimeras die of a selective erythroid defect

Hematologic analyses performed 6 to 8 weeks after transplantation in mice engrafted with $Hbb^{th3/th3}$ FLCs revealed severe anemia (2.8 ± 0.8 g/dL of Hb levels, versus 13.2 ± 1.0 in 11 $Hbb^{+/+}$ chimeras and 11.1 ± 2.1 in 24 $Hbb^{th3/+}$ chimeras). Low red blood cell (RBC) counts, hematocrit values, and reticulocyte counts, together with high levels of serum erythropoietin (S.R., M.S., unpublished observations), further confirmed the development of a profound erythroid deficiency. Platelet counts (Figure 2), as well as neutrophil counts (data not shown), were comparable in experimental and control groups, excluding aplasia or graft failure as the cause of death. Analyses of murine globin transcripts in BM, spleen, and blood by primer extension analysis showed that murine β-globin (muβ) expression was extremely low or undetectable in bone marrow, spleen, and blood of mice engrafted with $Hbb^{th3/th3}$ FLCs (data not shown). The presence of the β-globin transcript further confirmed that these chimeras developed a selective erythroid defect caused by the lack of β-globin expression. The profound anemia settled in after 50 days, consistent with the clearance rate of the recipient's normal RBCs.[17]
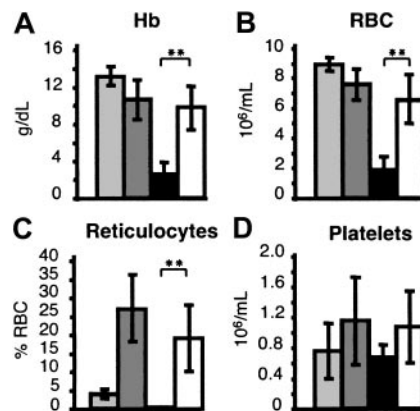

Figure 2. Hematologic parameters in hematopoietic chimeras 6-8 weeks after transplantation. (A) Hb: Hemoglobin levels. (B) RBC: Red blood cell counts. (C) Reticulocytes are represented as percentage of RBC. (D) Platelet counts. Donors: light gray indicates $Hbb^{+/+}$ FLCs; dark gray, $Hbb^{th3/+}$ FLCs; black, $Hbb^{th3/th3}$ FLCs; and white, TNS9-transduced $Hbb^{th3/th3}$.

$Hbb^{th3/th3}$ chimeras develop pathologic features that are characteristic of severe thalassemia

After 6 to 7 weeks, mice engrafted with $Hbb^{th3/th3}$ FLCs exhibited massive splenomegaly, far exceeding that found in mice engrafted with $Hbb^{th3/+}$ FLCs (Figure 1C). The massive splenomegaly in the $Hbb^{th3/th3}$ chimeras was due to marked erythroid hyperplasia consisting mostly of immature nucleated erythroid cells that obliterated the normal microscopic architecture of the spleen. Mature RBCs, still present in the $Hbb^{th3/+}$ chimera albeit in reduced numbers compared with $Hbb^{+/+}$ chimera, were virtually absent in their bone marrow and spleen (Figure 3A-B). EMH was absent in the liver of $Hbb^{+/+}$ chimeras, moderate in $Hbb^{th3/+}$ chimeras, and extensive in $Hbb^{th3/th3}$ chimeras (Figure 3C). We also examined hepatic iron accumulation, which is a major characteristic of the systemic disease found in patients with severe thalassemia, arising as a consequence of hemolysis, ineffective erythropoiesis, and increased gastrointestinal iron uptake. In $Hbb^{+/+}$ and $Hbb^{th3/+}$ chimeras, iron accumulation was, respectively, absent and moderate, predominantly within Kupffer cells (Figure 3D). In the $Hbb^{th3/th3}$ chimeras, iron deposition in the liver was found as early as 6 weeks after transplantation, with preferential accumulation in the cytoplasm of the hepatocytes around the portal tracts (Figure 3D). Altogether, these pathologic findings confirmed the presence of severe ineffective erythropoiesis, extensive EMH, and rapid iron accumulation.

