A Highly Efficient, Stable, and Rapid Approach for *Ex Vivo* Human Liver Gene Therapy Via a FLAP Lentiviral Vector

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Allogenic hepatocyte transplantation or autologous transplantation of genetically modified hepatocytes has been used successfully to correct congenital or acquired liver diseases and can be considered as an alternative to orthotopic liver transplantation. However, hepatocytes are neither easily maintained in culture nor efficiently genetically modified and are very sensitive to dissociation before their reimplantation into the recipient. These difficulties have greatly limited the use of an *ex vivo* approach in clinical trials. In the present study, we have shown that primary human and rat hepatocytes can be efficiently transduced with a FLAP lentiviral vector without the need for plating and culture. Efficient transduction of nonadherent primary hepatocytes was achieved with a short period of contact with vector particles, without modifying hepatocyte viability, and using reduced amounts of vector. We also showed that the presence of the DNA FLAP in the vector construct was essential to reach high levels of transduction. Moreover, transplanted into uPA/SCID mouse liver, lentivirally transduced primary human hepatocytes extensively repopulated their liver and maintained a differentiated and functional phenotype as assessed by the stable detection of human albumin and antitrypsin in the serum of the animals for months. In conclusion, the use of FLAP lentiviral vectors allows, in a short period of time, a high transduction efficiency of human functional and reimplantable hepatocytes. This work therefore opens new perspectives for the development of human clinical trials based on liver-directed *ex vivo* gene therapy. (HEPATOLOGY **2003;38:114-122.**)

Orthotopic liver transplantation is the only effective therapy for several congenital and acquired liver diseases. However, this therapeutic surgical approach has been limited mainly by the shortage of donor livers, the prohibitive cost, and the requirement of lifelong immunosuppressive treatment. Segmental transplants or living-donor liver transplantation has only marginally impacted on organ scarcity, and 10% of patients still die while on waiting lists for liver transplantation. Hepatocyte transplantation can be considered in some cases as "bridging therapy" to enable a short-term improvement of liver functionality until a donor liver becomes available.[1] Hepatocyte transplantation has been used successfully in humans as an alternative therapy to orthotopic liver transplantation for the treatment of some metabolic diseases such as Crigler-Najjar syndrome type I and glycogen storage disease type IA.[2,3] Liver gene therapy based on transplantation of genetically engineered hepatocytes should be considered as an alternative strategy applicable to liver disorders as well as to metabolic disorders nondirectly involving the liver. *Ex vivo* cell transduction before transplantation into the host avoids systemic dissemination of viral particles, ensures a tissue-specific expression of the transgene, and markedly reduces the amount of vector particles required for direct *in vivo*

Abbreviations: HIV, human immunodeficiency virus; uPA, urokinase plasminogen activator; Hα1-AT, human α_1-antitrypsin; GFP, green fluorescent protein; BrdU, bromodeoxyuridine; VSV-G, vesicular stomatitis virus-G.

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transduction. Moreover, a highly expressed therapeutic gene in a limited number of cells may be sufficient to achieve normal secretion of the corresponding protein in the systemic circulation and to allow the correction of a metabolic disorder.[4-6] In this line, the hepatocyte constitutes an ideal target for gene therapy thanks to its endogenous secretory role.

For long-term correction of metabolic deficiencies, stable and continuous expression of the transgene is required. Oncoretroviral-based vectors have been preferentially used until now for this purpose.[4,7] A therapeutic effect has been reported in a human clinical trial of hypercholesterolemia after autotransplantation of hepatocytes retrovirally transduced with the low-density lipoprotein receptor gene.[8] However, the requirement of hepatocyte division to achieve efficient gene transfer with oncoretroviral vectors has restricted their use in *ex vivo* liver gene therapy.

Considerable improvements have recently been achieved in vector technology with the introduction of human immunodeficiency virus (HIV)-1–derived lentiviral vectors.[9,10] These vectors maintain the capacity of HIV to efficiently transduce nondividing cells and can be obtained at high titers. After systemic administration, VSV-G pseudotyped lentiviral vector can transduce a broad spectrum of target cell types with extensive transduction of several other organs (namely, spleen, bone marrow, blood cells, and even gonads).[11] However, only a few reports have been published on the use of lentiviral vectors for *in vivo* or *ex vivo* liver targeting. Moreover, discordant results have been obtained concerning the requirement of cell division to reach an efficient *in vivo* lentiviral transduction of the hepatocytes.[12-15]

In the present study, we show that primary human and rat hepatocytes can be transduced very efficiently with a FLAP lentiviral vector without plating and culture. The presence of DNA FLAP sequences within the vector construct (central poly-purine tract sequence and central termination sequence) was essential for efficient transduction of nonadherent hepatocytes. In addition, after genetic modification, adult primary human hepatocytes were able to repopulate large areas of uPA/SCID mouse liver, thus emphasizing their ability to proliferate and to maintain a differentiated phenotype. These results open new perspectives to liver-directed human *ex vivo* gene therapy.

