Correction of Sickle Cell Disease in Transgenic Mouse Models by Gene Therapy

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Science **294**, 2368 (2001);
DOI: 10.1126/science.1065806

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MIPS (www.mips.biochem.mpg.de/). Yeast genetic and protein-interaction data sets were obtained from YPD and MIPS databases. Cellular role and biochemical functions were obtained from YPD.

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28. Because double mutants are created by meiotic crossover, a set of gene deletions that are linked to the query gene, which we refer to as the "linkage group," form double mutants at reduced frequency. For example, the gene-deletion mutations of 13 different genes that are linked to *BNI1* and contained on the array [*YNL253W*, *YNL254C*, *YNL255C*, *YNL257C*, *YNL259C*, *YNL264C*, *YNL265C*, *YNL266W*, *YNL268W*, *YNL270W*, *BNI1* (*YNL271W*), *YNL273W*, and *YNL275W*] appeared to be synthetically lethal with the *bni1*Δ query mutation. A list of the gene deletions that were linked to the query gene and appeared to be synthetically lethal with the query gene is provided as supplementary material (*16*). Thus, systematic random spore analysis with the set of gene-deletion mutants provides a method for genetically mapping mutations that are linked to a dominant selectable marker. Similarly, alleles that are associated with a gain-of-function phenotype can also be mapped. For example, through identification of linkage groups that are defective for the gain-of-function phenotype, we should be able to map mutations that result in filamentous growth or high-temperature growth, neither of which are associated with the S288C strains within the deletion array, or suppressors of the lethality associated with temperature-sensitive alleles linked to a dominant selectable marker.
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30. Each of the deletion mutations is marked with two unique oligonucleotide bar codes that were integrated along with common flanking primer sites for PCR amplification (*34*). Because the bar codes allow the growth rate of all the deletion mutants to be followed within a population of cells, the steps for creating double mutants outlined in Fig. 1 could be carried out with a pool of deletion mutants. In this scheme, synthetic lethality or slow growth of the resulting double mutants would be analyzed by PCR amplification of the bar codes and subsequent hybridization to a bar-code microarray, such that the intensity of the signal observed for an element on the bar-code array reflects the representation of the double-mutant meiotic progeny (*35*). As another means of large-scale synthetic lethal analysis, a collection of deletion mutant strains can be transformed en masse with a gene-deletion cassette and the lethal double-mutant transformants predicted by bar-code microarray analysis (*36*).
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37. We thank B. Drees, S. Fields, and J. Friesen for advice on the robotic manipulation of yeast arrays; C. Udell for construction of the *MFA1pr-HIS3* reporter; P. Jorgensen for discussions concerning SGA mapping of gain-of-function mutations; and G. Brown, N. Davis, D. Durocher, S. Gasser, S. Fields, T. Hughes, and T. Roemer for comments on the manuscript. Supported by grants from the Canadian Institute of Health Research (B.A., C.B., C.H., and M.T.), an operating grant from the National Cancer Institute of Canada (C.B.), and an operating grant from the Natural Sciences and Engineering Research Council of Canada (H.B.).

28 August 2001; accepted 13 November 2001

Correction of Sickle Cell Disease in Transgenic Mouse Models by Gene Therapy

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Sickle cell disease (SCD) is caused by a single point mutation in the human β^A globin gene that results in the formation of an abnormal hemoglobin [HbS $(\alpha_2\beta^S{}_2)$]. We designed a β^A globin gene variant that prevents HbS polymerization and introduced it into a lentiviral vector we optimized for transfer to hematopoietic stem cells and gene expression in the adult red blood cell lineage. Long-term expression (up to 10 months) was achieved, without preselection, in all transplanted mice with erythroid-specific accumulation of the antisickling protein in up to 52% of total hemoglobin and 99% of circulating red blood cells. In two mouse SCD models, Berkeley and SAD, inhibition of red blood cell dehydration and sickling was achieved with correction of hematological parameters, splenomegaly, and prevention of the characteristic urine concentration defect.