TNS9 rescues lethal β⁰-thalassemia

The establishment of a novel adult model of severe β-thalassemia allowed us to investigate the efficacy of lentivirus-mediated globin gene transfer in correcting the lethal phenotype and the anemia. $Hbb^{th3/th3}$ as well as control $Hbb^{+/th3}$ and $Hbb^{+/+}$ FLCs were transduced with either the TNS9 vector or a control lentiviral vector encoding eGFP. All mice engrafted with eGFP-transduced $Hbb^{th3/th3}$ FLCs (n = 20) died as expected within 60 days (Figure 4). In contrast, all mice engrafted with TNS9-transduced $Hbb^{th3/th3}$ FLCs (n = 13) survived at least 4 months ($P < .01$, TNS9 versus eGFP-transduced $Hbb^{th3/th3}$; Figure 4), thus confirming the deficit in β-globin synthesis to be the cause of death. These mice showed a remarkable improvement of their hematologic parameters (Figure 2) 8 weeks after transplantation, with Hbb^{hu} accounting for 30%-65% of total Hb. Of the 13 mice TNS9-treated $Hbb^{th3/th3}$ chimeras, 10 stably produced Hb consisting predominantly or
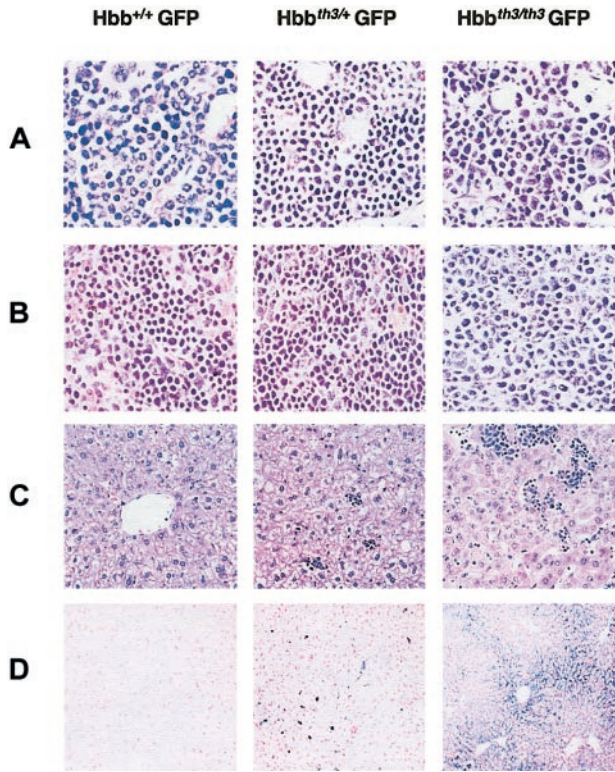
Figure 3. Histopathologic analysis of hematopoietic tissues in Hbb⁺/⁺, Hbbᵗʰ³/⁺, and Hbbᵗʰ³/ᵗʰ³ chimeras. (A) Bone marrow (original magnification, × 60). Left: Hbb⁺/⁺ control. The marrow shows various stages of normal erythroid maturation. Megakaryocytes as well as a significant numbers of maturing myeloid cells are present. Center: Hbbᵗʰ³/⁺ control. The marrow shows a relative increase in erythroid elements; some myeloid cells are still present. Right: Hbbᵗʰ³/ᵗʰ³ chimera. Misshapen erythroid cells and red cell fragments are present in the sinuses. Some of the immature erythroid cells have dysmorphic nuclei. Mature erythroid cells are decreased. (B) Spleen (original magnification, × 60). Left: Hbb⁺/⁺ control. The red pulp contains a mixture of erythroid and myeloid cells. The sinuses are open. Center: Hbbᵗʰ³/⁺ control. Significant expansion of red pulp compressing the sinuses. Right: Hbbᵗʰ³/ᵗʰ³ chimera. The red pulp consists primarily of immature erythroid cells. Mitotic figures are easily identified. The immature erythroid cells often have abnormal nuclei. (C) Liver (original magnification, × 20). Left: Hbb⁺/⁺ control. EMH is absent. Center: Hbbᵗʰ³/⁺ control. Moderate EMH is present. Right: Hbbᵗʰ³/ᵗʰ³ chimera: Extensive EMH is present. (D) Liver (original magnification, × 20), Gomori stain). Left: Hbb⁺/⁺ control: undetectable iron. Center: Hbbᵗʰ³/⁺ control. Moderate iron deposits predominantly in Kuppfer cells. Right: Hbbᵗʰ³/ᵗʰ³ chimera: Diffuse iron accumulation, concentrated in the cytoplasm of the hepatocytes preferentially around the portal tracts. Control tissues from mice engrafted with eGFP-transduced Hbb⁺/⁺ or Hbbᵗʰ³/⁺ FLCs were harvested 6 and 8 months after transplantation. Tissues from control Hbbᵗʰ³/ᵗʰ³ chimeras were harvested 8 weeks after transplantation. Stained with hematoxylin and eosin (A-C).

exclusively of Hbbʰᵘ. Over 8 months (Figure 5A-B), 6 consistently showed levels of Hbbʰᵘ above 95% of total Hb levels, therefore surviving on the sole basis of sustained production of lentivirus-encoded human β-globin. Chimeras with more than 5% murine Hb were excluded from further analyses to rule out any contribution of residual host erythropoiesis to their pathology and survival. Secondary bone marrow chimeras derived 5 and 6 months after transplantation from 4 of the 6 healthy mice with more than 95% Hbbʰᵘ expressed high levels of the human β-globin levels in peripheral blood after 2-4 months (data not shown). These findings, consistent with our results obtained in normal mice,[9] indicated that TNS9-treated chimeras were rescued by transduced fetal liver HSCs.