Materials and Methods

Primary Hepatocyte Isolation. Primary rat hepatocytes were isolated by liver perfusion with a collagenase blend (Liberase; Roche Molecular Biochemicals, Mannheim, Germany) as previously described.[16]

Primary human hepatocytes were isolated as described elsewhere[17] from the healthy liver tissue of surgical liver biopsy specimens (approximately 20-25 cm^3) collected after informed consent had been obtained from patients undergoing therapeutic partial hepatectomy for liver metastasis and benign hepatic tumor. Subjects with viral infections (hepatitis C virus, hepatitis B virus, HIV), cirrhosis, and primary hepatic carcinomas were not considered. Briefly, liver lobes were resected at a distance of at least 3 cm from the metastasis. Collagenase H (Roche Molecular Biochemicals) perfusion (500 μg/mL, 2.4 mg/mL $CaCl_2$ in HEPES buffer, pH 7.4) was preceded by extensive washing of the liver tissue with HEPES/ethylenediaminetetraacetic acid buffer (pH 7.4) using a catheter inserted into the vessels on the cut surface of the resected fragment. The flow rate of the perfusate was 15 to 18 mL/min, and all solutions were prewarmed at 37°C. Cells were then washed twice at 100*g* for 2 minutes, and hepatocytes were separated from nonparenchymatous cells by Percoll fractionation (30% isotonic Percoll solution, centrifuged at 450*g* for 4 minutes). Viable cells were determined by trypan blue exclusion.

Lentiviral Vectors. TRIP-ΔU3-CMV-GFP (referred in the text as TRIP-GFP) and HR-GFP vectors have been described elsewhere.[10,18] Vector particles were produced by the transient calcium phosphate cotransfection of 293T cells as previously described.[19] The concentrations of vectors were normalized according to the p24 (HIV-1 capsid protein) content of supernatants. The titer of the TRIP-GFP vector, measured on MT4 cell line (a very permissive lymphocyte cell line), was 10^{10} transducing units/mL, and the p24 concentration was 97 ng p24 antigen/μL.

In all experiments, the vectors were concentrated by ultracentrifugation except when otherwise mentioned ("nonconcentrated medium"). In the case of a nonconcentrated vector supernatant, Dulbecco's modified Eagle medium was replaced by serum-free Williams medium in 293T cells 24 hours after transfection. The vector supernatant was collected after a further 24 hours, cleared by centrifugation (800*g* for 10 minutes), aliquoted, and stored at −80°C until use. The p24 antigen concentration of nonconcentrated vector stock was 740 ng/mL.

Nonadherent Transduction and Culture of Primary Hepatocytes. Immediately after isolation, hepatocytes were incubated at 37°C in Williams medium (10^7 cells/mL) to which lentiviral vector stock solutions were added and maintained under rolling agitation. In case of nonconcentrated vectors, hepatocytes were directly resuspended in vector supernatant. After the time intervals

indicated, the cells were washed twice and resuspended in complete Williams medium (1 mg/mL bovine serum albumin, 100 μg/mL streptomycin, 100 U/mL penicillin, 25 nmol/L dexamethasone, and 5 μg/mL bovine insulin) with 10% fetal calf serum (FCS) and plated at a density of 2 to 3 × 10^4 cells/cm^2. After 5 hours, serum-containing medium was removed and cells were cultured in complete Williams medium, with or without mitogens (human epidermal growth factor, 25 ng/mL; sodium pyruvate, 20 mmol/L). At the indicated time points, the cells were washed twice with phosphate-buffered saline and fixed with a 4% paraformaldehyde solution at 4°C for 15 minutes. Hoechst staining was performed by incubating fixed cells for 10 minutes in the presence of a 0.5 μg/mL solution of Hoechst 33258 in phosphate-buffered saline. The results presented herewith correspond to the average of 3 independent experiments; at least 500 cells were analyzed per condition during each experiment.