Sickle cell disease (SCD) is one of the most prevalent autosomal recessive disorders worldwide. In 1957, SCD became the first genetic disorder for which a causative mutation was identified at the molecular level: the substitution of valine for glutamic acid in human β^A-globin codon 6 (*1*). In homozygotes, the abnormal hemoglobin (Hb) [HbS $(\alpha_2\beta^S{}_2)$] polymerizes in long fibers upon deoxygenation within red blood cells (RBCs), which become deformed ("sickled"), rigid, and adhesive, thereby triggering microcirculation occlusion, anemia, infarction, and organ damage (*2, 3*).

Human γ-globin is a strong inhibitor of HbS polymerization, in contrast to human β^A-globin, which is effective only at very high concentrations (*4*). Hence, gene therapy of SCD was proposed by means of forced expression of human γ-globin or γ/β hybrids in adult RBCs after gene transfer to hematopoietic stem cells (HSCs) (*5–11*).

Although the discovery of the human β-globin locus control region (LCR) held promise to achieve high globin gene expression levels (*12, 13*), the stable transfer of murine onco-retroviral vectors encompassing minimal core elements of the LCR proved especially challenging (*14–20*). To allow the transfer of larger LCR and globin gene sequences, we proposed the use of RNA splicing and export controlling elements that include the Rev/R responsive element (RRE) components of human immunodeficiency virus (HIV) (*21*), and an RRE-bearing HIV-based lentiviral vector recently resulted in substantial amelioration of β-thalassemia in transplanted mice (*22*). However, gene expression remained heterocellular, and the amount of human β^A-globin found incorporated in Hb tetramers in a nonthalassemic background is unlikely to be therapeutic for SCD (*22*). Here, a lentiviral vector was optimized to express an antisickling protein at therapeutic levels in virtually all circulating RBCs of SCD mouse models.

We constructed a human β^A-globin gene variant mutated at codon 87 to encode the amino-acid residue believed to be responsible

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for most of the antisickling activity of γ-globin [β^{A87} Thr:Gln ($\beta^{\text{A-T87Q}}$)] (*23*). To assess first its antisickling capacity and oxygen-binding affinity, we generated transgenic mice expressing both human $\beta^{\text{A-T87Q}}$- and α-globins, but neither mouse α- nor mouse β-globin. These mice had normal hematological parameters and viability, and the $\beta^{\text{A-T87Q}}$-globin variant extracted from their RBCs was found to be almost as potent an inhibitor of HbS polymerization as γ-globin in vitro and much more so than β^{A}-globin (Supplementary fig. 1A) (*24*). Whole-blood analysis of p50, the pO_2 at which 50% of the Hb molecules are oxygenated, showed that the oxygen-binding affinity of $\beta^{\text{A-T87Q}}$ Hb was well within the range observed with wild-type β^{A} Hb in mice: 31.1 ± 0.2 mm Hg (standard error = SE) versus 32.7 ± 1.8 mm Hg (SE), respectively (*24*).

The $\beta^{\text{A-T87Q}}$-globin gene variant was then inserted in a lentiviral vector we optimized for transfer to HSCs and erythroid-specific expression. The central polypurine tract/DNA flap of HIV-1 (*25*) was incorporated in the construct to increase viral titers and transduction of HSCs after pseudotyping with the vesicular stomatitis virus glycoprotein G (VSV-G) and concentration (Supplementary fig. 1C) (*24*). Specific LCR elements were chosen on the basis of results of single integrants in erythroid cells assessed by recombinase-mediated cassette exchange (*26*).

The $\beta^{\text{A-T87Q}}$-globin lentivirus was first analyzed in lethally irradiated normal syngeneic C57BL/6 recipient mice in the absence of any selection (*24*). Proviral transfer was stable with an average copy number of 3.0 ± 0.5 (SE) per genome of peripheral nucleated blood cells 3 months after transplantation (Supplementary fig. 1B) (*24*). At 10 months after transplantation, all mice expressed human $\beta^{\text{A-T87Q}}$-globin protein with up to 99% [mean 96 ± 0.9% (SE)] of their RBCs staining positive with an antibody that specifically recognizes human β-globin, in this case, the $\beta^{\text{A-T87Q}}$ variant (Fig. 1A) (*24*). No $\beta^{\text{A-T87Q}}$-globin expression was detected in other blood lineages by antibody staining. Human $\beta^{\text{A-T87Q}}$-globin mRNA reached up to 107% [mean 71 ± 15% (SE)] of endogenous mouse β-single globin mRNA transcripts (Fig. 1B) (*24*). Human $\beta^{\text{A-T87Q}}$-globin protein represented up to 22.5% [mean 16 ± 3.1% (SE)] of endogenous mouse β-chains in recipients of $\beta^{\text{A-T87Q}}$-globin lentivirus-transduced bone marrow, as determined by high-performance liquid chromatography (HPLC) (Fig. 1C) (*24*). The fourfold discrepancy between human $\beta^{\text{A-T87Q}}$-globin mRNA and protein levels is consistent with differences observed in mice transgenic for the β^{A}-globin gene (*27*).