Correction of anemia and ineffective erythropoiesis in TNS9-treated Hbbᵗʰ³/ᵗʰ³ chimeras

Our long-term studies focused on chimeras with less than 5% residual murine Hb levels in the 8 months following transplantation
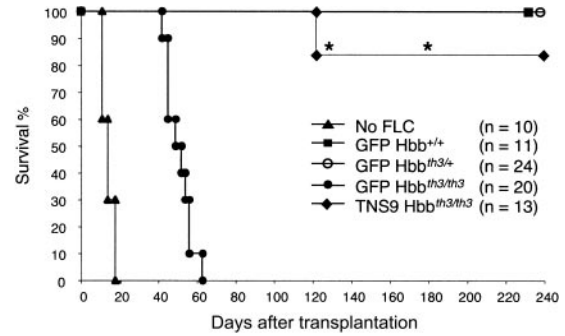

Figure 4. Lentivirus-mediated globin gene transfer rescues Hbbᵗʰ³/ᵗʰ³ hematopoietic chimeras from death. Donor Hbb⁺/⁺, Hbbᵗʰ³/⁺, and Hbbᵗʰ³/ᵗʰ³ FLCs harvested from ED 14.5 embryos were transduced with TNS9 or a control lentivirus encoding the eGFP protein. Of the 13 TNS9-treated chimeras, 6 stably and predominantly expressed Hbbʰᵘ (> 95% of total Hb). The asterisk indicates that healthy Hbb⁺/⁺, Hbbᵗʰ³/⁺, and Hbbᵗʰ³/ᵗʰ³ FLCs recipient mice were killed at 4 or 6 months after transplantation to perform pathologic and molecular analysis (see "Results" and Table 1). The remaining mice were killed 8 months after transplantation to complete the analysis. Two mice died 4 months after transplantation, showing profound anemia. Measurement of vector copy number in bone marrow, spleen, and peripheral blood showed, respectively, undetectable and very low vector copy number, thus establishing that these 2 chimeras had been temporally rescued by genetically modified, short-lived erythroid progenitor cells.

(n = 6). Over this period the mice showed stable and long-term expression of Hbbʰᵘ, averaging 6.5 ± 2.9 g/dL. This level approximates that of hemizygous Hb production (8.1 ± 0.3 g/dL in Hbbᵗʰ³/⁺ chimeras, Table 1). Pathologic examination performed between 5 and 8 months after transplantation showed variable degrees of ineffective erythropoiesis, commensurate to the degree of anemia. Tissue sections from 2 mice, one with a level of 4.7 g/dL Hb and the other with a level of 12 g/dL are shown in Figure 6. The findings in the latter are similar to the eGFP-transduced Hbb⁺/⁺ control (Figure 3), except for small and rare foci of EMH in the liver (Figure 6 right column). Virtually no iron deposition was detected. The long-term chimera with 4.7 g/dL Hb levels showed features intermediate between control Hbbᵗʰ³/ᵗʰ³ and Hbbᵗʰ³/⁺ chimeras (Figure 6 left column). To further demonstrate the restoration of erythroid maturation, we analyzed splenic erythroid populations by fluorescence-activated cell-sorter scanner (FACS)
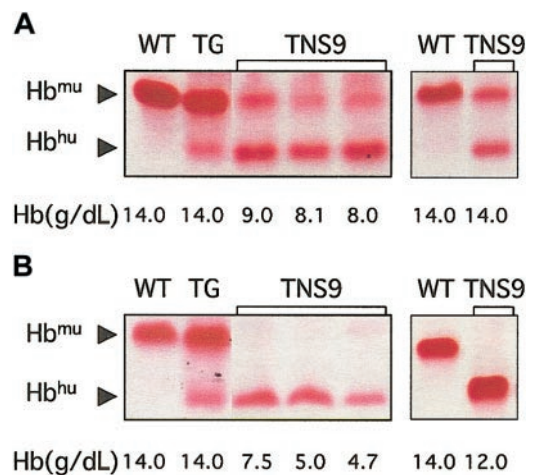
Figure 5. Hb analysis in TNS9-rescued mice. Cellulose acetate electrophoresis of peripheral blood lysates was performed monthly. Hb levels (g/dL) are indicated at the bottom of each lane. Hbᵐᵘ indicates mouse Hb tetramer; Hbʰᵘ, Hb tetramer composed of mouse α and human β chains; WT, wild-type mouse, showing Hb single; and TG, transgenic mouse blood,[9,10,15] showing hemoglobin tetramers that incorporate human βᴬ (Hbbʰᵘ, 14% of total Hb). TNS9: 4 TNS9-transduced Hbbᵗʰ³/ᵗʰ³ FLCs mice (3 on one gel on the left and one on another gel to the right) 8 weeks (A) and 4 months (B) after transplantation.

Table 1. Vector copy number (VCN) and Hb levels in long-term hematopoietic chimeras

Group genotype	n	Months after transplantation	VCN BM	VCN blood	Hb g/dL	% Hbᵸᵘ	Spleen mg
GFP$^{+/+}$	5	4-8	NA	NA	13.8 ± 1.0	0	108 ± 25
GFP$^{+/-}$	5	4-8	NA	NA	8.1 ± 0.3	0	231 ± 58
GFP$^{-/-}$	5	1.5-2	NA	NA	2.8 ± 0.8	0	800 ± 338
TNS9$^{-/-}$	6	4-8	1.6 ± 0.6	1.2 ± 0.5	6.5 ± 2.9	> 95	777 ± 382
TNS9-1$^{-/-}$	1	4	1.7	1.6	5.7	> 95	550
TNS9-2$^{-/-}$	1	8	2.4	2.0	4.7	> 97	1450
TNS9-3$^{-/-}$	1	4	1.0	1.0	4.2	> 97	829
TNS9-4$^{-/-}$	1	5	0.9	1.0	5.0	> 97	850
TNS9-5$^{-/-}$	1	6	1.4	0.7	7.5	> 97	650
TNS9-6$^{-/-}$	1	6	2.2	1.1	12.0	> 97	330