Generation of uPA/SCID Mice. To generate the uPA/SCID mice, we crossed the Alb-uPA (Jackson Laboratories, Bar Harbor, ME) and SCID mouse lines (IFFA-CREDO, Lyon, France) and, through selective backcrossing, bred SCID trait to homozygosity. uPA transgenic mice were identified by polymerase chain reaction of mouse-tail DNA with the following nucleotide sequences: p1, 5′-ATTCTGGAGGACCGCTTATCTGT-3′; p2, 5′-CTTGAACCCAGGAGGCGGAGATT-3′. Hemizygous mice were used for transplantation experiments. Homozygosity of the SCID trait was confirmed by quantification of total serum immunoglobulin G using a sandwich enzyme-linked immunosorbent assay technique.

Liver Cell Transplantation. uPA/SCID mice were anesthetized, the spleen was exteriorized through a small, left flank incision, and a syringe with a 30-gauge needle was used to inject 100 μL of the cell suspension (5-6 × 10^5 viable human hepatocytes) in Williams medium (Invitrogen, Carlsbad, CA). The spleen was replaced in the abdominal cavity, and the incision site was closed with sutures. Serum samples were collected every 3 weeks until week 12.

uPA/SCID mice were killed at 4 (n = 3), 8 (n = 3), and 12 weeks (n = 5) after the transplantation of hepatocytes transduced with green fluorescent protein (GFP)-expressing lentiviral vectors.

Enzyme-Linked Immunosorbent Assay for Human Albumin and α_1-Antitrypsin. Human albumin and human α_1-antitrypsin (hα1-AT) levels in uPA/SCID mouse sera were quantified by a standard sandwich enzyme-linked immunosorbent assay. Briefly, mouse anti-human albumin (0.1 μg/mL, clone HSA-9; Sigma Chemical Co., St Louis, MO) and goat anti–hα1-AT (11 μg/mL; Rockland, Gilbertsville, PA) were used as antigen-specific capture antibodies. After overnight coating, nonspecific binding was blocked by 1 hour of incubation with 1% bovine serum albumin at 37°C. After washing, 75 μL/well of 1:10 diluted mouse sera was added and incubated overnight at 4°C. Rabbit anti-human albumin (0.16 μg/mL; Sigma Chemical Co.) and rabbit anti–hα1-AT (2 μg/mL; Sigma Chemical Co.) were used as antigen-specific indicator antibody. Horseradish peroxidase–conjugated anti-rabbit antibodies were purchased from Sigma Chemical Co. The chromogen and the substrate were used according to the manufacturer's indications (Sigma Chemical Co.). Absorbance values (405 nm) were converted to micrograms per milliliter by the comparison with a standard curve performed by using serial dilution of purified human albumin and hα1-AT (Sigma Chemical Co.).

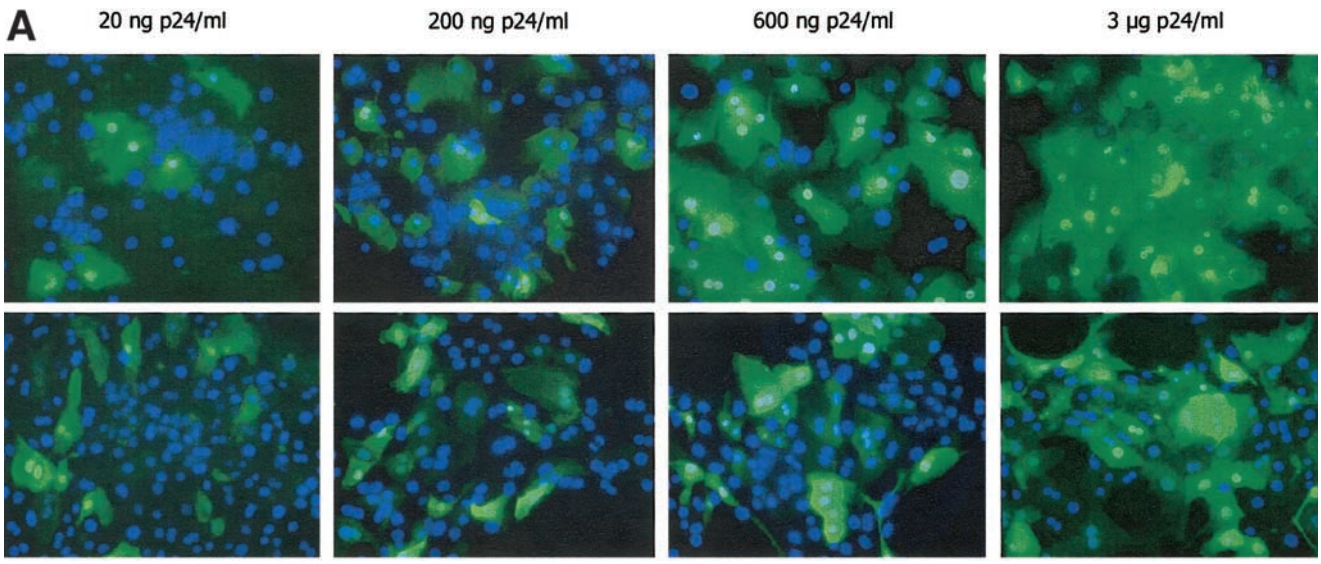
Immunohistochemical Staining for hα1-AT. Frozen liver sections (7 μm) were immediately fixed for 10 minutes at 4°C in 4% paraformaldehyde solution and then permeabilized by incubation in 0.2% Triton X-100 and 10% goat serum solution for 30 minutes. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in phosphate-buffered saline for 5 minutes at room temperature and then incubated with rabbit anti–hα1-AT antibodies (Dako, Glostrup, Denmark) for 1 hour at room temperature. Slides were developed using a secondary anti-rabbit antibody conjugated with horseradish peroxidase (EN-VISION-kit; Dako) following the manufacturer's instructions. After immunohistochemistry, sections were counterstained with Meyer's hematoxylin (Dako).

