

Gene Therapy of a Mouse Model of Protoporphyrin with a Self-Inactivating Erythroid-Specific Lentiviral Vector without Preselection

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Successful treatment of blood disorders by gene therapy has several complications, one of which is the frequent lack of selective advantage of genetically corrected cells. Erythropoietic protoporphyria (EPP), caused by a ferrochelatase deficiency, is a good model of hematological genetic disorders with a lack of spontaneous *in vivo* selection. This disease is characterized by accumulation of protoporphyrin in red blood cells, bone marrow, and other organs, resulting in severe skin photosensitivity. Here we develop a self-inactivating lentiviral vector containing human ferrochelatase cDNA driven by the human ankyrin-1/ α -globin HS-40 chimeric erythroid promoter/enhancer. We collected bone marrow cells from EPP male donor mice for lentiviral transduction and injected them into lethally irradiated female EPP recipient mice. We observed a high transduction efficiency of hematopoietic stem cells resulting in effective gene therapy of primary and secondary recipient EPP mice without any selectable system. Skin photosensitivity was corrected for all secondary engrafted mice and was associated with specific ferrochelatase expression in the erythroid lineage. An erythroid-specific expression was sufficient to reverse most of the clinical and biological manifestations of the disease. This improvement in the efficiency of gene transfer with lentiviruses may contribute to the development of successful clinical protocols for erythropoietic diseases.

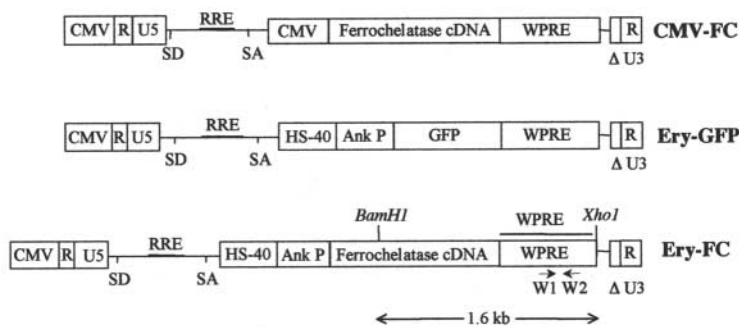
Key words: ankyrin-1 promoter, erythroid specificity, ferrochelatase, gene transfer, gene therapy, hematopoietic stem cell, lentiviral vectors, porphyria, skin photosensitivity

INTRODUCTION

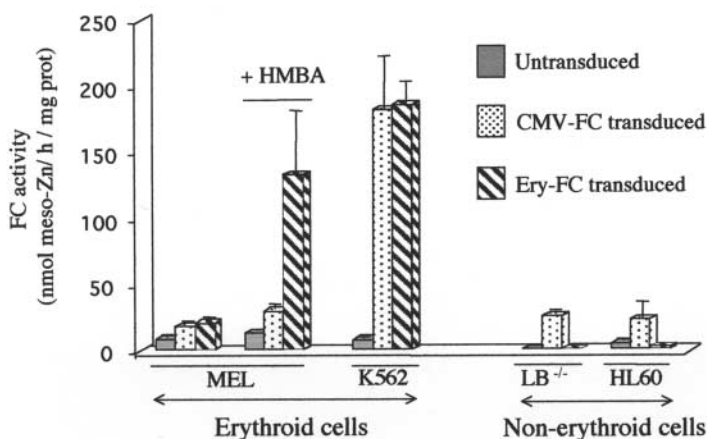
Gene therapy using hematopoietic stem cells (HSCs) is rapidly becoming a powerful approach to cure hematopoietic diseases in which a selective advantage is conferred by transgene expression [1]. For most blood disorders, however, there is no selective advantage of genetically corrected HSCs and a full correction of the disease phenotype continues to be a challenge. Erythropoietic porphyrias (EP) are good models of hematological genetic disorders with a lack of spontaneous *in vivo* selection. In such diseases, the activities of the enzymes of the heme biosynthetic pathway are partially deficient, leading to tissue accumulation of toxic porphyrins [2]. The three major forms of EP are hepatoerythropoietic porphyria (HEP), congenital erythropoietic porphyria (CEP), and erythropoietic protoporphyria (EPP). Knowledge of the molecular biology and

pathology of each enzymatic defect, which began with HEP [3], is the prerequisite for any gene therapy. CEP is the most severe porphyria and the most widely studied for preclinical gene therapy studies [4–8]. On the other hand, EPP is particularly amenable to gene therapy experiments given the availability of a mouse model [9–10]. It is an inherited disease that does not strictly follow recessive or dominant rules and is caused by a deficiency of ferrochelatase (FECH; EC 4.99.1.1), the terminal enzyme of the heme biosynthetic pathway [11]. It is characterized by accumulation of protoporphyrin in red blood cells (RBCs), bone marrow (BM), and other organs resulting in a painful skin photosensitivity. A rare complication is liver failure, which may require liver transplantation [12]. The mouse model reproduces the human disease, but with constant liver cirrhosis. The first report of a definitive cure of this

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B



model was obtained with a mouse oncoretroviral vector co-expressing the enhanced green fluorescent protein (GFP) and the therapeutic *FECH* cDNA after transplanting GFP⁺ selected cells [13]. To avoid the presence of a potentially harmful gene marker, we developed an alternative preselective gene therapy of this EPP mouse using flow cytometric separation of genetically corrected cells from deficient ones based on the absence of porphyrin fluorescence [14]. In both reports, however, a preselection of transduced cells was mandatory with these vectors for full correction of the disease. This may lead to several problems regarding the number of cells available for BM transplantation and safety considerations for human clinical applications. Indeed, loss of provirus-containing cells due to host immune response against selectable markers has been reported [15–16].