NA indicates not applicable.

analysis. In control eGFP-Hbb$^{+/+}$ chimeras, the majority (72% ± 7%) of Ter-119$^+$ cells corresponded to erythrocytes and reticulocytes,[18] shown in the top left box of each quadrant in Figure 7. This fraction decreased to 52% ± 13% in eGFP-transduced Hbb$^{th3/+}$ chimeras and was virtually absent in recipients of eGFP-Hbb$^{th3/th3}$ FLCs. In TNS9-treated Hbb$^{th3/th3}$ chimeras, this fraction was restored to 25% ± 8% (n = 4), thus directly demonstrating restoration of erythroblastic maturation.
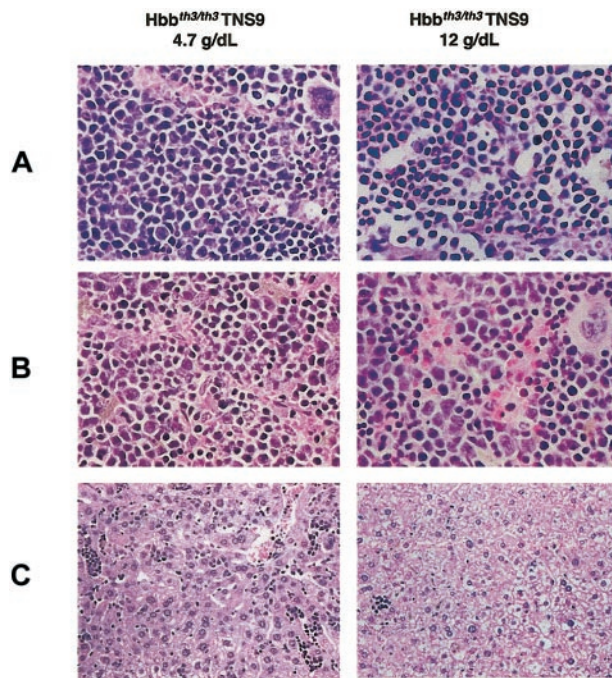

Figure 6. Histopathologic analysis of hematopoietic tissues in long-term TNS9-treated chimeras. Left: 4.7 g/dL Hb, 8 months after transplantation. Right: 12.0 g/dL Hb, 6 months after transplantation. (A) Bone marrow (original magnification, × 60). Left: There is still a relative increase in the erythroid lineage but with more evidence of maturation than in untreated chimeras (Figure 3). Myeloid maturation is seen. Right: Normal erythroid maturation is present; the erythroid precursors are not as densely packed. Myeloid maturation, still decreased compared with normal, is more frequently seen than in the mouse with the lower Hb level (left). (B) Spleen (original magnification, × 60). Left: There is more maturation and less degenerative changes in the erythroid lineage than in the untreated mice (Figure 3B, right). Right: In comparison to the mouse with lower Hb level, the sinuses appear to be somewhat less compressed. The sinusoidal erythroid cells are more eosinophilic, suggesting better hemoglobinization. The nuclei are not as irregular as in either the mouse with lower Hb levels (left) or in the Hbb$^{th3/th3}$ chimera. (C) Liver (original magnification, × 20). Left: Residual EMH, greater than in control Hbb$^{th3/+}$ chimeras (Figure 3C, center) and approaching that of the Hbb$^{th3/th3}$ chimeras (Figure 3C, left). Right: Minimal residual EMH, considerably less than in the Hbb$^{th3/+}$ controls (Figure 3C, center). Stain: hematoxylin and eosin. Hepatic iron deposition, as detected by a Gomori iron stain: the 4.7 g/dL Hb chimera was similar to the Hbb$^{th3/+}$ chimera (Figure 3D; center) and was minimal in the 12.0 g/dL Hb chimera (data not shown).

Discussion

The thorough evaluation of globin gene transfer as a potential treatment for severe hemoglobinopathies requires murine models of relevant severity in which the impact of gene transfer efficiency, transgene expression, chimerism, and the recipient's genetic background can be evaluated. Using the TNS9 vector, we previously demonstrated efficient gene transfer in bone marrow hematopoietic stem cells, tissue-specific transgene expression, and long-term correction in a mouse model of β-thalassemia intermedia.[9,10] In order to fully evaluate the therapeutic potential of TNS9 and its potential clinical applicability to the most severe hemoglobinopathies, it is essential to investigate its efficacy in the context of a fatal anemia. Here we address this requirement by establishing a novel mouse model for the most severe form of β-thalassemia, Cooley anemia. We demonstrate that mice die as a consequence of extreme ineffective erythropoiesis and that they can be rescued and cured by lentivirus-mediated transfer of the human β-globin gene.