Bromodeoxyuridine Staining. Primary human hepatocytes were transduced in a nonadherent manner with 1.5 μg p24 antigen/mL of TRIP-GFP vectors, plated, and cultured for 96 hours with 10 μmol/L bromodeoxyuridine (BrdU) in the absence or presence of 25 ng/mL epidermal growth factor (as previously described). Cells were washed twice and then fixed and stained following the manufacturer's instructions (BrdU labeling and detection kit I; Roche Molecular Biochemicals). BrdU positivity of cultured hepatocytes was assessed by light microscopy. At least 500 cells were analyzed per data point. Presented data represent the average of 3 independent experiments.

Statistical Analysis. *P* values were determined using Fisher's exact test.

Results

Efficient Transduction of Nonadherent Human and Rat Primary Hepatocytes Using FLAP Lentiviral Vectors. To analyze the transduction rate of human and rat primary hepatocytes with the HIV-1–derived FLAP lentiviral vector, we incubated freshly isolated hepatocytes



B

	20 ng p24/ml	200 ng p24/ml	600 ng p24/ml	1.5 µg p24/ml	3 µg p24/ml
Human	13.2	32.1	64.5	96	100
Rat	13.8	41	47.2	58.2	70.8

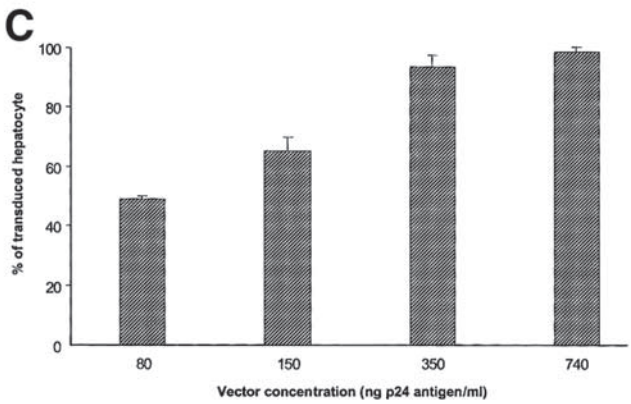


Fig. 1. Efficient transduction of nonadherent, primary human and rat hepatocytes using FLAP lentiviral vectors. (A) Direct fluorescence images of human (**upper panels**) and rat (**lower panels**) primary hepatocytes transduced using a nonadherent protocol with concentrated TRIP-GFP vectors. Green fluorescence corresponds to GFP-expressing cells, whereas nuclei are counterstained in blue with Hoechst 33258 (original magnification ×250). (B) Summary table showing the percentage of transduced human and rat hepatocytes with different concentrations of TRIP-GFP vector. Cells were incubated for 2 hours with vector solutions before washing and plating and were analyzed 4 days after transduction. (C) Histogram representing transduction efficiency of primary human hepatocytes with indicated concentrations of nonconcentrated vector supernatant. Hepatocytes were transduced for 2 hours in a nonadherent manner (see Materials and Methods).

