Priming of Hepatocytes Enhances *In Vivo* Liver Transduction with Lentiviral Vectors in Adult Mice

Virginie Pichard,^{1,2} Sébastien Boni,^{1,2} William Baron,^{1,2} Tuan Huy Nguyen,^{1,2,*} and Nicolas Ferry^{1,2,*}

Abstract

Lentiviral vectors are promising tools for liver disease gene therapy, because they can achieve protracted expression of transgenes in hepatocytes. However, the question as to whether cell division is required for optimal hepatocyte transduction has still not been completely answered. Liver gene-transfer efficiency after *in vivo* administration of recombinant lentiviral vectors carrying a green fluorescent protein reporter gene under the control of a liver-specific promoter in mice that were either hepatectomized or treated with cholic acid or phenobarbital was compared. Phenobarbital is known as a weak inducer of hepatocyte proliferation, whereas cholic acid has no direct effect on the cell cycle. This study shows that cholic acid is able to prime hepatocytes without mitosis induction. Both phenobarbital and cholic acid significantly increased hepatocyte transduction six- to ninefold, although cholic acid did not modify the mitotic index or cell-cycle entry. However, the effect of either compound was weaker than that observed after partial hepatectomy. In no cases was there a correlation between the expression of cell-cycle marker and transduction efficiency. We conclude that priming of hepatocytes should be considered a clinically applicable strategy to enhance *in vivo* liver gene therapy with lentiviral vectors.

Introduction

THE LIVER IS THE TARGET ORGAN of many inherited metabolic diseases, some of which are amenable to gene therapy. It is particularly suitable for *in vivo* gene transfer, because it is an organ readily accessible via the bloodstream through fenestrations of the hepatic sinusoid endothelium. Recombinant lentiviruses are powerful vectors for gene transfer and have the ability to integrate their transgene into the genome of infected host cells. This ensures long-term expression of the transgene, in particular in the case of cell division. Another stated advantage of lentivirus-based vectors, in contrast to other retroviral vectors such as Moloney murine leukemia virus vectors, is their ability to infect non-proliferating cells in addition to dividing cells (Naldini *et al.*, 1996; Zufferey *et al.*, 1997; Uchida *et al.*, 1998). However, some exceptions may exist, for example, lentiviral vectors pseudotyped with a vesicular stomatitis virus G protein (VSV-G) envelope gene do not easily transduce G₀-arrested hematopoietic cells, such as quiescent lymphocytes or macrophages (Zack *et al.*, 1990; Kootstra *et al.*, 2000).

The adult liver is formed of hepatocytes that are in G₀, and only a limited proportion of hepatocytes are transduced in adult rodents after systemic injection of lentiviral vectors.

Induction of cell proliferation by mitogens or by partial hepatectomy dramatically improves liver transduction (Park *et al.*, 2000; Ohashi *et al.*, 2002; Picanço-Castro *et al.*, 2008). In mice, using lentiviral vectors carrying LacZ cDNA, there was a nearly 30-fold increase in X-Gal-positive hepatocytes in hepatectomized animals, resulting in up to 60% hepatocyte transduction, compared with less than 3% in non-hepatectomized controls (Park *et al.*, 2000). Most of the transduced hepatocytes (>80%) were bromodeoxyuridine (BrdU)-positive. Similarly, the use of mitogenic compounds known to induce liver hyperplasia, such as 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), resulted in a 30-fold increase in hepatocyte transduction in mice (Ohashi *et al.*, 2002). Along the same line, a number of studies have revealed that lentiviral vector transduction efficiency was 10 times higher in young and newborn rodents, for which the physiological turnover of hepatocytes is higher than in adult animals (Park *et al.*, 2003; Nguyen *et al.*, 2005, 2007). However, some studies demonstrate that hepatocyte cell division is not a prerequisite for *in vivo* lentiviral gene transfer (Pfeifer *et al.*, 2001; Tsui *et al.*, 2002; VandenDriessche *et al.*, 2002). Administration of BrdU by different techniques [osmotic pump, intraperitoneal (i.p.) injection] during the 3–7 days following vector injection shows in each case an absence of

¹INSERM U948, Biothérapies hépatiques, CHU Hotel Dieu, 44093 Nantes Cedex, France.

²Université de Nantes, CHU Hotel Dieu, 44093 Nantes Cedex, France.

*These two authors contributed equally to this work (co-last authors).

colocalization of BrdU and green fluorescent protein (GFP) staining in most lentivirally transduced cells, indicating that passage through the S phase is not mandatory to achieve effective transduction. Therefore, questions pertaining to the correlation between cellular division and hepatocyte transduction remain. In addition, besides the difficulty of reproducing these methods of stimulating hepatocyte transduction, in a clinical setting, a recent study showed that lentiviral vectors are less prone to integrate in transcription units in nondividing cells than in actively dividing cells (Bartholomae *et al.*, 2011). Improving lentiviral transduction of hepatocytes without inducing liver regeneration is therefore important in terms of efficacy as well as safety of gene therapy.