In this new model, a severe anemia gradually develops, reaching 2-3 g/dL of Hb levels within 6 to 9 weeks. These kinetics
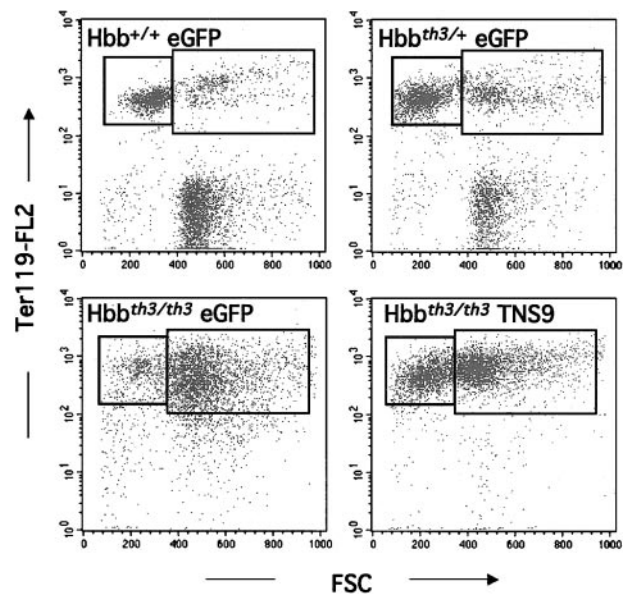
Figure 7. FACS analysis of splenic erythroid populations in long-term chimeras. Y-axis: Ter-119 expression (BD PharMingen); X-axis: cell size (forward scatter). The left box corresponds to the smaller, more mature fraction of erythroid cells. Ter119$^+$ cells represented, respectively, 47% ± 10% and 79% ± 18% of all spleen cells in eGFP-Hbb$^{+/+}$ (n = 5) and eGFP-Hbb$^{th3/+}$ (n = 5) chimeras. In recipients of eGFP-transduced Hbb$^{th3/th3}$ (n = 4), erythroid cells represented 93% ± 2% of splenocytes, and in TNS9-transduced Hbb$^{th3/th3}$, 93% ± 5% (n = 4).

are consistent with the disappearance of host RBCs[17] and the inability to generate functional RBCs from the graft. Moreover, these mice rapidly develop massive EMH and iron overload, features that are characteristic of severe β-thalassemia. This model should therefore prove useful to investigate novel treatments for congenital anemias, including pharmacologic and genetic approaches. Genetic approaches based on vectors encoding β-like globin genes, either β, γ, or mutants thereof, can be thoroughly tested in this extreme form of β-thalassemia. Lacking expression of any adult endogenous β-globin, this model provides a gold standard for quantifying globin transgene expression and comparing therapeutic vectors. By introducing selected genomic regions of the human β-like globin gene domain, in particular the γ-globin region, pharmacologic treatments for reactivating human fetal hemoglobin[19-21] also could be investigated in this adult model of disease.

Our results establish lentivirus-mediated gene transfer as an effective, expeditious, and broadly applicable approach to study and rescue lethal hematopoietic phenotypes in animal models. In the absence of any selection, fetal liver stem cells were efficiently transduced with a lentiviral vector encoding a regulated, tissue-specific gene. Complex vectors like TNS9 provide a powerful tool for rescuing lethal phenotypes obtained by targeted gene disruption as well as a novel approach for investigating gene regulation. In contrast to the multicopy concatamers typically found in transgenic mice following pronuclear DNA injection,[22] lentivirus-mediated gene transfer will be of great use in gene expression studies by enabling titration of gene delivery and thus facilitate the generation of single-copy mice. The combination of highly efficient gene transfer,[23] the ability to package large genomic elements and thus enhance gene regulation,[9] and the preferential integration into sites of active chromatin[24] make lentiviral vectors a major tool for experimental gene transfer. The same vectors can be used to either generate hematopoietic chimeras or transgenic animals through infection of embryonic stem cells.[25,26] As shown here, fetal liver cell transduction is highly efficient, enabling rapid and effective hematopoietic reconstitution and thus providing a significant practical advantage over transgenesis.

Using this model, we could determine for the first time the absolute level of human β chain production obtained in vivo in the absence of competing murine β chains. Unlike transgenic mice, hematopoietic chimeras harbor variable mixtures of transduced and nontransduced cells, with the former also varying in their vector copy number. Chimeras with endogenous repopulation in excess of 5% were not included in these analyses to rule out any contribution of residual host erythropoiesis to their pathology and survival. In the 6 long-term chimeras with negligible residual host erythropoiesis (< 5% Hbb^mu), the mean Hb levels were 6.5 ± 2.9 g/dL, in conjunction with an average vector copy number of 1.6 ± 0.6. In one chimera showing stable production of 12 g/dL Hbb^hu, the vector copy number was 2.2-2.4 in the different hematopoietic tissues. This mouse was virtually cured of anemia. The persistent, moderate EMH noted in this mouse, greater than expected for this Hb level,[10] suggests that the chimeric Hbb^hu may not be functionally equivalent to Hbb^mu.