Lentiviral vectors based on human immunodeficiency virus (HIV) have inherent advantages in transducing non-dividing cells. So far, most HIV-based vector systems have used reporter genes and have proven efficient in transducing human and mouse HSCs [17–22]. Previously, therapeutic hemoglobin synthesis in β -thalassemic mice was obtained with a lentivirus-encoded human β -globin gene [23]. In this model, some degree of competitive advantage was observed after gene transfer, leading to an increase in

FIG. 1. SIN lentiviral constructs and erythroid-specific expression of *FECH* in cell lines transduced with Ery-FC vector. (A) Different transfer vector constructs: CMV-FC, ubiquitously expressing control vector with *FECH* cDNA under the internal human cytomegalovirus immediate-early promoter (CMV); Ery-GFP and Ery-FC, erythroid-specific expression vectors with GFP or *FECH* cDNA under the control of ankyrin-1 promoter and HS-40 enhancer. WPRE, woodchuck hepatitis virus post-transcriptional regulatory element. (B) *FECH* activity in cell lines transduced with CMV-FC or Ery-FC. Erythroid (MEL, K562) and non-erythroid (FECH-deficient lymphoblastoid or LB^{-/-}, and myeloid HL-60) cell lines were transduced with both lentiviral vectors at equal amounts of p24 antigen concentration. For MEL cells, erythroid differentiation was induced in the presence of 5 mM *N,N*-hexamethylene bisacetamide (HMBA) for 5 days.

red cell survival. Unlike β -thalassemia, CEP and EPP do not offer an *in vivo* selective advantage [8,13,14]. Therefore, here we tested whether the high transduction efficiency of HSCs with lentiviral vectors was sufficient to cure the EPP mouse model without any preselection of transduced HSCs. Another advantage of lentiviral vectors over conventional murine leukemia vectors is that self-inactivation eliminates transcription from the viral long terminal repeat (LTR) promoter/enhancer without loss of viral titers [24,25]. Such vectors are therefore safer and suitable for lineage-specific expression. Using GFP as a reporter gene, we previously showed that a modular use of ery-

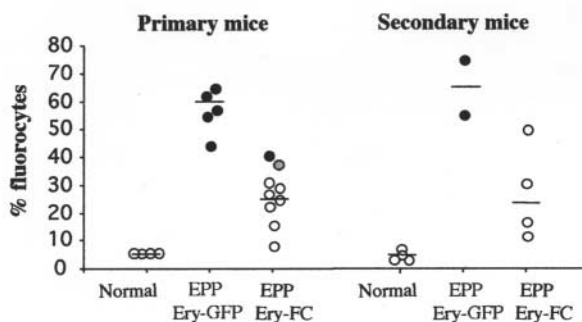
throid promoters and enhancers in a self-inactivating (SIN) lentiviral vector can result in stably transmitted, high-level erythroid-specific expression in mouse HSCs [26]. For gene therapy of the mouse model of EPP, we replaced the GFP marker with human *FECH* cDNA in one of the lentiviral vector constructs previously screened for high-level erythroid expression of GFP. Here, we report that the preselection can be overcome using a SIN lentiviral vector. Furthermore, our findings demonstrate that high-level, long-term gene expression limited to erythroid progeny is possible *in vivo* with SIN lentiviral vectors and is sufficient to reverse most of the biological and clinical manifestations of the disease, especially skin photosensitivity.

RESULTS

Erythroid-Specific Expression from Lentiviral Vectors in Hematopoietic Cell Lines

We cloned the human *FECH* cDNA sequence in a SIN lentiviral vector downstream of a 400-bp human ankyrin-1 promoter gene [24] (Fig. 1A). The HS-40 erythroid enhancer of the human α -globin gene used here contained a mutation at the 3' NF-E2/AP1 transcription factor binding site that confers position-independent expression in transgenic mice [27]. A SIN lentiviral vector containing

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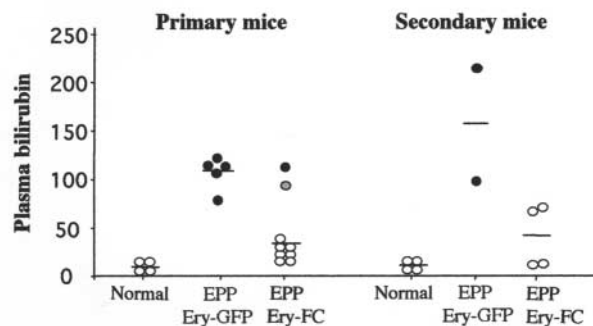


FIG. 2. Metabolic and enzymatic correction in primary and secondary mice 4 months after BM transplantation. (A) Fluorocytes were measured in peripheral blood by flow cytometry analysis. Cells were excited using the UV laser and cells emitting protoporphyrin fluorescence (fluorocytes) were quantified. A threshold was placed at the point showing 5% of the most fluorescent RBCs from normal mice. (B) Total plasma bilirubin level. Filled circles, mice with severe skin photosensitivity; gray circle, mouse with mild photosensitivity and inflammatory infiltrates in epidermis and dermis; open circles, mice without any macroscopic or microscopic lesions.