In the present study, we studied the impact of two compounds already used in the clinic on the lentiviral transduction of hepatocytes in 10-week-old adult mice: phenobarbital, which is known to enhance cell proliferation in the liver (Busser and Lutz, 1987; Smith *et al.*, 1991), and cholic acid, a bile acid that could have a key role in liver regeneration and may behave as a primer for hepatocytes (Huang *et al.*, 2006; Dong *et al.*, 2010). Hepatocyte priming has been defined, initially by Fausto and colleagues, as a specific and reversible condition of hepatocytes during which they are responsive to cytokines and other growth factors that stimulate cell-cycle entry (Webber *et al.*, 1994). However, priming *per se* is not sufficient to trigger mitosis completion. Here we report that injection of phenobarbital or cholic acid treatment increased hepatocyte transduction to the same level, supporting a future clinical application of this strategy for liver gene therapy.

Materials and Methods

Animals

We used male congenic C57BL/6 mice, aged 10 weeks (weighing 25 ± 1 g), obtained from Iffa Credo (I'Abresle, France) and maintained under a 12-hr light/dark illumination cycle with food and water *ad libitum*. All surgical procedures were conducted according to the guidelines of the French Ministère de l'Agriculture on animals deeply anesthetized by using isoflurane inhalation (3% vol/vol in air). Hepatectomies were performed according to the procedure of Higgins and Anderson (1931). Phenobarbital was given by i.p. injection at a daily dose of 80 mg/kg at 10 A.M. for 4 days. Cholic acid was incorporated at 0.2% (wt/wt) in the pellet chow. Recombinant lentiviral vectors [2×10^{10} transducing units (TU)/kg] were injected in the penile vein at the different time points indicated. Viral vectors were injected at doses of 2×10^{10} or 7×10^{10} TU/kg of body weight. Assuming that the liver weight of a mouse is 1 g and contains 1×10^8 hepatocytes (Seglen, 1973; Sohlenius-Sternbeck, 2006), the corresponding multiplicities of infection (MOIs) were 2 and 7 for the low and high doses, respectively.

Lentiviral vectors

High-titer lentiviral vector stocks were generated by calcium phosphate-mediated transient transfection of 293T cells (2×10^8 cells) in 10-chamber Corning CellSTACK large culture vessels with the vector transfer plasmid (1 mg), the packaging plasmid psPAX2 (0.75 mg), and the VSV-G en-

velope protein-coding plasmid pMD2G (0.5 mg). Cells were transiently transfected at 70% confluence. Twenty-four hours after transfection, fresh Advanced DMEM (Dulbecco's modified Eagle's medium) (Invitrogen Cergy-Pontoise, France) medium containing 2 mM glutamine and antibiotics (100 IU/ml penicillin, 100 mg/ml streptomycin) was added, and viral supernatant was collected at 24-hr intervals for 2 consecutive days. Viruses were concentrated by high-speed centrifugation in a JA10 rotor at 10,000 rpm (17,700 g; Avanti J-26 XP centrifuge, Beckman Coulter, Inc.) for 16 hr at 4°C, and the viral pellet was resuspended in Advanced DMEM. Concentrated vector aliquots were snap-frozen and stored at -80°C .

These vectors harbored the GFP gene or macaque erythropoietin (EPO) cDNA under the control of a liver-specific murine transthyretin (mTTR) promoter formed with an mTTR promoter fused to a synthetic hepatocyte-specific enhancer. They contained four copies of a perfectly matched miR142-3p target sequence (kindly provided by Dr. Luigi Naldini) to decrease immune response (Brown *et al.*, 2006; Schmitt *et al.*, 2010). They also harbored the posttranscriptional regulatory element from the woodchuck hepatitis virus (WPRE), and a matrix attachment region, which increased transgene expression.

To determine the titers of GFP-transducing vectors, serial dilutions of vector stock were used to transduce HeLa cells in six-well plates. DNA was extracted and vector titers were determined by real-time quantitative PCR (qPCR) as described below. Vector titers were routinely 5×10^9 TU/ml.

Real-time qPCR

High-molecular-weight DNA was extracted from total liver using the standard phenol-chloroform method. For qPCR, 100 ng of DNA was added to a solution containing the primers and the SYBR Green PCR Master Mix and loaded to a 96-well plate. Reaction conditions were 10 min at 95°C for activation followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The values were normalized to the mouse or human β -actin gene. A standard curve was generated by using dilutions of lentiviral vector plasmid in genomic DNA extracted from liver mouse or for titration of lentivirus from HeLa cells. The points ranged from 0 to 100 copies of plasmid per genome.

The sequences of the forward and reverse primers were as follows: GAG-F, GGAGCTAGAACGATTCCGAGTGA; GAG-R, GGTGTAGCTGTCCCAGTATTGTGTC; mouse β -actin-F, ATCCTGTGGCATCCATGAAACTAC; mouse β -actin-R, GGAGCCAGAGCAGTAATCTCCTTC; human β -actin-F, TCCGTGTGGATCGGCGGCTCCA; human β -actin-R, CTGCTTGCTGATCCACATCTG.