in the presence of increasing concentrations of lentiviral vector particles expressing GFP under the control of the cytomegalovirus promoter (Fig. 1). Human hepatocytes were transduced while still in suspension during a 2-hour period. Efficiency close to 100% was obtained with a viral vector particle concentration corresponding to 1.5 μg p24 antigen/mL (Fig. 1A and B). Primary rat hepatocytes were less permissive to transduction. At 1.5 μg p24 antigen/mL, approximately 60% of rat hepatocytes were transduced. A plateau was reached at 70% efficiency for a vector particle concentration of 3μg p24 antigen/mL. These results indicate that primary rat and human hepatocytes are efficiently transduced by FLAP lentiviral vectors without the need for plating or culture. In human and rat cells, we did not observe any toxicity induced by the vector, even at the highest doses tested (5 μg/mL of p24 antigen, data not shown).

A 100% hepatocyte transduction was achieved using nonultracentrifugated vector supernatant conditioned in Williams medium (see Materials and Methods) (Fig. 1C). Interestingly, transduction to homogeneity was achieved at lower vector doses (350-740 ng p24 antigen/mL) when compared with experiments performed using concentrated vector stocks (1,500 ng p24 antigen/mL).

To confirm that lentiviral vectors are able to transduce nondividing hepatocytes, freshly isolated, nonadherent human and rat hepatocytes were transduced (1.5 μg p24 antigen/mL of TRIP-GFP) and further cultured either with or without epidermal growth factor. BrdU was added in the medium and maintained throughout the

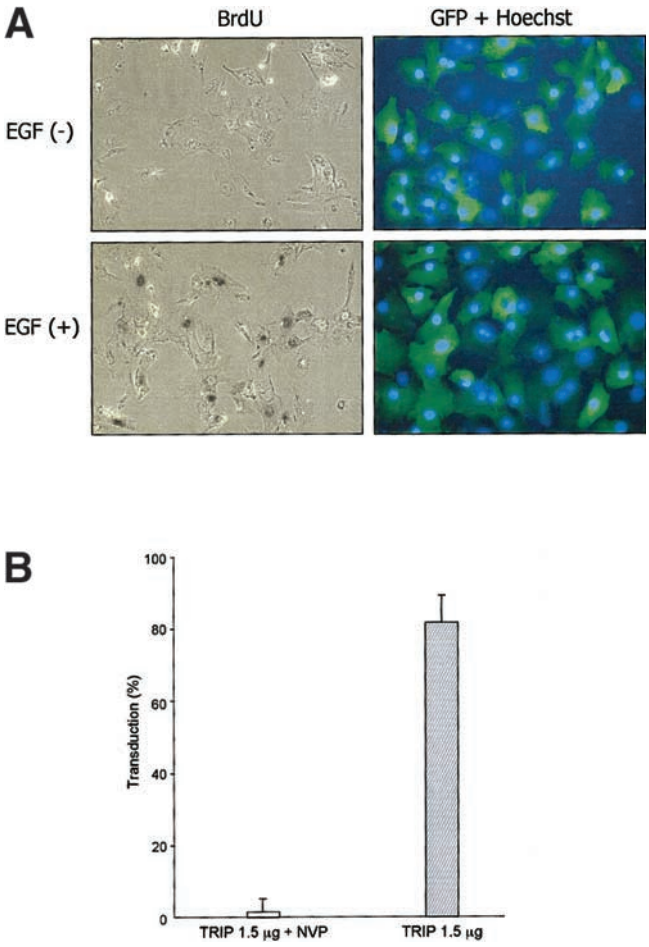


Fig. 2. Efficient transduction of nonreplicating primary human hepatocytes by FLAP lentiviral vectors. (A) BrdU incorporation (**left panels**) and GFP expression (**right panels**) in primary human hepatocytes transduced with 1.5 μg p24 antigen/mL of TRIP-GFP vectors after 96 hours of culture in the absence (**upper panels**) or presence (**lower panels**) of mitogenic stimulation (epidermal growth factor). In the right panels, nuclei are counterstained in blue with Hoechst 33258 (original magnification $\times$250). (B) Effect of treatment with nevirapine on primary human hepatocyte transduced with 1.5 μg p24 antigen/mL. Cells were treated (**white bar**) or not (**gray bar**) with nevirapine (NVP). At least 500 cells were analyzed for GFP fluorescence per data point; presented data represent the average of 3 independent experiments.

experiment (96 hours) as a marker for dividing cells. Incorporation of BrdU was found in 63% $\pm$ 7.5% of hepatocytes when cultured in the presence of epidermal growth factor and in 7.6% $\pm$ 4.4% when cultured in complete Williams medium without mitogenic stimulation. An equivalent transduction efficiency of human hepatocytes was obtained in both culture conditions whether cells had incorporated BrdU or not (96.4% $\pm$ 3.6% vs. 97.1% $\pm$ 2.9% of GFP-positive cells) (Fig. 2A).