FECH cDNA under the control of the CMV promoter (CMV-FC) served as a lineage nonspecific control for *in vitro* studies. All three vectors contained a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) to improve the level of expression [28]. The HIV-based lentiviral vectors were pseudotyped with the vesicular stomatitis virus (VSV) glycoprotein. Viral particles were obtained by a three-plasmid expression system and concentrated. We determined viral productions by HIV-1 p24 antigen level measurement (ELISA test) in the concentrated viral supernatants. To investigate the erythroid specificity of the vector-encoded human *FECH* cDNA (Ery-FC), we compared *FECH* activities in lymphoblastoid (LB) cells established from a patient with EPP, myeloid HL-60 cells, erythroid human K562, and mouse erythroleukemia (MEL) cells transduced with Ery-FC and CMV-FC lentiviral vectors (Fig. 1B). We carried out all transductions in triplicate at the same concentration of HIV-1 p24 antigen (400 ng p24/ml) with an estimated multiplicity of infection (MOI) of 10. All five cell lines transduced by the standard CMV-FC vector expressed *FECH*, but overexpression from the erythroid-specific vector Ery-FC was observed only in K562 and MEL cells, particularly after erythroid induction (Fig. 1B). This experiment demonstrated the erythroid specificity of *FECH* expression from the Ery-FC vector in mouse and human hematopoietic cell lines.

Metabolic and Phenotypic Correction of EPP Primary Mice after BM Transduction and Transplantation

To investigate the efficiency of these vectors *in vivo*, we transduced and transplanted mouse BM cells from male donor EPP mice into lethally irradiated syngenic female recipient mice. We carried out transductions at 1000 ng/ml p24 antigen concentration three times at 12-hour intervals in the presence of a combination of cytokines with an

estimated MOI of 150. On day 2, two groups of lethally irradiated syngenic EPP female mice were engrafted with BM cells without any preselection of transduced cells; a group of five EPP mice was engrafted with BM cells transduced with the control Ery-GFP vector; and a group of nine mice was engrafted with BM cells transduced with the therapeutic Ery-FC vector. Four wild-type BALB/c mice neither irradiated nor engrafted served as a normal control group. At 4 months after BM transplantation, the 14 mice presented a high chimerism (mean of $91\% \pm 13\%$) as determined by FISH analysis [29]. We next determined therapeutic efficiency. Protoporphyrin fluorescence in individual RBCs was determined by flow cytometry as described [29]. We placed a threshold at the point showing 5% of the most fluorescent RBCs from normal mice. When we examined peripheral blood from Ery-GFP/EPP mice, the fluorescing RBCs (fluorocytes) represented a median of 57% of the cells. In contrast, blood samples from Ery-FC/EPP mice had a median of only 26% fluorocytes ($P < 0.001$ by the Student's *t*-test; Ery-FC/EPP mice, $n = 9$, versus Ery-GFP/EPP mice, $n = 5$; Fig. 2A, left). To confirm the level of metabolic correction, we measured RBC protoporphyrin concentration and observed a 43% decrease in the protoporphyrin level in the RBCs of Ery-FC/EPP compared with Ery-GFP/EPP mice (median of 6.5 versus 11.3 μmoles protoporphyrin/L RBC; normal mice, 2.4 μmoles protoporphyrin/L RBC).

To study whether this decrease in protoporphyrin level was sufficient to reverse skin photosensitivity, we carried out an assay in all groups of mice as described [14] (Fig. 3). At 5 days after light irradiation, there were no lesions on normal mice. In contrast, skin damage in Ery-GFP/EPP mice was very severe. Of nine Ery-FC/EPP mice, seven did not present any macroscopic or histological damage, as observed in normal mice. We observed a relationship between severity of skin lesions and the percentage of fluorocytes (Fig. 2A).