Histological analysis and immunohistochemistry

Liver samples were formalin-fixed, embedded in paraffin, and cut into 5- μm sections. Morphological analysis was performed after hematoxylin and eosin staining. Mitotic index was calculated after microscopic examination of at least 10 fields at $40\times$ magnification (one field contained 300 hepatocytes). The presence of Ki67- and GFP-positive hepatocytes was assessed by immunohistochemistry on formalin-fixed/paraffin-embedded sections using anti-GFP antibodies or the TEC-3 antibody, which recognizes the Ki67 antigen. Sections were deparaffinized and rehydrated. For Ki67

immunostaining, antigen retrieval was carried out by microwave slides in antigen retrieval buffer (1.8 mM citric acid, 8.2 mM sodium citrate, pH 6) for a total of six cycles of 5 min. Endogenous peroxidase activity was blocked by incubation for 10 min in a 3% H₂O₂ solution in methyl alcohol. Blocking of nonspecific activity was carried out by incubation for 90 min in 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Monoclonal primary rabbit anti-GFP antibody and monoclonal primary rat anti-Ki67 antibody were applied for 2 hr at room temperature. The dilutions were 1:200 for anti-GFP and 1:50 for anti-Ki67 antibodies diluted in PBS containing 2% BSA and 0.1% Tween 20. Positive cells were revealed with biotinylated goat anti-mouse immunoglobulin and streptavidin-peroxidase using diaminobenzidine as a substrate. After counterstaining with hematoxylin, mitosis and the percentage of positive cells were counted after microscopic examination of at least 10 fields at 40 $\times$ magnification, corresponding approximately to at least 3,000 hepatocytes.

Hematocrit and EPO monitoring

Hematocrit values were measured by microcapillary centrifugation. Serum EPO levels were measured by enzyme-linked immunosorbent assay (ELISA) (Quantikine IVD; R&D Systems) according to the instructions provided by the manufacturer.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software and the Mann-Whitney *U* test to compare quantitative data. Data are presented as mean values $\pm$ SEM or SD.

Results

First, the basal turnover of hepatocytes in male C57BL/6 mice aged 3.5, 5.5, 7.5, 9.5, and 11.5 weeks ($n=3$ mice/time point) was evaluated. The mitotic index was low after 5.5 weeks and not significantly different from that of older mice. Immunohistological detection of the Ki67 antigen, which is expressed during all active phases of the cell cycle (G₁, S, G₂, and M), revealed a higher labeling in C57BL/6 mice aged 3.5 weeks that was significantly different from that in older mice. The labeling index was the lowest ($0.015 \pm 0.017\%$) in mice aged 9.5 weeks, and thus 10-week-old mice were chosen for this study to ensure quiescence of the hepatocytes.

Next, regeneration triggered by partial hepatectomy was evaluated. A first cohort of 24 mice (10 weeks old) was sacrificed at different time points ($n=4$ mice/time point) after a two-thirds or one-third partial hepatectomy to evaluate the kinetics of hepatocyte proliferation. At 41 hr after a two-thirds hepatectomy, a peak of mitosis ($4.2 \pm 0.1\%$) together with a strong increase in the Ki67-positive cells ($75 \pm 7\%$) was observed. After a one-third hepatectomy, the proliferation peak occurred at the same time (41 hr), but with a 15–20-fold lower intensity (mitotic index, $0.27 \pm 0.28\%$; Ki67 labeling, $3.9 \pm 2.1\%$) (Fig. 1A and B). In both instances, at 24 hr after hepatectomy, the Ki67 labeling was low and not significantly different from the control value, as shown in Fig. 1B. These results agree with those reported in previous studies showing a DNA synthesis peak at 40 hr after partial hepatectomy in mice (Yamada *et al.*, 1997).

Liver cell proliferation after administration of phenobarbital or cholic acid was then assessed. Twenty-seven mice received phenobarbital intraperitoneally (80 mg/kg daily for up to 4 days), and a second cohort of 27 mice was fed cholic acid (0.2% in the pellet chow). Animals were sacrificed at 48, 72, and 96 hr after the first injection of phenobarbital or after the beginning of the cholic acid diet ($n=9$ at each time point). After phenobarbital treatment, we observed a statistically significant increase in mitotic index: ($0.15 \pm 0.13\%$ and $0.13 \pm 0.13\%$ at 72 hr and 96 hr, respectively; Fig. 1A). Similarly, Ki67 labeling at both time points was higher than that in control animals ($3.7 \pm 1.5\%$ and $3.5 \pm 2.3\%$ at 72 hr and 96 hr, respectively; Fig. 1B). With the cholic acid diet, the mitotic index and Ki67 staining were not significantly different from the control values at all time points (Fig. 1A and B).