A direct delivery of GFP, referred to as a pseudotransduction mechanism, could have explained these results. To test this hypothesis, cells were treated with 1 μmol/L nevirapine (Boehringer Ingelheim, Ridgefield, CT), a nonnucleoside HIV-1 reverse-transcriptase inhibitor, leading to a dramatic decrease in transduction efficiency (Fig. 2B).

Altogether, these results confirm the ability of lentiviral vectors to transduce primary hepatocytes with equal efficiency, whether or not they are induced to proliferate.

Efficient Transduction of Human and Rat Primary Hepatocytes Can Be Achieved Using a Rapid Transduction Protocol. The time required for *ex vivo* manipulation of primary hepatocytes (isolation, transduction, washing, and transplantation) has to be optimally minimized in the view of a clinical liver gene therapy trial. Therefore, we studied the kinetics of transduction of human and rat liver cells while in suspension with a suboptimal dose of vector particles as determined previously (600 ng p24 antigen/mL). Thirty minutes of contact with vector particles was sufficient to obtain 70% of the maximum value of transduction (34.6% $\pm$ 4.1% at 30 minutes vs. 47.7% $\pm$ 2% at 240 minutes and 43.7% $\pm$ 4.9% at 30 minutes vs. 59% $\pm$ 8.7% at 240 minutes for rat and human hepatocytes, respectively). This proportion increased with time of contact and reached a plateau after 2 hours of incubation (43.7% $\pm$ 1.2% and 55% $\pm$ 7.3% for rat and human hepatocytes, respectively) (Fig. 3B).

The permissiveness of rat hepatocytes to transduction differed from that seen in human cells but exhibited similar transduction kinetics (Fig. 3B). Interestingly, the viability of nonadherent human and rat primary hepatocytes, as determined by trypan blue exclusion, was not substantially modified by incubation with lentiviral vectors, even after the longest period tested (90% $\pm$ 1.4% and 92% $\pm$ 1.2% of viable hepatocytes for rat and human, respectively) (Fig. 3C).

Internal FLAP Is Essential for the Efficient Transduction of Nonadherent Primary Human Hepatocytes. To determine the impact of DNA FLAP on the efficiency of primary human hepatocyte transduction, these cells were transduced using vectors containing the DNA FLAP (TRIP-GFP) or not (HR-GFP). Transductions were conducted with vector concentrations, which increased from 75 to 3,000 ng p24 antigen/mL (Fig. 4). For each concentration, the TRIP vector was found to be more efficient than the HR vector for nonadherent hepatocyte transduction, with this improvement ranging from 8-fold (at 3,000 ng p24 antigen/mL; 98.8% $\pm$ 1.1% vs. 12.7% $\pm$ 1.9%) to 20-fold (at 250 ng p24 antigen/mL; 50% $\pm$ 10.6% vs. 2.5% $\pm$ 1.7%). This improvement was statistically significant for each vector concentration ($P <$ .01). Moreover, TRIP-transduced hepatocytes exhibited much brighter GFP fluorescence, suggesting multiple vector genome integrations per cell using high vector doses (data not shown).