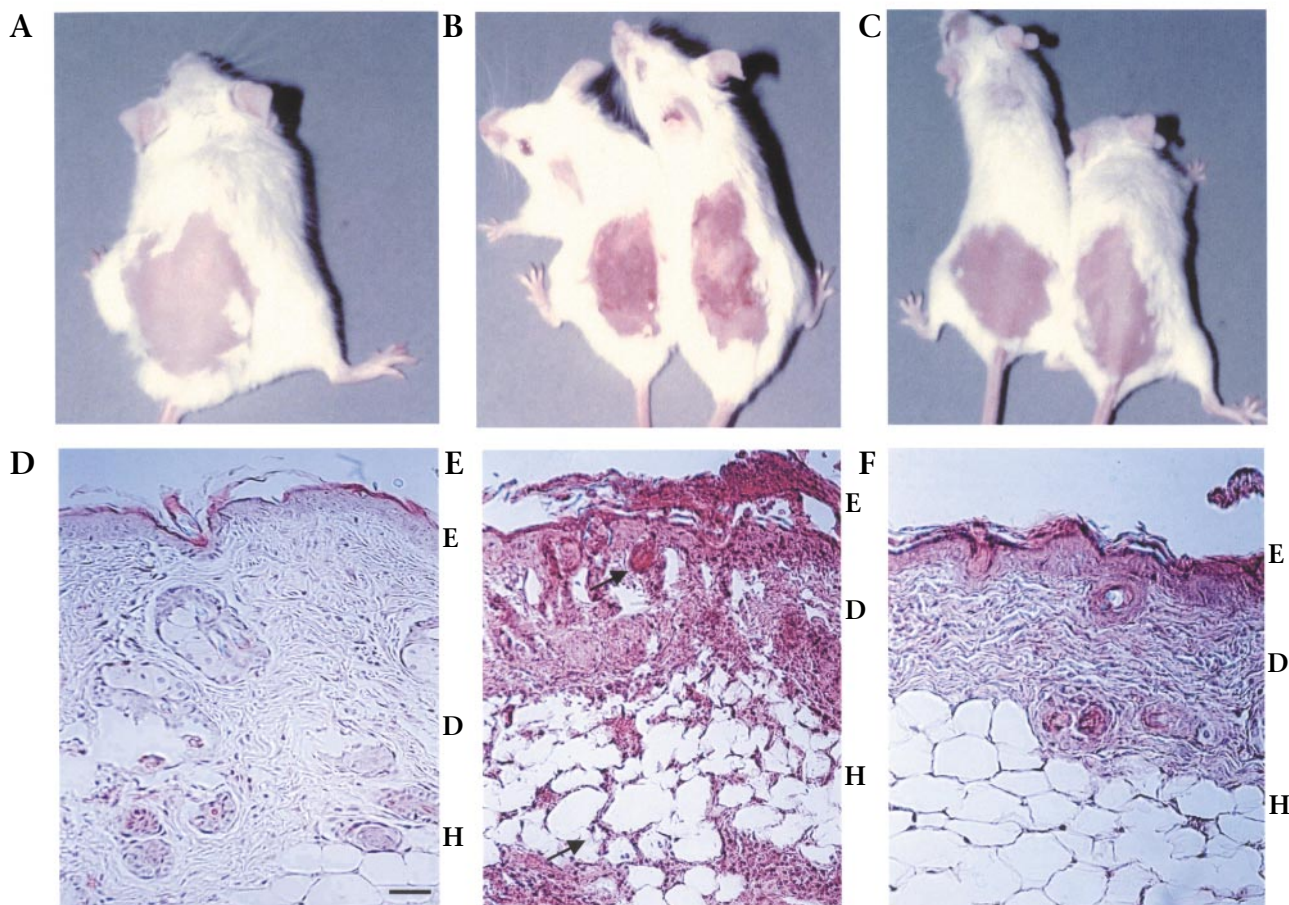


FIG. 3. Skin photosensitivity and histological analysis. Macroscopic observations and skin histology of representative primary mice 5 days following UV irradiation. (A) Wild-type normal mice presented no macroscopic skin lesions and transparent ears. (B) Ery-GFP/EPP mice with numerous macroscopic skin lesions and yellow ears. (C) Ery-FC/EPP mice with no macroscopic skin lesions. Light microscopic analyses were performed on skin lesions stained with hematoxylin and eosin. (D) Wild-type mice showed neither necrosis nor apoptosis in epidermis nor any dermal inflammatory infiltrate. (E) Irradiated Ery-GFP/EPP mouse showed an epidermal disorganization and a cleavage between epidermis and dermis. An inflammatory infiltrate in the dermis and hypodermis was also seen (arrows). (F) Representative Ery-FC/EPP mouse with normal skin. The apparent difference in skin thickness (D–F) is due to the angle of the section. E, epidermis; D, dermis; H, hypodermis. Scale bar, 50 μ m.

Only one mouse was not cured and showed the highest percentage of fluorocytes (40%). We carried out a PCR proviral integration analysis on colony-forming cells (CFCs) derived from the BM. Although a high percentage of proviral integration (mean of $75\% \pm 22\%$, $n = 9$) was observed for all Ery-FC/EPP mice, the mouse with the lowest percentage of transduced CFCs (30%) was the only one not cured. Biochemical plasma analysis showed an elevated total bilirubin in Ery-GFP/EPP mice compared with normal mice (Fig. 2B). After gene therapy, we observed a significant decrease in bilirubin ($P < 0.01$ by Student's *t*-test; Ery-FC/EPP mice, $n = 9$, versus Ery-GFP/EPP mice, $n = 5$). However, plasmatc alanine transaminase (ALAT) and alkaline phosphatase (ALP) remained at the same high levels (Ery-FC/EPP mice, ALAT 805 ± 350 , ALP 680 ± 285 , $n = 9$; Ery-EGFP/EPP

mice, ALAT 773 ± 350 , ALP 1007 ± 377 , $n = 5$; normal mice, ALAT 128 ± 43 , ALP 96 ± 13 , $n = 4$). Persistent hepatomegaly and hepatic regenerative nodules confirmed the irreversibility of the liver cirrhosis in our experimental conditions (data not shown).