Next, the impact of cell-cycle activation on hepatocyte lentiviral transduction was evaluated. A first series of seven control mice, aged 10 weeks, were injected with mTTR-GFP lentiviral vectors at a unique dose of 2×10^{10} TU/kg, corresponding to an MOI of 2. At day 7 after injection, the animals were sacrificed and the number of GFP-expressing hepatocytes was measured using immunohistochemistry. As shown in Figs. 1C and 2, $0.14 \pm 0.07\%$ of GFP-positive hepatocytes was observed. Furthermore, the vector copy number in whole liver was determined by qPCR; this was 0.73 ± 0.17 genome copy (gc) per haploid genome, twofold higher than that of the control mice (Fig. 3).

A second series of mice ($n=4$ mice/time point) were injected with the same amount of vector 24 hr or 41 hr after a two-thirds hepatectomy. At the time of sacrifice, 1 week later, the animals that had been injected 24 hr after hepatectomy showed the highest level of GFP-positive hepatocytes ($4.3 \pm 1.10\%$) together with a genome copy number (1.3 ± 0.5 gc/haploid genome) statistically different from controls (Figs. 1C and 2). For animals injected 41 hr after surgery, a statistically significant increase in positive hepatocytes ($2.05 \pm 1.2\%$) was observed (Figs. 1C and 2). However, although there was an increase in genome copy numbers, the difference was not statistically significant (Fig. 3).

A third group of mice ($n=3$ mice/time point) received the same lentiviral dose at 30 min, 24 hr, or 41 hr after a one-third hepatectomy. Transduction efficiency was significantly higher than in the control group at all time points and varied between $0.92 \pm 0.18\%$ (30 min) and $1.3 \pm 0.3\%$ (41 hr) as shown in Figs. 1C and 2. The amount of vector copies was higher than in the control group at all time points, but was statistically significant only when vector delivery occurred 41 hr after hepatectomy (Fig. 3).

Next, the efficiency of gene transfer after treatment with phenobarbital was investigated. The groups of animals received mTTR-GFP lentiviral vectors 48 hr, 72 hr, or 96 hr after the first phenobarbital injection ($n=8$ for each time point). After vector injection, phenobarbital treatment was stopped. At sacrifice, a significantly higher proportion of GFP-positive hepatocytes than in controls was observed for all time points, and transduction efficiency varied from $0.36 \pm 0.16\%$ and $1.29 \pm 0.92\%$, as shown in Figs. 1C and 2. The highest value corresponds to lentiviral injection performed 48 hr after treatment with a ninefold increase in hepatocyte transduction. No statistical difference was observed in the genome vector copy numbers between phenobarbital-treated and control mice (Fig. 3).

FIG. 1. Evaluation of the mitotic index (A) and Ki67 labeling (B) in the various groups of animals. Mitotic index was evaluated by counting the number of mitotic figures after hematoxylin and eosin staining of paraffin sections. Ki67 staining was performed by immunohistochemistry. (C) Evaluation of lentiviral transduction in mouse liver. Seven days after virus injection, mice were sacrificed, and the number of GFP-positive hepatocytes was scored by the immunohistochemical labeling on paraffin sections. *Statistically different from control value ($p < 0.05$) using the Mann-Whitney U test. Data are represented as means $\pm$ SD.

A last group of animals was fed cholic acid and received mTTR-GFP lentiviral vectors 48 hr, 72 hr, or 96 hr after the beginning of the diet ($n=8$ for each time point). At day 7, the animals were sacrificed, and a statistically significant increase in the percentage of transduction was observed for all time points. Transduction efficiency varied between $0.42 \pm 0.23\%$ and $0.76 \pm 0.49\%$, as shown in Figs. 1C and 2. The highest value was achieved when lentiviral injection was performed 48 hr after the beginning of the diet. Genome vector copy numbers determined by qPCR were not different from those of the control mice (Fig. 3). It is noteworthy that it was possible to achieve significant hepatocyte transduction in animals in which Ki67 staining and the mitotic index were similar to control values. To further determine whether the induction of cell cycle was correlated to transduction efficiency, the values of Ki67 staining and GFP transduction

obtained in all animals were compared. The correlation coefficient was low ($r^2=0.04106$), indicating the absence of any correlation between the two parameters. In contrast, Ki67 labeling was strongly correlated to mitotic index, as expected ($r^2=0.999$). Therefore, these data strongly suggest that cell-cycle entry is not mandatory for efficient transduction with lentiviral vectors.

In addition, a cohort group of mice ($n=10$) was injected with a higher dose of vector. Five mice were injected with mTTR-GFP lentiviral vectors at a unique dose of 7×10^{10} TU/kg, corresponding to an MOI of 7. Five other animals received the same dose of vectors after administration of phenobarbital (80 mg/kg i.p. daily for 2 days). At day 7 after injection, the animals were sacrificed, and the number of GFP-expressing hepatocytes was measured using immunohistochemistry. As shown in Fig. 4, we observed $27.5 \pm 7.6\%$

FIG. 2. Histochemical detection of GFP. (A, F) Control mouse. (B) Two-thirds hepatectomized mouse (sacrificed at 41 hr). (C) One-third hepatectomized mouse (sacrificed at 41 hr). (D, G) Phenobarbital-fed mouse sacrificed at 48 hr. (E) Cholic acid-treated mouse sacrificed at 48 hr. Hematoxylin counterstain. Original magnification: $\times 20$ (A–E) and $\times 4$ (F, G).

of GFP-positive hepatocytes in control mice that received the vector with no pretreatment ($n=5$). In phenobarbital-treated mice ($n=5$), an average of $51.65 \pm 19.9\%$ of GFP-positive hepatocytes, with up to 70% of transduced hepatocytes in one animal, was observed. The values were significantly different between the two groups ($p < 0.05$).