Long-term secondary transplants were also performed with bone marrow from a representative primary recipient killed 5 months after

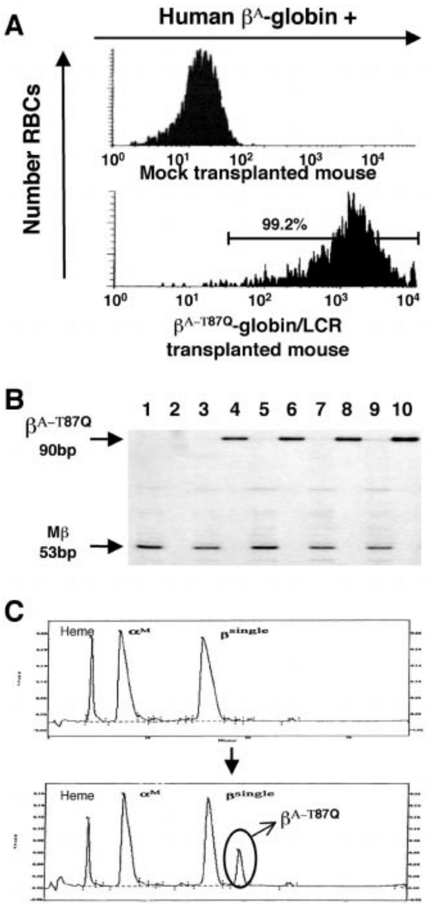

Fig. 1. Analysis of human $\beta^{\text{A-T87Q}}$-globin gene expression in C57BL/6 recipient mice 5 months after transplantation. (**A**) Circulating RBCs from recipient mice were fixed, permeabilized, stained with a FITC-labeled antibody that specifically recognizes human β-globin (Perkin-Elmer Wallac, Norton, Ohio), and subsequently analyzed by FACS (*24*). Top: representative mouse transplanted with mock-transduced bone marrow cells. Bottom: representative mouse transplanted with bone marrow transduced with the $\beta^{\text{A-T87Q}}$-globin lentivirus. (**B**) Primer extension analysis of peripheral blood RNA (*24*). Lanes 1, 3, 5, 7, and 9: amplification with primers specific for the endogenous murine β-single globin mRNA generating a 53–base pair (bp) DNA fragment. Lanes 2, 4, 6, 8, and 10: amplification with primers specific for the human $\beta^{\text{A-T87Q}}$-globin mRNA generating a 90-bp DNA fragment. Lanes 1 and 2: mock-transduced mouse. Lanes 3 and 4: transgenic control mouse expressing 86% of human β-globin mRNA. Lanes 5 to 10: three C57BL/6 recipients of $\beta^{\text{A-T87Q}}$-globin–transduced bone marrow cells (lanes 5 and 6, mouse #1; 7 and 8, mouse #2; 9 and 10, mouse #3). (**C**) HPLC profiles of globin chains extracted from RBCs of a mock-transduced mouse (top) and a recipient of human $\beta^{\text{A-T87Q}}$-globin–transduced bone marrow (bottom) (*24*).