Results Obtained from Secondary EPP Mice

To confirm that HSCs were transduced and to study long-term expression of the transgene, we carried out secondary transplants using BM primary recipients (five EPP mice from three Ery-FC/EPP BM and two EPP mice from one control Ery-GFP/EPP BM). We analyzed metabolic and phenotypic corrections 4 months later. The two Ery-GFP/EPP secondary mice showed severe skin photosensitivity associated with a high percentage of fluorocytes and ele-

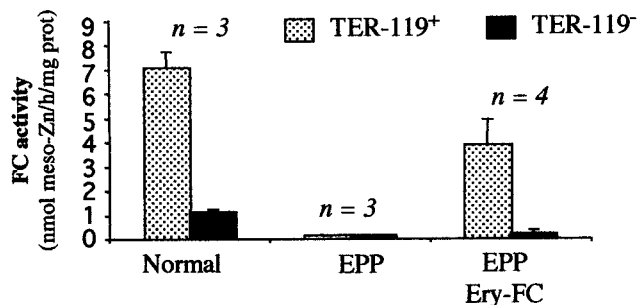


FIG. 4. FECH activity in TER-119⁺ and TER-119⁻ cell populations of BM cells from normal, EPP, and Ery-FC/EPP secondary mice. The positive and negative cells for the TER-119 antigen were isolated from 20 to 30 × 10⁶ whole BM mononuclear cells using anti-TER119-PE antibody, anti-PE microbeads, and Mini-MACS columns. A total of 1 to 10 × 10⁶ selected cells were used for FECH assay.

vated total bilirubin as compared with normal mice (Figs. 2A and 2B). Of the five Ery-FC/EPP secondary mice, four presented a high chimerism (mean of 90% ± 7%), while the fifth was not correctly engrafted (38%). Thus, this mouse was not retained for analysis. The four highly engrafted mice did not present any skin lesions, a finding in accordance with the decrease in the percentage of fluorocytes and in total plasma bilirubin level (Figs. 2A and 2B). We also analyzed CFCs by PCR and an integrated provirus was detected in 82% ± 17% of CFCs derived from BM of Ery-FC/EPP secondary mice.

Long-Term *in Vivo* Erythroid-Specific Expression of Lentiviral Vectors

To demonstrate that phenotypic correction was associated with specific expression in the erythroid lineage, we isolated TER-119-positive and -negative BM cells from normal, EPP, and Ery-FC/EPP secondary mice (Fig. 4). FECH activity was sevenfold higher in erythroid TER-119⁺ cells from normal mice compared with TER-119⁻ cells ($P < 0.001$ by Student's *t*-test; TER-119⁻ versus TER-119⁺) thereby confirming the erythroid expression of the mouse endogenous *FECH* [30,31]. As expected, FECH activity was very low in both cellular populations from EPP mice. By contrast, in Ery-FC/EPP secondary mice, FECH activity in TER-119⁺ cells represented 53% of the normal erythroid activity. On the other hand, we detected only a very low FECH activity in TER-119⁻ compared with TER-119⁺ cells ($P < 0.001$ by the Student's *t*-test). These results demonstrate the tissue-specific expression of the Ery-FC vector *in vivo*. Moreover, FECH expression limited to the erythroid lineage was sufficient to correct photosensitivity in EPP mice.

Integration of SIN Erythroid-Specific Lentiviral Vectors in HSCs

Finally, Southern blot analysis of genomic DNA from BM of secondarily transplanted Ery-FC/EPP mice demonstrated the presence of a single, 1.6-kb band, indicating unarranged provirus (Fig. 5A). The proviral copy number per cell ranged from 1.6 to 2.1 copies per cell. In addition, a single digestion within the provirus demonstrated several identical proviral integrants in BM and spleen, consistent with the transduction of HSCs at an oligoclonal level (Fig. 5B).

DISCUSSION

Bone marrow (from children) and mobilized peripheral blood (from young adults) are considered as good sources of hematopoietic cells for retrovirus-mediated gene therapy of hematological genetic diseases such as hemoglobinopathies, hemolytic anemias, or erythropoietic porphyrias. The most feasible approach for gene therapy is to introduce *ex vivo* a normal counterpart of the defective gene into the HSCs and to return them to the patient. CEP is the most severe porphyria, and is treated by allogeneic BM or cord blood transplantation [32–34]. Because HLA-identical donors are often not available, this disease is a good candidate for gene therapy. The demonstration of successful gene therapy in an animal model is a prerequisite for a gene therapy clinical trial. We chose EPP for gene transfer experiments because of the availability of the mouse model. Using Moloney murine retroviral vectors, a long-term cure of photosensitivity was previously obtained in this model by a preselective gene therapy protocol using GFP [13]. In our previous study using the same model, we used a fluorescence-based selection of retrovirus-mediated corrected cells, an approach theoretically applicable to humans [14]. In these previous studies, which used both Moloney-murine-based vectors, a preselection of transduced HSCs was required before transplantation, leading to a time-consuming and more complicated procedure than a protocol without selection.