To confirm the above results using a therapeutic transgene, a lentiviral vector carrying the EPO cDNA under the

transcriptional control of the same mTTR liver-specific promoter was constructed. Five mice, aged 10 weeks, were injected with mTTR-EPO viral infectious particles at a unique dose of 2×10^{10} TU/kg infectious particles, corresponding to an MOI of 2. Five other mice also received lentiviral vectors, but 48 hr after being injected daily with 80 mg/kg i.p. phenobarbital. One week after lentiviral injection, blood hematocrit had increased in phenobarbital-treated mice

FIG. 3. Evaluation of vector genome copy numbers in high-molecular-weight DNA by real-time qPCR. *Statistically different from control value using the Mann-Whitney test. Data are represented as means $\pm$ SD.

compared with noninjected controls (Fig. 5). At 1 week, there was a statistically significant difference in blood hematocrit between phenobarbital-treated mice and mice that received the vector with no pretreatment ($61.6 \pm 3.2\%$ vs. $55.2 \pm 4.6\%$; $p=0.04$). At 2 weeks, blood hematocrit was increased and the values were still significantly different ($67.5 \pm 2.4\%$ vs. $72.5 \pm 2.6\%$; $p=0.048$). We also measured serum EPO by ELISA, and the values were also significantly different between the two groups ($25.98 \pm 4.56\%$ vs. $69.53 \pm 44.02\%$; $p=0.03$; Fig. 5).

Discussion

In the present study, the correlation between cell proliferation and transduction in the liver after *in vivo* delivery of recombinant lentiviral vectors via a peripheral vein has been studied using adult mice. In young animals, liver growth is responsible for a background level of hepatocyte division that may complicate the interpretation of transduction experiments. The existence of binucleated hepatocytes and the polyploidization process that takes place after weaning may have led to the misinterpretation of previous results. As shown in Table 1, the majority of hepatocytes do not become quiescent before 8 weeks of age. Therefore, in previous studies carried out in younger animals, the transduction levels that were observed may have been related to hepatocytes that were not completely quiescent.

In the present study, older animals with fully developed livers were used, and it was verified that the hepatocytes were quiescent at 10 weeks of age. After a two-thirds partial hepatectomy, there was a dramatic increase in Ki67 staining and mitotic index. Similarly, hepatectomy improved the level of hepatocyte transduction, with a 30-fold increase in GFP-positive hepatocytes as compared with control, non-hepatectomized animals. This ratio is similar to the one reported in a previous study (Park *et al.*, 2000). The ability of two chemical compounds, phenobarbital and cholic acid, to trigger cell-cycle entry and to increase *in vivo* transduction of adult hepatocytes was compared next. Phenobarbital can increase the mitotic index, and after phenobarbital adminis-

tration, a ninefold increase in GFP-transduced hepatocytes following administration of vector at 2×10^{10} TU/kg was detected using immunohistochemistry. A similar enhancement of lentiviral transduction of hepatocytes with up to 70% of transduced hepatocytes in phenobarbital-treated animals was also observed at a higher vector dose (7×10^{10} TU/kg). At this high dose, the basal level of transduction in non-treated animals was also higher, in comparison with the low dose, indicating that the amount of vector administered is also a critical parameter in achieving high transduction levels. This is consistent with previous results reported by Park *et al.* (2000). Finally, we demonstrated that phenobarbital enhanced the hematocrit level and serum EPO obtained after administration of a single dose of EPO lentiviral vectors, using EPO cDNA as a therapeutic transgene. This result also showed that the stimulating effect of phenobarbital on lentiviral transduction of hepatocytes is not transgene-dependent.

Cholic acid treatment did not elicit any significant modification of Ki67 labeling or the mitotic index. However, there was a sixfold increase in GFP-positive hepatocytes when lentiviral vectors were injected in cholic acid-treated mice. This demonstrated that priming of hepatocytes was sufficient, in itself, to make a significant impact on transduction with lentiviral vectors without any requirement for cell division. However, the effects of both phenobarbital and cholic acid were weaker than that of a two-thirds hepatectomy.

qPCR showed that copy number did not correlate with transduction level. Indeed the number GFP-positive hepatocytes increased 30-fold in two-thirds hepatectomized mice compared with the control, although a minor difference in vector copy number (twofold increase) was detected. Similarly, in cholic acid- and phenobarbital-treated mice, no difference in the vector copy numbers was detected compared with the control, whereas the proportion of GFP-positive hepatocytes was significantly higher. To determine whether the high number of vector copies in control mice was due to a carryover of the plasmid DNA used for vector production, a real-time PCR was carried out to detect any ampicillin resistance gene, present in the plasmids only. Plasmid DNA in