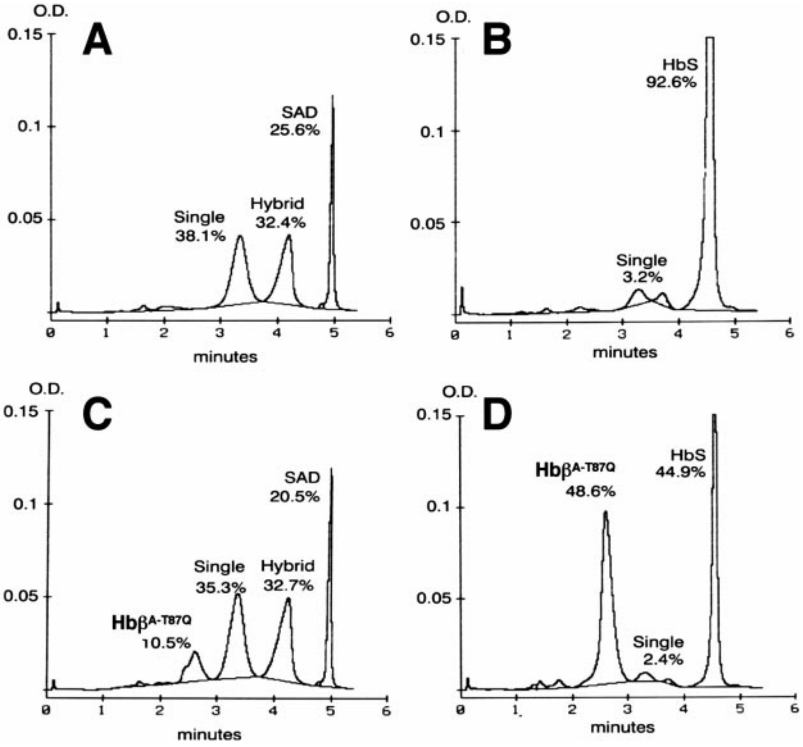

Fig. 2. HPLC profiles of Hb extracted from RBCs of mouse recipients of (**A**) mock-transduced SAD, (**B**) mock-transduced BERK, (**C**) $\beta^{\text{A-T87Q}}$-globin–transduced SAD, and (**D**) $\beta^{\text{A-T87Q}}$-globin–transduced BERK bone marrow cells (*24*).