We now report a definitive cure of this EPP mouse model in the absence of any preselective gene therapy with a SIN erythroid-specific lentiviral vector. Our results demonstrate that the high efficiency of lentiviral transduction of mouse HSCs precludes the need for any preselection of the cells required with onco-retroviruses. We observed a high transduction efficiency of HSCs with this vector, as demonstrated in primary and secondary mice by proviral PCR analysis of CFCs. The metabolic correction limited to the erythroid lineage was sufficient to reverse the skin phenotype and demonstrates a transduction of very primitive cells with a long-term *in vivo* expression of the transgene. FECH is expressed in all tissues at a basal level but is upregulated during erythropoiesis for the synthesis of hemoglobin [30,31]. We demonstrated a strong erythroid-enhanced expression of the endogenous *FECH* by comparing the enzymatic activity in TER-119⁺ and TER-119⁻ cells from normal BM. Transgene expression in deficient BM cells, 8 months after initial transduction, showed a profile close to that of the normal gene. This expression was limited to erythroid progeny of HSCs and is sufficient to reverse the clinical manifestation of the disease. A high

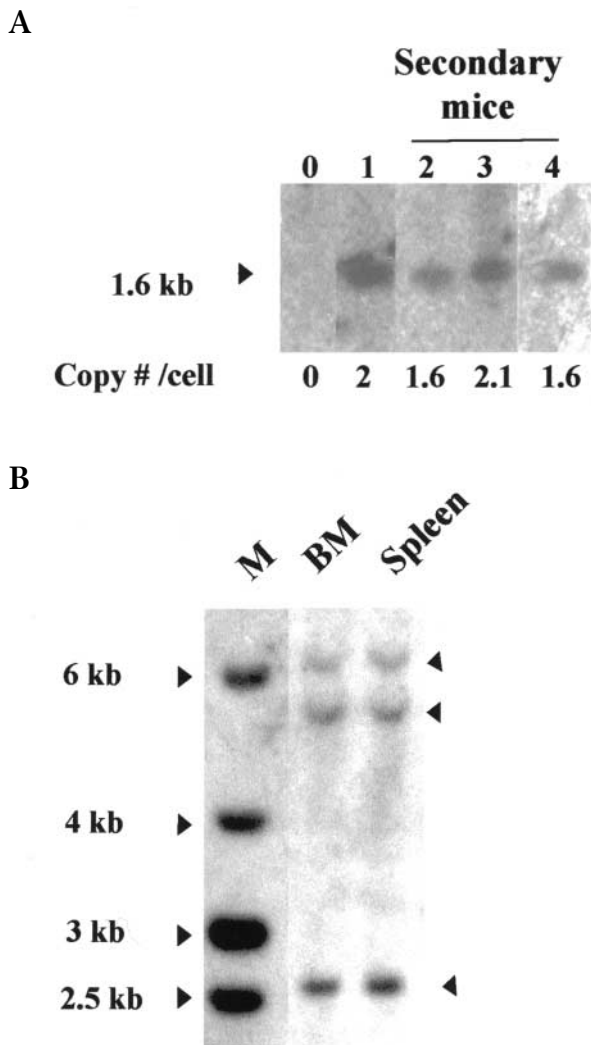


FIG 5. Proviral integration analyses. (A) Southern blot analysis was carried out on transduced secondary BM cells to test the integrity of the Ery-FC provirus by digestion with *XhoI* and *BamHI*. Digested DNA was gel-fractionated, blotted, and hybridized. Lane 0, DNA from non-transduced BM mouse; lane 1, 28 pg of Ery-FC DNA plasmid mixed with 10 μ g of non-transduced DNA, corresponding to 2 copies per cell. Lanes 2–4, 10 μ g of BM DNA from three representative secondary mice; a single proviral band of 1.6 kb was found. (B) Integration site analysis was carried out by a single restriction cut with *BamHI* on BM and spleen DNA from a representative secondary mouse. Both blots were hybridized with a 32 P-labeled WPRE probe (Fig. 1). M, 1-kb size marker. Arrowheads show multiple proviral integrations.

applications. First, a self-inactivating vector precludes the risk of replication-competent recombinant viruses emerging. Second, the high efficiency of gene transfer circumvents the need for preselection of transduced cells by co-expression of potentially immunogenic selectable genes. Third, an expression occurring only during erythroid differentiation meets the most stringent safety requirements for clinical applications as it prevents any unnecessary gene expression in white blood cells. Indeed, an expression limited to the erythroid progeny reduces the likelihood of a putative oncogene enhancement occurring after random proviral integration, as erythroid cells become progressively transcriptionally inactive with differentiation, and finally extrude their nucleus. These findings therefore form the basis for the development of future vectors for the gene therapy of severe forms of erythropoietic porphyrias, particularly CEP and major red cell disorders such as the hemoglobinopathies.