FIG. 4. Evaluation of phenobarbital to mediate lentiviral transduction into mouse liver cells. Mice were treated or not with phenobarbital and received GFP-lentiviral vectors. Seven days after virus injection, mice were sacrificed, and the number of GFP-positive hepatocytes was scored by the immunohistochemical labeling on paraffin sections. Histochemical detection of GFP in vehicle (A, B) or phenobarbital-treated mouse livers (C, D) is shown. (E) Example of a mouse liver section from a mouse injected with PBS. (F) Percentage of GFP-positive hepatocytes. *Statistically different from control value ($p < 0.05$) using the Mann-Whitney U test. Data are represented as means $\pm$ SD. Hematoxylin counterstain. Original magnification: $\times 20$ (B, D) and $\times 4$ (A, C, E).

the genomic DNA from the mice was not detected (data not shown). The high level of vector copies in the experimental animals likely resulted from efficient transduction of nonparenchymal liver cells that did not express GFP under the control of a liver-specific promoter. Indeed, van Til and colleagues previously reported a higher transduction rate of nonparenchymal cells as compared with hepatocytes (van Til *et al.*, 2005). Kupffer-cell depletion led to an 80% decrease in the viral copy number as assessed by qPCR (van Til *et al.*,

2005). In another study, in which 26% of liver cells were transduced with GFP recombinant lentiviral vectors, the majority (78.7%) of the positive cells were nonhepatocytes (Pfeifer *et al.*, 2001). This could indicate that, when looking at copy numbers, a high background level of nonparenchymal cell transduction (Kupffer cells and endothelial cells) may have diluted the positive effect of phenobarbital and cholic acid on hepatocyte transduction. In contrast, because a liver-specific promoter was used, transduced nonparenchymal

FIG. 5. Evaluation of therapeutic efficacy after lentiviral liver transduction in mice. **(A)** Hematocrit was evaluated at 7 and 14 days after lentiviral injection in mice treated or not with phenobarbital (PB). **(B)** Level of serum EPO was evaluated at day 14 post injection by ELISA. *Statistically different ($p < 0.05$) using the Mann-Whitney U test. The data shown are means $\pm$ SEM. Each plot corresponds to one experimental animal.

cells were not detected after GFP immunohistochemistry. Finally, it cannot be excluded that some unintegrated copies of the genome may still be present in the cells as one long terminal repeat (LTR) or two LTR circles. These forms may also account for the high qPCR values observed.

The reasons why lentiviral vectors at low MOI do not easily transduce differentiated adult hepatocytes in the G_0 state of the cell cycle are still unknown. Previous studies have demonstrated that lentiviral vectors can achieve high transduction efficiency in hepatocytes in primary culture and isolated liver biopsies resected from patients with liver diseases (Seppen *et al.*, 2002; Maruyama *et al.*, 2003; Nguyen *et al.*, 2006). However, such isolated cells are no longer fully quiescent, but in the early G_1 phase (Skouteris and Schroder, 1996). Regarding G_0 -arrested cells of the hematopoietic system, like resting B cells, it has been shown recently that lentiviral vectors pseudotyped with the Edmonston measles virus hemagglutinin (H) and fusion (F) proteins could efficiently and stably transduce quiescent B cells or B-cell chronic lymphocytic leukemia cells that were not transduced with VSV-G pseudotyped lentiviral vectors (Frecha *et al.*, 2009). The same was true for resting T lymphocytes (Frecha *et al.*, 2010). Most importantly, it appeared that a high level of gene transfer in the resting T and B cells was not at the cost of cell-cycle entry or cell activation. Therefore, we believe that hepatocyte priming may similarly alleviate certain ill-defined restriction points and make hepatocytes more susceptible to lentiviral vector transduction. It is worth noting that a minimal, transient damage to the liver (an increase in serum alanine aminotransferase) after vector injection has been observed in some studies, but transduction of hepatocytes was inefficient (Tsui *et al.*, 2002; VandenDriessche *et al.*, 2002).

A number of authors conclude that transduction efficiency relies much more on the design of the lentiviral backbone. Insertion of a central polypurine tract (cPPT) resulted in a 50% reduction in the requirement for cell cycling of hepatocytes *in vivo* (Park and Kay, 2001). Also, the same study demonstrated an increase in transduction when a matrix attachment region was inserted in the viral backbone. Other studies have shown that the incorporation of cPPT, as well as the presence of a WPRE, in third-generation self-inactivating lentivectors could achieve transduction levels in the range of 2–5% in adult livers (Follenzi *et al.*, 2002; VandenDriessche *et al.*, 2002).