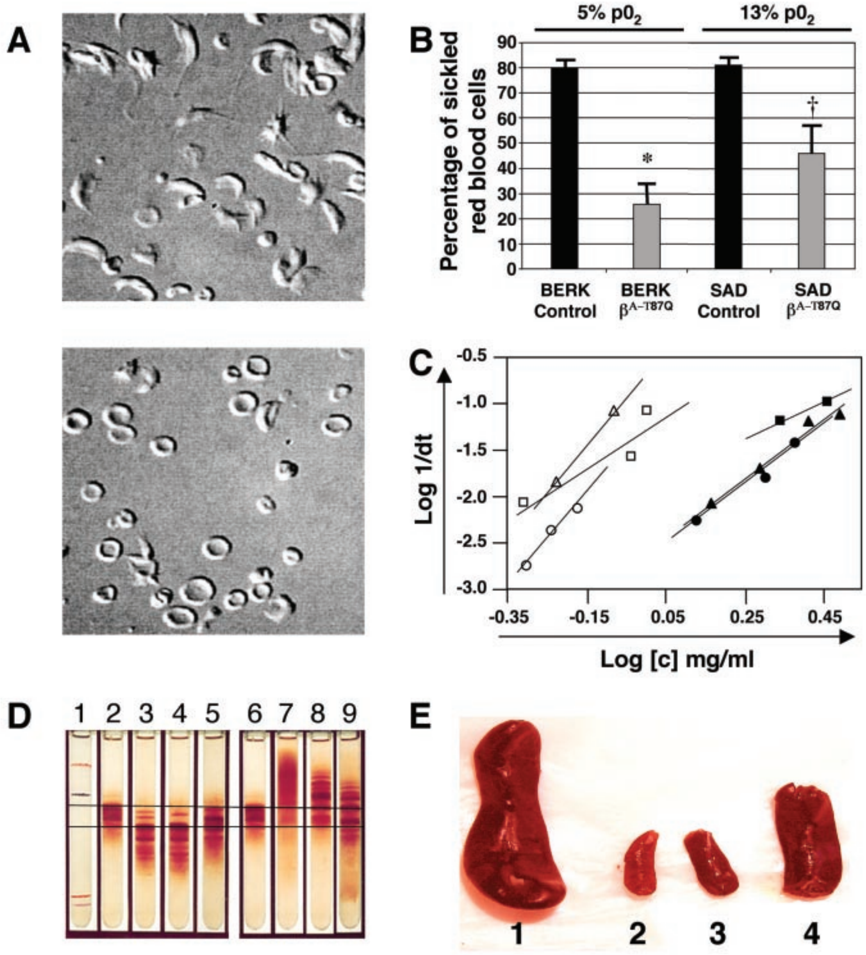

Fig. 3. Correction of SCD pathology. (**A**) Nomarski optics microscopy of RBCs from mice transplanted with either (top) mock- or (bottom) $\beta^{A\text{-}T87Q}$-globin lentivirus–transduced BERK bone marrow cells under 5% pO_2 3 months after transplantation (*24*). (**B**) Quantification of the percentage of sickle RBCs from recipients of mock-transduced and $\beta^{A\text{-}T87Q}$-globin–transduced BERK or SAD bone marrow under 5% or 13% oxygen conditions, respectively (*24*). Error bars indicate SE; *, $P = 0.01$; †, $P = 0.03$. (**C**) Relationship between log of reciprocal delay time (dt) of HbS polymerization and Hb concentration of RBC lysates. Time courses of Hb polymerization in lysates were performed at various concentrations by the temperature jump method (*24*). △, lysate from a homozygote SS patient; ▲, lysate from an asymptomatic AS sickle cell trait patient; □, lysate from a mouse recipient of mock-transduced SAD marrow; ■, lysate from a mouse recipient of β^{A-T87Q}-globin–transduced SAD marrow; ○, lysate from a mouse recipient of mock-transduced BERK marrow; ●, lysate from a mouse recipient of β^{A-T87Q}-globin–transduced BERK marrow. (**D**) Percoll-Larex continuous density gradients from blood of recipient mice (*24*). Lane 1, density marker beads; lanes 2 and 6, C57BL/6 controls; lanes 3 and 7, SAD and BERK controls, respectively; lanes 4 and 5, C57BL/6 recipients of mock-transduced or $\beta^{A\text{-}T87Q}$-transduced SAD bone marrow, respectively; lane 8, C57BL/6 recipient of $\beta^{A\text{-}T87Q}$-transduced BERK bone marrow; lane 9 transgenic BERK mouse expressing human γ-globin at ~100% of β^S-globin. (**E**) Spleens from nontransplanted (1) BERK and (2) C57BL/6 mice, or C57BL/6 mice transplanted with either (3) $\beta^{A\text{-}T87Q}$-transduced or (4) mock-transduced BERK bone marrow.

transplantation (*24*). Fluorescence-activated cell sorting (FACS) analysis of peripheral blood samples of secondary recipients 4 months after transplantation showed that 87 ± 2.3 (SE) of RBCs expressed high levels of human $\beta^{A\text{-}T87Q}$-globin protein, thus demonstrating that transduction of true HSCs was achieved. Analysis of position effect variegation suggested that pancellular expression was the result of balanced expression from polyclonal stem cell reconstitution with multiple chromosomal integration sites rather than true position-independent expression (*24*).

Because no transgenic mouse model perfectly recapitulates the exact disease characteristics of human SCD patients (*28*–*33*), we investigated the efficacy of the $\beta^{A\text{-}T87Q}$-globin lentiviral vector in two different SCD transgenic mouse models: SAD (*29*) and Berkeley (BERK) (*31*). SAD mice express human α-globin together with a "super S" globin resulting from two point mutations added to the human β^S gene (*29*), whereas BERK mice, which express human α- and human β^S-globulins, do not express any murine globins because of complete disruption of both mouse α- and β-globin gene loci (*31*). The phenotype of BERK mice is overall more severe than that of SAD mice, although some of the hematological abnormalities in BERK mice are caused by an associated β-thalassemic syndrome due to suboptimal expression of the transgenic human β^S gene (*28*).

SAD and BERK bone marrow was transduced with the $\beta^{A\text{-}T87Q}$-globin lentiviral vector and transplanted into lethally irradiated syngeneic C57BL/6 mouse recipients (*24*). Transduced SAD marrow was also transplanted into lethally irradiated syngeneic SAD recipients. Three months after transplantation, reconstitution of recipient C57BL mice with donor BERK or SAD bone marrow was essentially complete for all mice, as determined by quantification of murine β-single Hb by HPLC (Fig. 2) (*24*).