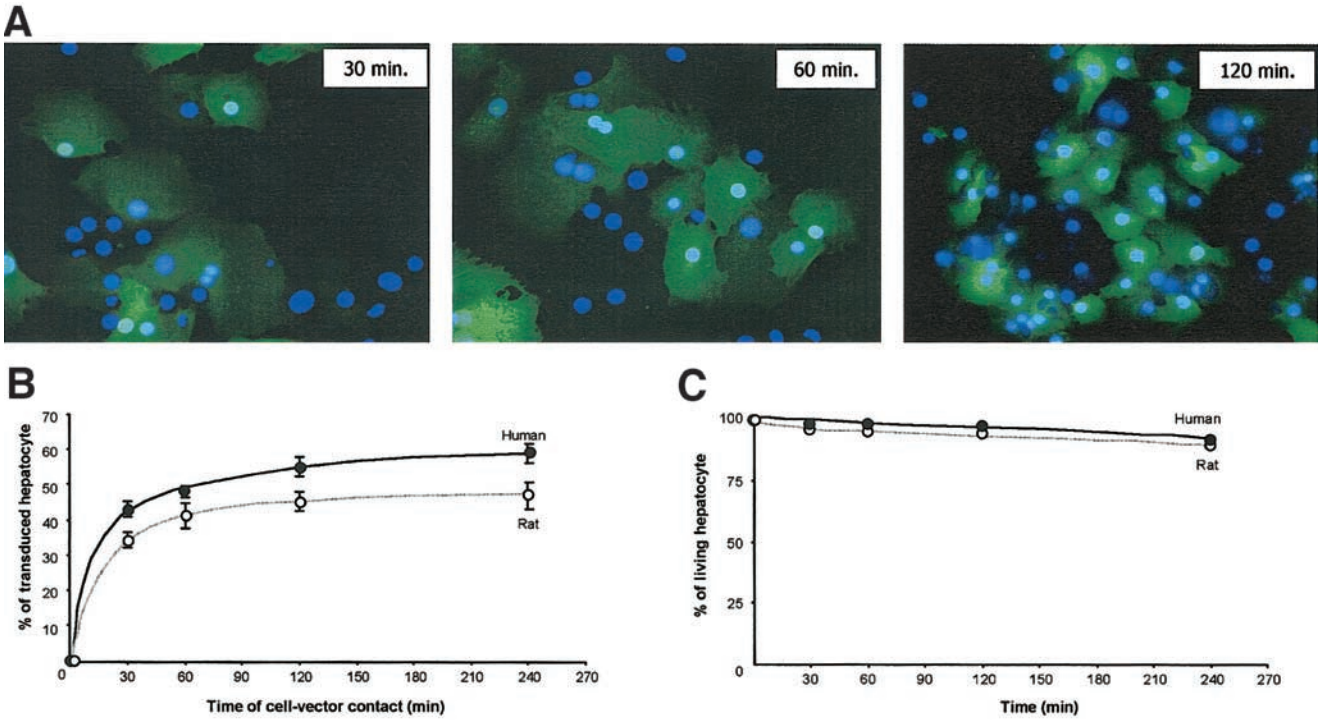


Fig. 3. Efficient transduction of primary human and rat hepatocytes can be achieved using a rapid transduction protocol. (A) Direct fluorescence images of human primary hepatocytes incubated with TRIP-GFP vectors at 600 ng p24 antigen/mL for indicated times and then plated and cultured for 96 hours. Green fluorescence corresponds to GFP-positive cells; nuclei are counterstained in blue with Hoechst 33258 (original magnification: left and center panels, ×200; right panel, ×100). (B) Transduction efficiency of human (**black lines**) and rat (**gray lines**) primary hepatocytes incubated with 600 ng p24 antigen/mL of TRIP-GFP vectors for indicated times. At least 500 cells were analyzed for GFP fluorescence per data point; presented data represent the average of 3 independent transduction experiments. (C) Viability of human (**black lines**) and rat (**gray lines**) primary hepatocytes incubated with 600 ng p24 antigen/mL of TRIP-GFP vectors for indicated times. Tests were performed in triplicate for each data point. Presented results correspond to the average of 3 independent transductions. Standard error was always less than 1.5%.

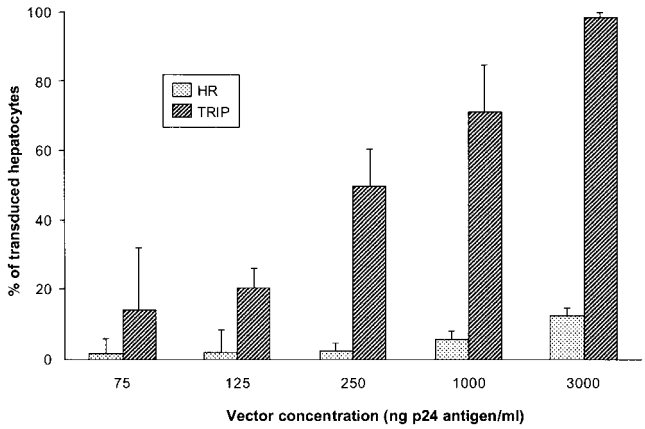


Fig. 4. Internal FLAP is essential for the efficient nonadherent transduction of primary human hepatocytes. Percentage of GFP-positive human primary hepatocytes transduced in a nonadherent manner for 2 hours with HR-GFP or TRIP-GFP lentiviral vectors at indicated concentrations. At least 500 cells were analyzed for GFP fluorescence per data point; presented data represent the average of 3 independent experiments.