For clinical applications of lentiviral mediated gene transfer in adults, it is conceivable that a therapeutic level of transduction will not be reached after *in vivo* administration of low doses of vectors. Therefore, other strategies aimed at increasing liver transduction will have to be developed. Increasing the doses of vectors may prove to be difficult, because clinical-grade lentiviral vectors are still difficult to produce in large quantities. Moreover, dissemination of the vector in the body may also be increased when using high doses. Most of the methods that have been described so far to enhance lentiviral transduction (partial hepatectomy, treatment with potentially carcinogenic chemical compounds) are not fully compatible with a clinical application. Recently, a 10-fold increase in liver transduction was achieved in mice that were pretreated with a chimeric hyper-interleukin-6 cytokine (Picanço-Castro *et al.*, 2008). This cytokine is known to participate in the initiation of liver regeneration as a priming agent, but is devoid of the potency to cause full cell replication (Webber *et al.*, 1994). We believe that our present results, obtained with drugs already used clinically, may help in the design of future protocols in which low vector

TABLE 1. COMPARISON OF MITOTIC INDEX AND Ki67 LABELING IN THE LIVER OF MICE ($N=15$) AT VARIOUS AGES

Weeks	3½	5½	7½	9½	11½
Mitotic index	0.75 $\pm$ 0.1 ^a	0.04 $\pm$ 0.075	0.02 $\pm$ 0.03	0.02 $\pm$ 0.03	0.02 $\pm$ 0.03
Ki67 labeling	23.8 $\pm$ 5.1 ^a	0.59 $\pm$ 0.19	0.75 $\pm$ 0.15	0.15 $\pm$ 0.07	0.31 $\pm$ 0.10

Mitotic index is expressed as percentage of mitotic figures in hepatocytes and Ki67 as percentage of immunohistologically labeled hepatocytes. Data shown are means $\pm$ SD.

^aStatistically different from values from other cohort of mice ($p < 0.05$) using the Mann-Whitney U test.

doses will be used while maintaining therapeutic efficacy, and thus allow lentiviral vectors to enter the field of clinical gene therapy for liver diseases.

Acknowledgments

This work was supported by grants from the Association Française contre les Myopathies, the Association Francophone des Glycogénoses, the Agence Nationale de la Recherche (ANR-07-MRAR_CRINAGENETHER), and the Région Pays de Loire.

Author Disclosure Statement

All authors have nothing to disclose.