Isoelectric focusing electrophoresis of blood samples from mice 3 months after transplantation showed all of the expected species of Hb (Supplementary fig. 2) (*24*). The amount of $\beta^{A\text{-}T87Q}$-globin expressed in the transplanted mice, as measured by Hb HPLC, was up to 108% [mean 75.5 ± 17.1% (SE)] and 51% [mean 42.5 ± 5.5% (SE)] of the transgenic HbS for recipients of $\beta^{A\text{-}T87Q}$-globin lentivirus–transduced BERK and SAD bone marrow, respectively (Fig. 2, C and D) (*24*). These values correspond to up to 52% and 12% of the total Hb of BERK and SAD mice, respectively. The greater amount of $\beta^{A\text{-}T87Q}$-globin–containing Hb observed in erythrocytes derived from transduced bone marrow cells of BERK mice as compared to SAD mice may be explained by the absence of the murine β-globulin mRNA and the associated thalassemic phenotype of BERK mice, which favors translation of the added $\beta^{A\text{-}T87Q}$-globin mRNA species (*28*).

To determine whether $\beta^{A\text{-}T87Q}$-globin was capable of inhibiting HbS polymerization in vivo in transplanted SCD mouse models, the morphology of RBCs from transplanted mice was analyzed as a function of oxygen pressure in vitro (*24*). Examination of the obtained sigmoid sickling curves showed a marked change in the proportion of sickled cells (Fig. 3, A and B). For recipients of $\beta^{A\text{-}T87Q}$-globin lentivirus–transduced BERK marrow, the greatest difference occurred at 5% pO_2, with 80 ± 1.7% (SE) versus 26 ± 7.5% (SE) ($P = 0.01$) sickle cells for mock-transduced and $\beta^{A\text{-}T87Q}$-globin lentivirus–transduced marrow, respectively. In comparison, analysis of RBCs from humans with sickle trait, who are heterozygous for the β^S allele and asymptomatic, showed ~40% sickled cells at 5% pO_2. For SAD marrow, the greatest difference occurred at 13% pO_2, with 81 ± 3% (SE) versus 46 ± 11% (SE) ($P = 0.03$) sickle cells for mock-transduced and $\beta^{A\text{-}T87Q}$-globin lentivirus–transduced marrow, respectively. Examination of peripheral blood

Table 1. Correction of hematological abnormalities and urine concentrating defect in recipients of $\beta^{A\text{-}T87Q}$-globin–transduced BERK bone marrow. RBCs, red blood cells; Hb, hemoglobin; ISCs, irreversibly sickled cells. Values shown with SE and statistical significance established by Student's *t* test.

Mice*	RBCs ($10^6/\mu l$)	Hb (g/dl)	Reticulocytes (%)	ISCs† (‰)	Urine concentrations (mOsM) (number of mice)
C57Bl/6 controls ($n = 3$)	10.1 ± 0.3	15.0 ± 0.6	4.1 ± 0.6	–	3247 ± 500 ($n = 22$)
BERK controls ($n = 3$)	7.4 ± 0.6	9.4 ± 0.9	17.8 ± 0.6	16.0‡	1452 ± 331 ($n = 4$)
BERK $\beta^{A\text{-}T87Q}$ ($n = 3$)	10.1 ± 1.1§	13.0 ± 0.4‖	5.8 ± 1.8¶	2.0#	3600 ± 381 ($n = 2$)**
SAD controls ($n = 4$)	8.4 ± 0.6	13.0 ± 0.6	3.4 ± 1.2	2.6#	3840 ± 175 ($n = 3$)
SAD $\beta^{A\text{-}T87Q}$ ($n = 3$)	8.7 ± 0.1	13.7 ± 0.2	2.8 ± 0.1	0	3920 ± 326 ($n = 3$)

**n* is the number of mice for RBCs, Hb, and reticulocytes. †A total of 2000 RBCs were examined from BERK control and BERK $\beta^{A\text{-}T87Q}$ mice ($n = 2$) and 3000 RBCs were examined from SAD control and SAD $\beta^{A\text{-}T87Q}$ mice ($n = 2$). ‡Mostly dehydrated ISCs. §$P = 0.15$ with substantial correction of anisocytosis and poikilocytosis. ‖$P = 0.01$. ¶$P = 0.05$. #Only hydrated ISCs. **$P = 0.01$.

smears at ambient pO_2 showed an eightfold decrease in the proportion of irreversibly sickled cells (ISCs) in mice transplanted with $\beta^{A\text{-}T87Q}$-globin lentivirus–transduced BERK marrow with complete disappearance of highly dehydrated ISCs. For SAD mice, no ISCs could be detected after $\beta^{A\text{-}T87Q}$-globin lentivirus–transduction (Table 1).