METHODS

Construction and production of lentiviral vectors. Vectors were generated using the SIN lentiviral vector, CS. Ery-FC vector was derived from Ery-GFP (previously described as HKGW) [26] by replacing GFP with the human *FECH* cDNA. Briefly, a 400-bp ankyrin-1 promoter was previously obtained by PCR from human genomic as described [26]. The mutant form of the human α -globin locus-specific regulatory element HS-40 was provided by the pHS40 z600-GH plasmid [27]. All details of cloning strategies of these vectors are available on request. Human kidney 293T cells (5×10^6) were plated on 10-cm plates, transfected the following day with the packaging construct pCMV Δ P8.91 (10 μ g), VSV-G pMD.G (5 μ g), and a vector construct (15 μ g) by calcium phosphate DNA precipitation and induced with 10 mM sodium butyrate (Sigma, St. Louis, MO). Viral supernatant was collected, filtered through 0.22- μ m filters, and concentrated by ultra-centrifugation [21]. To determine viral production, HIV-1 p24 antigen levels were measured in the concentrated viral supernatants by ELISA assay (Innotest HIV p24 INGEN SA, Rungis, France). Viral titers were estimated by comparing p24 antigen levels of each lentiviral supernatant with a CMV-GFP lentiviral supernatant produced simultaneously and titered as described [21]. Lentiviral vector preparations were tested for the presence of replication-competent lentivirus (RCL) as described [26] and were found to be free of RCL.

Cell line transduction. Human (K562, HL60, LB *Fcgh*^{-/-}) hematopoietic cell lines were maintained in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (FCS, BioWhittaker, Emerainville, France), 100 units/ml streptomycin, and 0.1 mg/ml penicillin at 37°C in 5% CO₂ atmosphere. We transduced 50,000 cells with Ery-FC and CMV-FC lentiviral vectors in the presence of 8 μ g/ml Polybrene (Sigma). Transductions were carried out in triplicate at the same concentration of HIV-1 p24 antigen (400 ng p24/ml). MEL cells were maintained in DMEM (Gibco, Grand Island,

percentage of gene transfer in CFCs derived from BM secondary mice was always associated with both metabolic and phenotype corrections, thus indicating an absence of downregulation of the transgene. This transgene stability over time may be due to the use of a mutant form of the NF-E2/AP1 sequence motif, which converts HS-40 into an erythroid-specific LCR [27]. The human ankyrin-1 promoter gene has shown direct position-independent and copy number-dependent expression of a linked γ -globin gene in transgenic mice [35]. New experiments will be necessary to study the respective contribution of the mutant form of the HS-40 enhancer and the ankyrin-1 promoter in the prevention of vector silencing. Furthermore, a lack of viral promoter/enhancer elements in SIN lentiviral vectors may prevent vector silencing from erythroid-lineage-specific regulatory elements [36].

Such erythroid-specific lentiviral vectors represent a possible breakthrough for the safety of therapeutic

NY), 10% FCS, and were induced to differentiate in the presence of 5 mM *N,N*-hexamethylene bisacetamide (HMBA, Sigma) for 5 days. On day 8 following transduction, all cell lines were collected and analyzed for FECH activity.

Transduction of BM cells from deficient mice. The *Fech^{mlpas}/Fech^{mlpas}* mice (BALB/cJ) were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were kept under pathogen-free conditions in air-filtered cages and were provided with autoclaved food and water. Heterozygous (*Fech^{mlpas}/+*) males were crossed with heterozygous females (*Fech^{mlpas}/+*) and homozygous pups were characterized by genomic DNA PCR followed by restriction analysis as described [10]. All animal experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Donor male mice (8 to 12 weeks old) were intraperitoneally injected with 5-fluorouracil (150 mg/kg per mouse). Five days after injection the donor mice were killed, then BM cells were collected by flushing the marrow from the two femurs and tibias. Cells were dispersed using a 21-gauge needle and RBCs were eliminated by hypotonic lysis. Remaining BM cells were washed twice with phosphate buffered saline (PBS) and were incubated in Myelocult medium (Stem Cell Technologies, Inc., Vancouver, Canada) supplemented with the following cytokines: 10 ng/ml recombinant human (rh) interleukin 6 (rhIL-6), 20 ng/ml recombinant murine stem cell factor (rmSCF), 50 ng/ml thrombopoietin (rhTPO), 10% Wehi medium (to provide mIL-3), 10 μ M dNTPs, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in 5% CO₂ atmosphere. Cells were transduced in 6-well plates coated with the CH-296 fragment of recombinant fibronectin (Takara Shuzo, Otsu, Japan) in the presence of Polybrene (4 μ g/ml) with viral supernatants from the Ery-FC or Ery-GFP three times at 12-h intervals using similar concentrations of p24 antigen (1000 ng/ml) with an estimated multiplicity of infection of 50. On day 2, 3 \times 10⁵ hematopoietic cells per mouse were injected into the tail vein of 8- to 16-week-old female EPP mice that had been irradiated with two splits of 6-Gy, 24 h apart to suppress the endogenous hematopoiesis. Four months after BM transplantation, animals were sacrificed and peripheral blood and BM cells harvested. For transplantation of secondary mice, 5 \times 10⁶ BM cells from primary mice were collected, washed twice in PBS containing 2 mM EDTA, resuspended in PBS, and injected into the tail vein of 8- to 16-week-old female EPP mice that had been irradiated with two splits of 6-Gy, 24 h apart.