References

- Bartholomae, C.C., Arens, A., Balaggan, K.S., *et al.* (2011). Lentiviral vector integration profiles differ in rodent postmitotic tissues. *Mol. Ther.* 19, 703–710.
- Brown, B.D., Venneri, M.A., Zingale, A., *et al.* (2006). Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. *Nat. Med.* 12, 585–591.
- Busser, M.T., and Lutz, W.K. (1987). Stimulation of DNA synthesis in rat and mouse liver by various tumor promoters. *Carcinogenesis* 8, 1433–1437.
- Dong, X., Zhao, H., Ma, X., and Wang, S. (2010). Reduction in bile acid pool causes delayed liver regeneration accompanied by down-regulated expression of FXR and c-Jun mRNA in rats. *J. Huazhong Univ. Sci. Technol. Med. Sci.* 30, 55–60.
- Follenzi, A., Sabatino, G., Lombardo, A., *et al.* (2002). Efficient gene delivery and targeted expression to hepatocytes *in vivo* by improved lentiviral vectors. *Hum. Gene Ther.* 13, 243–260.
- Frecha, C., Costa, C., Levy, C., *et al.* (2009). Efficient and stable transduction of resting B lymphocytes and primary chronic lymphocyte leukemia cells using measles virus gp displaying lentiviral vectors. *Blood* 114, 3173–3180.
- Frecha, C., Levy, C., Cosset, F.L., and Verhoeyen, E. (2010). Advances in the field of lentivector-based transduction of T and B lymphocytes for gene therapy. *Mol. Ther.* 18, 1748–1757.
- Higgins, G.M., and Anderson, R.M. (1931). Experimental pathology of the liver. *Arch. Pathol.* 187–202.
- Huang, W., Ma, K., Zhang, *et al.* (2006). Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration. *Science* 312, 233–236.
- Kootstra, N.A., Zwart, B.M., and Schuitemaker, H. (2000). Diminished human immunodeficiency virus type 1 reverse transcription and nuclear transport in primary macrophages arrested in early G₁ phase of the cell cycle. *J. Virol.* 74, 1712–1717.
- Maruyama, M., Kobayashi, N., Okitsu, T., *et al.* (2003). Successful lentivirus-based delivery of NLS-LACZ gene into porcine hepatocytes. *Transplant. Proc.* 35, 435–436.
- Naldini, L., Blomer, U., Gallay, P., *et al.* (1996). *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 272, 263–267.
- Nguyen, T.H., Bellodi-Privato, M., Aubert, D., *et al.* (2005). Therapeutic lentivirus-mediated neonatal *in vivo* gene therapy in hyperbilirubinemic Gunn rats. *Mol. Ther.* 12, 852–859.
- Nguyen, T.H., Birraux, J., Wildhaber, B., *et al.* (2006). *Ex vivo* lentivirus transduction and immediate transplantation of uncultured hepatocytes for treating hyperbilirubinemic Gunn rat. *Transplantation* 82, 794–803.
- Nguyen, T.H., Aubert, D., Bellodi-Privato, M., *et al.* (2007). Critical assessment of lifelong phenotype correction in hyperbilirubinemic Gunn rats after retroviral mediated gene transfer. *Gene Ther.* 14, 1270–1277.
- Ohashi, K., Park, F., and Kay, M.A. (2002). Role of hepatocyte direct hyperplasia in lentivirus-mediated liver transduction *in vivo*. *Hum. Gene Ther.* 13, 653–663.
- Park, F., and Kay, M.A. (2001). Modified HIV-1 based lentiviral vectors have an effect on viral transduction efficiency and gene expression *in vitro* and *in vivo*. *Mol. Ther.* 4, 164–173.
- Park, F., Ohashi, K., Chiu, W., *et al.* (2000). Efficient lentiviral transduction of liver requires cell cycling *in vivo*. *Nat. Genet.* 24, 49–52.
- Park, F., Ohashi, K., and Kay, M.A. (2003). The effect of age on hepatic gene transfer with self-inactivating lentiviral vectors *in vivo*. *Mol. Ther.* 8, 314–323.
- Pfeifer, A., Kessler, T., Yang, M., *et al.* (2001). Transduction of liver cells by lentiviral vectors: analysis in living animals by fluorescence imaging. *Mol. Ther.* 3, 319–322.
- Picanço-Castro, V., Fontes, A.M., Heinz, S., *et al.* (2008). The chimeric cytokine Hyper-IL-6 enhances the efficiency of lentiviral gene transfer in hepatocytes both *in vitro* and *in vivo*. *Biotechnol. Lett.* 30, 215–220.
- Schmitt, F., Remy, S., Dariel, A., *et al.* (2010). Lentiviral vectors that express UGT1A1 in liver and contain miR-142 target sequences normalize hyperbilirubinemia in Gunn rats. *Gastroenterology* 139, 999–1007.
- Seglen, P.O. (1973). Preparation of rat liver cells. 3. Enzymatic requirements for tissue dispersion. *Exp. Cell Res.* 82, 391–398.
- Seppen, J., Rijnberg, M., Cooreman, M.P., and Oude Elferink, R.P. (2002). Lentiviral vectors for efficient transduction of isolated primary quiescent hepatocytes. *J. Hepatol.* 36, 459–465.
- Skouteris, G.G., and Schroder, C.H. (1996). C-myc is required for the G₀/G₁-S transition of primary hepatocytes stimulated with a deleted form of hepatocyte growth factor. *Biochem. J.* 316 (Pt 3), 879–886.
- Smith, P.F., O'Brien, K.A., and Keenan, K.P. (1991). Evaluation of bromodeoxyuridine labeling in hepatomegaly produced by peroxisomal proliferation or P-450 induction in rodents. *Prog. Clin. Biol. Res.* 369, 285–289.
- Sohlenius-Sternbeck, A.K. (2006). Determination of the hepatocellularity number for human, dog, rabbit, rat and mouse livers from protein concentration measurements. *Toxicol. In Vitro* 20, 1582–1586.
- Tsui, L.V., Kelly, M., Zayek, N., *et al.* (2002). Production of human clotting Factor IX without toxicity in mice after vascular delivery of a lentiviral vector. *Nat. Biotechnol.* 20, 53–57.
- Uchida, N., Sutton, R.E., Frier, A.M., *et al.* (1998). HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G₀/G₁ human hematopoietic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 95, 11939–11944.
- van Til, N.P., Markusic, D.M., van der Rijt, R., *et al.* (2005). Kupffer cells and not liver sinusoidal endothelial cells prevent lentiviral transduction of hepatocytes. *Mol. Ther.* 11, 26–34.
- VandenDriessche, T., Thorrez, L., Naldini, L., *et al.* (2002). Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce non-dividing hepatocytes and antigen-presenting cells *in vivo*. *Blood* 100, 813–822.

- Webber, E.M., Godowski, P.J., and Fausto, N. (1994). In vivo response of hepatocytes to growth factors requires an initial priming stimulus. *Hepatology* 19, 489–497.
- Yamada, Y., Kirillova, I., Peschon, J.J., and Fausto, N. (1997). Initiation of liver growth by tumor necrosis factor: deficient liver regeneration in mice lacking type I tumor necrosis factor receptor. *Proc. Natl. Acad. Sci. U.S.A.* 94, 1441–1446.
- Zack, J.A., Arrigo, S.J., Weitsman, S.R., *et al.* (1990). HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell* 61, 213–222.
- Zufferey, R., Nagy, D., Mandel, R.J., *et al.* (1997). Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat. Biotechnol.* 15, 871–875.

Address correspondence to:
Dr. Nicolas Ferry
 INSERM U948
 Biothérapies hépatiques
 CHU Hotel Dieu
 44093 Nantes Cedex
 France

E-mail: nicolas.ferry@inserm.fr

Received for publication January 17, 2011;
 accepted after revision August 22, 2011.

Published online: August 22, 2011.