Kinetic studies of HbS polymer formation by turbidimetry of RBC lysates from transplanted mice showed delayed HbS polymerization in lysates from mice transplanted with either SAD or BERK marrow transduced with the $\beta^{A\text{-}T87Q}$-globin lentivirus (Fig. 3C) (*24*). The change in kinetics paralleled what was observed with RBC lysates from homozygote SS patients versus asymptomatic AS heterozygotes (Fig. 3C).

We next examined the density of RBCs from transplanted SCD mouse models, since HbS polymerization causes an abnormally high cell density (*11, 28*). Whereas RBCs from control and mock-transduced SAD mice had a higher density than those of syngeneic C57BL/6 mice, mice completely reconstituted with $\beta^{A\text{-}T87Q}$-globin lentivirus–transduced SAD marrow showed a clear shift toward normal (Fig. 3D) (*24*). In BERK RBCs, the phenomenon was reversed, because the associated thalassemic phenotype decreases the mean corpuscular Hb concentration, resulting in lower cell density. The addition of $\beta^{A\text{-}T87Q}$-globin partially cured the thalassemia and resulted in higher cell density (Fig. 3D).

Unlike SAD mice, BERK mice have major alterations of their hematological parameters, as a consequence of both SCD and the associated thalassemia (*28, 31*). In mice transplanted with $\beta^{A\text{-}T87Q}$-globin lentivirus–transduced BERK marrow, RBC and reticulocyte counts were corrected with amelioration of Hb concentration, anisocytosis, and poikilocytosis (Table 1) (*24*).

We finally examined whether the SCD-associated splenomegaly and characteristic urine concentration defect in BERK mice (*28, 31*) could be ameliorated by gene therapy. Following transplantation of $\beta^{A\text{-}T87Q}$-globin lentivirus–transduced BERK bone marrow, both pathological features were corrected, whereas no effect was observed for recipients of mock-transduced BERK marrow (Table 1 and Fig. 3E) (*24*).

These data demonstrate that chromosomal integration of an antisickling globin gene variant in HSCs can result in its pancellular, erythroid-specific expression at levels sufficiently high to correct the main pathological features of SCD. In contrast to β-thalassemia, gene therapy of SCD requires expression of the therapeutic gene in most RBCs to prevent untoward vaso-occlusion by even a small fraction of sickle cells (*2, 3*). This criterion presented a major obstacle, since the LCR, even in its largest structural form, does not completely shield *cis*-linked genes from position-effect variegation in most settings in the absence of chromatin insulators (*34*). Here, structural optimization of the $\beta^{A\text{-}T87Q}$-globin gene/LCR lentivirus by recombination-mediated cassette exchange and incorporation of the central polypurine tract–DNA flap of HIV-1 resulted in very high viral titers yielding multiple events of chromosomal integration per HSC. This led to a state of balanced expression sufficiently high and homogeneous enough to surmount this hurdle and provide an overall protection similar to that observed in asymptomatic human AS heterozygotes.

Before gene therapy of SCD may be proposed to human patients on the basis of these preclinical results, achieving large-scale lentiviral production devoid of replication-competent retrovirus and bone marrow reconstitution with transduced stem cells in the absence of toxic myeloablation regimens remain desirable objectives.

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35. We thank A. Bank and S. Goff for HIV components and critical review of the manuscript; R. M. Bookchin and Z. Etzion for Bayer-Technicon red cell analysis; S. Pack, P. Rouyer-Fessard, and S. Suzuka for expert technical assistance; R. Nancel for art work; Supported by NIH grant HL554352 (M.E.F., I.M.L., C.E., R.K.H, R.L.N., P.L.), INSERM (Y.B., P.L.) and Association Française contre la Myopathie (Y.B., P.L.).

28 August 2001; accepted 30 October 2001