The TNS9 vector encodes the human β-globin gene, including an extended promoter and 2 proximal enhancers, 3 large genomic fragments derived from the human β-globin locus control region, and additional synthetic GATA-1 transcription factor binding sites. Globin transcriptional control elements have been extensively studied,[27,28] without conclusively defining what minimal segments and what combination thereof are best suited to generate a miniature β-globin transcription unit. The relative roles of the locus control region and of other genetic elements spanning the human β-globin locus and its chromatin domain in regulating globin gene expression remain controversial.[29-31] The combination adopted within the TNS9 vector results from a systematic analysis of numerous combinations (TNS 1 through 9; S.R., C.M., M.S., unpublished observations). As shown here, most Hbb^{th3/th3} chimeras were rescued by TNS9. This unequivocally demonstrates that a major therapeutic benefit can be achieved in a lethal hemoglobinopathy. Using a vector closely reproducing the structure of TNS9, termed β89, which differs in one amino acid in the β-globin gene, the promoter region (−615 from the cap site in TNS9 versus −266 in β89) and boundaries of the locus control region (LCR) elements (3.2 kb in TNS9 versus 2.7 kb in β89), Pawliuk et al achieved inhibition of red blood cell dehydration and sickling with correction of hematologic parameters and splenomegaly[11] in 2 murine SCD models, Berkeley[32] and SAD.[33] This result was obtained with an average of 3 copies of the vector per cell.[11] It is unclear whether such a high vector copy number is required to ameliorate anemia or increase lifespan in these models of SCD and whether this high vector copy number is also necessary to treat murine β-thalassemia intermedia or β-thalassemia major. It would be interesting to investigate β89 and other globin vectors in the present model of β⁰-thalassemia to determine what mean vector copy number is sufficient to rescue lethal anemia. Although seemingly minimal, the differences between TNS9 and β89 may well result in different levels of β-globin production and different susceptibility to position effects. In consideration of the potential risk of insertional oncogenesis associated with randomly integrated transgenes, it is essential in our view to optimize transgene expression so as to achieve therapeutic benefits with the lowest possible vector copy number.

Based on the results obtained in the model of Cooley anemia described here, we conclude that the TNS9 lentiviral vector expresses high enough levels of human β-globin to warrant its clinical evaluation. In addition to requiring elevated and regulated globin gene expression, the prospective clinical use of lentiviral vectors requires that other efficacy and safety features be ascertained. At present, the efficacy of lentiviral vectors in transducing human HSCs appears to be at least as good as that of oncoretroviral vectors. This matter is still under investigation,[34-42] including, importantly, marking studies in nonhuman primates.[43-45] The transduction efficiency of lentiviral vectors would have to prove to be vastly superior to that of oncoretroviral vectors to justify their clinical investigation for this reason alone. In the case of the severe hemoglobinopathies, the unparalleled efficacy of lentiviral vectors in permitting stable transfer of large genomic fragments and regulation of β-globin expression provides a powerful argument in favor of their use.[46] The major safety concerns relate to the risk of generating a replication-competent lentivirus (RCL) and to that of insertional oncogenesis. The former depends on the establishment of safe packaging cell lines and dependable RCL detection assays. The hitherto positive experience with oncoretroviral vectors[47] provides an encouraging precedent for the clinical investigation of lentiviral vector systems[48,49] and suggests that the generation of safe, stable, and high-titer lentiviral packaging cell lines should be feasible.

While insertional mutagenesis is unavoidable with retroviral vectors—as for any gene transfer method resulting in random integration—the risk of insertional oncogenesis is difficult to apprehend. Although insertional oncogenesis is deemed to be a rare event,[50] 2 recent reports[51,52] raise the concern that its frequency

may have been underestimated. In both instances, genes encoding molecules involved in the regulation of cell survival, ΔLNGFR[51,53] and γ_c,[52,54] were transduced in bone marrow cells under the nonspecific transcriptional control of the retroviral long terminal repeat (LTR). In both cases, integration was adjacent to oncogenes, respectively *Evi-1* and *LMO-2*. The complete analysis of the transforming events associated with leukemogenesis in both instances is still pending and therefore preclude definitive conclusions. Importantly, lineage-restricted vectors with U3-deleted LTRs provide in principle a mean to reduce the risk of insertional oncogenesis.[55] Globin vectors encompassing the β-globin promoter are transcribed only in late stages of proerythroblastic differentiation, prior to nuclear extrusion, except for possible low-level "leakiness" at some integration sites. While the risk of gene disruption is unchanged relative to LTR-driven vectors, the risk of oncogene activation would therefore be confined to a single lineage. The risk of promoter transactivation by vector-encoded enhancers could be further reduced by the incorporation into the vector of insulators or other elements with enhancer-blocking activity.[56,57] A comprehensive definition of the risk of insertional oncogenesis will require extensive studies over time. Ultimately, like any other medical decision, therapy will be best guided by a rational risk-benefit assessment. Based on our prior reports[9,10] and the present one, we conclude that lentiviral vectors are very promising drugs for the treatment of β-thalassemia intermedia and β-thalassemia major.