Ferrochelatase activity, porphyrin level, and fluorocytes analyses. FECH activity was measured as described [37]. Protoporphyrin levels in RBCs were determined spectrofluorimetrically using a Hitachi F-4500 fluorescence spectrophotometer (Braun Scientecet, Les Ulis, France; excitation, 405 nm, and emission 605 nm), after extraction with 1 mol/l HClO₄/CH₃OH (1:1, v/v). RBCs from normal and transplanted mice were diluted in PBS and analyzed by flow cytometry (Elite cytometer, Coultronics). Cells were excited using the UV laser (340 to 400 nm) and cells emitting protoporphyrin fluorescence (fluorocytes) were quantified (> 650 nm, channel FL3) as described [29].

Skin photosensitivity and morphological studies. Reversal of skin photosensitivity was assayed 4 months after transplantation using an irradiation protocol. Dorsal mouse skin was depilated to render mouse skin accessible to irradiation and the skin was irradiated at a dose of 8 J/cm² UVA with a Biotronic device (Vilber Lourmat, Marne La Vallée, France). The UVA lamp delivered UV in the range 312–400 nm. Skin pictures were taken 5 d after irradiation. Sheets of dorsal mouse skin were fixed in 4% formalin, dehydrated, embedded in paraffin, cut in 5 μ m sections, and stained with hematoxylin and eosin to visualize the irradiation-induced damage.

Hematological and biochemical measurements. Recipient mice were sacrificed 5 days after UVA exposure for biochemical, molecular, and histological analysis. The animals were anesthetized with a pentobarbital injection and killed by exsanguination. Skin, liver, spleen, and BM cells were immediately removed. Plasmatic total bilirubin, alanine transaminase, and alkaline phosphatase were determined by standard methods in a CX7 (Beckman Coulter, Paris, France).

Analysis of chimerism in transplanted mice. The use of males as donors and females as recipients made it possible to determine chimerism by fluorescence *in situ* hybridization (FISH). BM cells were hybridized with a Y

FITC-labeled paint probe for the mouse chromosome Y and a Cyanin-3-labeled paint probe for the mouse chromosome X (Valbiotech, Paris, France), as described [29]. A minimum of 300 cells was examined.

PCR proviral integration in CFCs. We plated 50,000 BM cells from grafted mice in a methylcellulose-based medium (Methocult GF M3434, Stem Cell Technologies, Inc.). CFCs were scored on day 14 and 20–30 colonies from each mouse were picked, washed twice with PBS, and frozen. Cells were then digested with proteinase K in lysis buffer (10 mmol/l Tris-Cl, pH 8.0, 50 mmol/l KCl, 2.5 mmol/l MgCl₂, 0.5% Tween-20, 100 μ g/ml proteinase K) at 50°C for 1 h, followed by a 10-min exposure at 95°C to inactivate the proteinase K. The presence of the provirus was characterized by PCR using specific primers designed in the WPRE sequence to generate a 220-bp fragment: W1, 5'-TGCTGTCTCTTTATGAGGAG-3', as a forward primer and W2, 5'-GAATTGTCAGTCCCCAACAG-3', as a reverse primer. Non-transduced colonies were also used as negative controls.

Southern blot analysis. DNA was extracted from BM and spleen of secondary mice 4 months after transplantation. For analysis of provirus integrity, 10 μ g genomic DNA was digested with *Xho*I and *Bam*HI, gel fractionated, blotted and hybridized with ³²P-labeled WPRE probe. Integration site analysis was performed by a single restriction cut with *Bam*HI on BM and spleen DNA from a representative secondary recipient mouse and probed with WPRE sequence.

TER-119⁺ and TER-119⁻ cell purification. BM cells from normal, EPP, and EPP/Ery-FC secondary mice were collected by flushing tibias and femurs. We collected 20 to 30 million whole BM cells, washed twice in PBS, and resuspended in PBS containing 2 mM EDTA with 5% HSA. Cells were stained with PE-labeled anti-mouse TER-119 antibody (BD Pharmingen, San Diego, CA), washed and incubated with MACS anti-PE microBeads (Miltenyi Biotec, Auburn, CA). Cells were then sorted for TER-119⁺ and TER-119⁻ fractions using Mini-MACS MS+ separation columns. More than 95% purity of both TER-119⁺ and TER-119⁻ fractions was obtained as assessed by flow cytometric analyses.

Statistical methods. Student's unpaired t-test was used for comparison of differences between controls and transduced cells. The null hypothesis was rejected when *P* < .05.

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