Acknowledgments

References

1. Forget BG. Molecular mechanism of β thalassemia. Steinberg MH, Forget BG, Higgs DR, Nagel RL, eds. Disorders of Hemoglobin: Genetics, Pathophysiology and Clinical Management. Cambridge, United Kingdom: Cambridge University Press; 2001:252-276.

2. Weatherall DJ. Phenotype-genotype relationships in monogenic disease: lessons from the thalassaemias. Nat Rev Genet. 2001;2:245-255.

3. Orkin SH, Nathan DG. Hematology of Infancy and Childhood. Philadelphia, PA: WB Saunders Co; 1998:811-886.

4. Weatherall D. The Molecular Basis of Blood Diseases. Philadelphia, PA: WB Saunders Co; 2001:183-226.

5. Cooley TB, Lee P. A series of cases of splenomegaly in children with anemia and peculiar bone changes. Trans Am Pediatr Soc. 1925;37:29.

6. Tisdale J, Sadelain M. Toward gene therapy for disorders of hemoglobin synthesis. Semin Hematol. 2001;38:382-392.

7. Rivella S, Sadelain M. Genetic treatment of severe hemoglobinopathies: the combat against transgene variegation and transgene silencing. Semin Hematol. 1998;35:112-125.

8. Persons DA, Nienhuis AW. Gene therapy for the hemoglobin disorders: past, present, and future. Proc Natl Acad Sci U S A. 2000;97:5022-5024.

9. May C, Rivella S, Callegari J, et al. Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin. Nature. 2000;406:82-86.

10. May C, Rivella S, Chadburn A, Sadelain M. Successful treatment of murine beta-thalassemia intermedia by transfer of the human beta-globin gene. Blood. 2002;99:1902-1908.

11. Pawliuk R, Westerman KA, Fabry ME, et al. Correction of sickle cell disease in transgenic mouse models by gene therapy. Science. 2001;294:2368-2371.

12. Skow LC, Burkhart BA, Johnson FM, et al. A mouse model for beta-thalassemia. Cell. 1983;34:1043-1052.

13. Shehee WR, Oliver P, Smithies O. Lethal thalassemia after insertional disruption of the mouse major adult beta-globin gene. Proc Natl Acad Sci U S A. 1993;90:3177-3181.

14. Yang B, Kirby S, Lewis J, Detloff PJ, Maeda N, Smithies O. A mouse model for beta 0-thalassemia. Proc Natl Acad Sci U S A. 1995;92:11608-11612.

15. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vectors achieve efficient gene delivery in vivo. Nat Biotechnol. 1997;15:871-875.

16. Porcu S, Kitamura M, Witkowska E, et al. The human beta globin locus introduced by YAC transfer exhibits a specific and reproducible pattern of developmental regulation in transgenic mice. Blood. 1997;90:4602-4609.

17. Franco RS, Lohmann J, Silberstein EB, et al. Time-dependent changes in the density and hemoglobin F content of biotin-labeled sickle cells. J Clin Invest. 1998;101:2730-2740.

18. Zhang D, Johnson MM, Miller CP, Pircher TJ, Geiger JN, Wojchowski DM. An optimized system for studies of EPO-dependent murine pro-erythroblast development. Exp Hematol. 2001;29:1278-1288.

19. Pace BS, Li Q, Stamatoyannopoulos G. In vivo search for butyrate responsive sequences using transgenic mice carrying A gamma gene promoter mutants. Blood. 1996;88:1079-1083.

20. Steinberg MH, Rodgers GP. Pharmacologic modulation of fetal hemoglobin. Medicine (Baltimore). 2001;80:328-344.

21. Rodgers GP, Saunthararajah Y. Advances in experimental treatment of beta-thalassaemia. Expert Opin Investig Drugs. 2001;10:925-934.

22. Ellis J, Pasceri P, Tan-Un KC, et al. Evaluation of beta-globin gene therapy constructs in single copy transgenic mice. Nucleic Acids Res. 1997;25:1296-1302.

23. Naldini L, Blomer U, Gallay P, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science. 1996;272:263-267.

24. Schroder A, Shinn P, Chen H, Berry C, Ecker J, Bushman F. HIV-1 integration in the human genome favors active genes and local hotspots. Cell. 2002;110:521-529.

25. Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science. 2002;295:868-872.

26. Pfeifer A, Ikawa M, Dayn Y, Verma IM. Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. Proc Natl Acad Sci U S A. 2002;99:2140-2145.

27. Molete JM, Petrykowska H, Bouhassira EE, Feng YQ, Miller W, Hardison RC. Sequences flanking hypersensitive sites of the beta-globin locus control region are required for synergistic enhancement. Mol Cell Biol. 2001;21:2969-2980.

28. Li Q, Harju S, Peterson KR. Locus control regions: coming of age at a decade plus. Trends Genet. 1999;15:403-408.

29. Higgs DR. Do LCRs open chromatin domains? Cell. 1998;95:299-302.

30. Grosveld F. Activation by locus control regions? Curr Opin Genet Dev. 1999;9:152-157.

31. Bulger M, Groudine M. Looping versus linking: toward a model for long-distance gene activation. Genes Dev. 1999;13:2465-2477.

32. Paszty C, Brion CM, Manci E, et al. Transgenic knockout mice with exclusively human sickle hemoglobin and sickle cell disease. Science. 1997;278:876-878.

33. Trudel M, Saadane N, Garel MC, et al. Towards a transgenic mouse model of sickle cell disease: hemoglobin SAD. EMBO J. 1991;10:3157-3165.

34. Case SS, Price MA, Jordan CT, et al. Stable transduction of quiescent CD34(+)CD38(-) human hematopoietic cells by HIV-1-based lentiviral vectors. Proc Natl Acad Sci U S A. 1999;96:2988-2993.

35. Sutton RE, Reitsma MJ, Uchida N, Brown PO. Transduction of human progenitor hematopoietic stem cells by human immunodeficiency virus type 1-based vectors is cell cycle dependent. J Virol. 1999;73:3649-3660.

36. Guenechea G, Gan OI, Inamitsu T, et al. Transduction of human CD34+ CD38- bone marrow and cord blood-derived SCID-repopulating cells with third-generation lentiviral vectors. Mol Ther. 2000;1:566-573.

37. Heim DA, Dunbar CE. Hematopoietic stem cell gene therapy: towards clinically significant gene transfer efficiency. Immunol Rev. 2000;178:29-38.

38. Sadelain M, Frassoni F, Riviere I. Issues in the manufacture and transplantation of genetically modified hematopoietic stem cells. Curr Opin Hematol. 2000;7:364-377.

39. Sirven A, Ravet E, Charneau P, et al. Enhanced transgene expression in cord blood CD34(+)-derived hematopoietic cells, including developing T cells and NOD/SCID mouse repopulating cells, following transduction with modified trip lentiviral vectors. Mol Ther. 2001;3:438-448.

40. Luther-Wyrsch A, Costello E, Thali M, et al. Stable transduction with lentiviral vectors and amplification of immature hematopoietic progenitors from cord blood of preterm human fetuses. Hum Gene Ther. 2001;12:377-389.

41. Donahue RE, Sorrentino BP, Hawley RG, An DS, Chen IS, Wersto RP. Fibronectin fragment CH-296 inhibits apoptosis and enhances ex vivo gene transfer by murine retrovirus and human lentivirus vectors independent of viral tropism in nonhuman primate CD34+ cells. Mol Ther. 2001;3:359-367.

42. Zielske SP, Gerson SL. Lentiviral transduction of P140K MGMT into human CD34(+) hematopoietic progenitors at low multiplicity of infection confers significant resistance to BG/BCNU and allows selection in vitro. Mol Ther. 2002;5:381-387.

43. Donahue RE, Dunbar CE. Update on the use of nonhuman primate models for preclinical testing

of gene therapy approaches targeting hematopoietic cells. Hum Gene Ther. 2001;12:607-617.

44. An DS, Kung SK, Bonifacino A, et al. Lentivirus vector-mediated hematopoietic stem cell gene transfer of common gamma-chain cytokine receptor in rhesus macaques. J Virol. 2001;75: 3547-3555.

45. Horn PA, Morris JC, Bukovsky AA, et al. Lentivirus-mediated gene transfer into hematopoietic repopulating cells in baboons. Gene Ther. 2002;9:1464-1471.

46. Rivella S, Sadelain M. Therapeutic globin gene delivery using lentiviral vectors. Curr Opin Mol Ther. 2002;4:505-514.

47. Cornetta K, Smith FO. Regulatory issues for clinical gene therapy trials. Hum Gene Ther. 2002;13:1143-1149.

48. Sadelain M, Riviere I. The advent of lentiviral vectors: prospects for cancer therapy. In: Gerson SL and Lattime EC, eds. Gene Therapy of Cancer. 2nd ed. New York, NY: Academic Press; 2002:109-124.

49. Salon P, Trono D. Lentiviral vectors for the gene therapy of lympho-hematological disorders. Curr Top Microbiol Immunol. 2002;261:211-227.

50. Stocking C, Bergholz U, Friel J, et al. Distinct classes of factor-independent mutants can be isolated after retroviral mutagenesis of a human myeloid stem cell line. Growth Factors. 1993;8:197-209.

51. Li Z, Dullmann J, Schiedlmeier B, et al. Murine leukemia induced by retroviral gene marking. Science. 2002;296:497.

52. Hacein-Bey-Abina S, von Kalle C, Schmidt M, et al. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med. 2003;348:255-256.

53. Sadelain M, Riviere I. Sturm und drang over suicidal lymphocytes. Mol Ther. 2002;5:655-657.

54. Hacein-Bey-Abina S, Le Deist F, Carlier F, et al. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. N Engl J Med. 2002;346:1185-1193.

55. Sadelain M. Globin gene transfer for the treatment of severe hemoglobinopathies: a paradigm for stem cell-based gene therapy. J Gene Med. 2002;4:113-121.

56. Rivella S, Callegari J, May C, Tan C, Sadelain M. The cHS4 insulator increases the probability of retroviral expression at random chromosomal integration sites. J Vir. 2000;74:4679-4687.

57. Emery DW, Yannaki E, Tubb J, Stamatoyannopoulos G. A chromatin insulator protects retrovirus vectors from chromosomal position effects. Proc Natl Acad Sci U S A. 2000;97:350-354.