These data show that the DNA FLAP structure is a critical determinant to achieve an efficient transduction of primary hepatocytes *ex vivo*.

Ex Vivo *Transduced Human Hepatocytes, Transplanted in Mice, Maintain Transgene Expression, Proliferation Potential, and Differentiation Status.* uPA/SCID mice have already been shown to provide an excellent model for xenogenic repopulation of their liver with human hepatocytes.[20] To show the ability of transduced adult human hepatocytes to engraft, maintain, and proliferate, we therefore transplanted primary adult human hepatocytes, transduced in a nonadherent manner with TRIP-GFP, into the spleen of uPA/SCID mice. Due to the continuous selective pressure induced by the cytotoxicity of uPA transgene expression in the liver, transduced human hepatocytes underwent strong proliferative stimulation and repopulated large portions of mouse liver, as shown by the presence of large clusters of hepatocytes expressing high levels of GFP (Fig. 5A). Successful transplantation was assessed by the presence of human hepatocyte-specific proteins in the serum of transplanted animals as well as the immu-

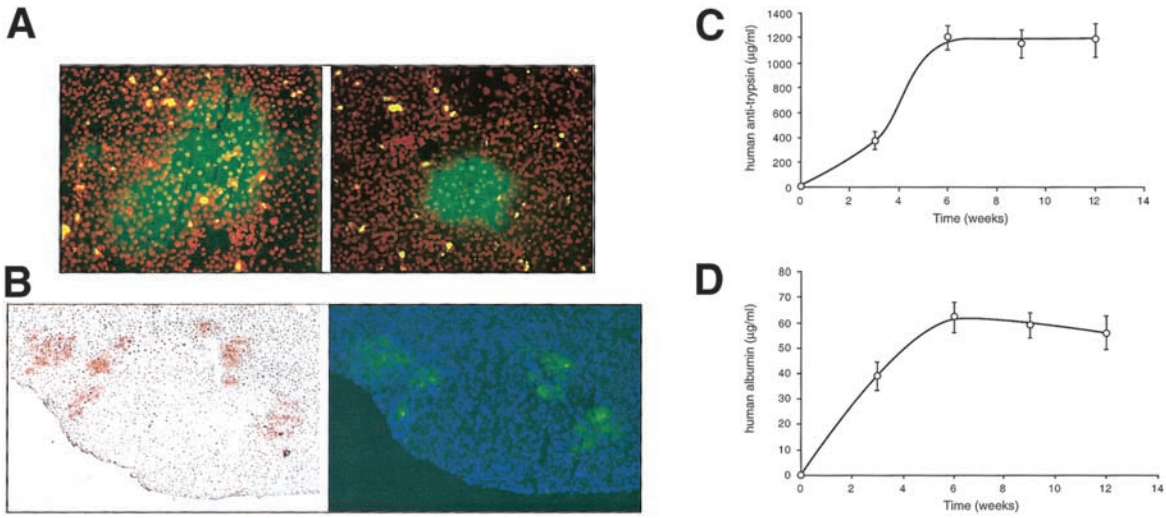


Fig. 5. Lentivirally *ex vivo* transduced human hepatocytes transplanted into uPA/SCID mice maintain transgene expression, proliferation potential, and differentiation status. (A) Direct fluorescence images of 2 representative clusters of GFP-positive human hepatocytes, *ex vivo* transduced with TRIP-GFP vectors (green cells), integrated in mouse liver parenchyma (counterstained in red by 7-amino-actinomycin D), and still present 3 months after human hepatocyte transplantation (frozen liver section, 7 μm). (B) Immunohistochemical staining of a representative uPA/SCID mouse frozen liver section with antibodies directed against hα-1 AT (**left panel**) and a serial section (**right panel**) analyzed by direct fluorescence after Hoechst 33258 counterstaining (animal killed 3 months after human hepatocyte transplantation). (C and D) Serum concentrations of (C) human albumin and (D) hα1-AT detected in the uPA/SCID mouse at indicated times. Results correspond to the average of the values found in 5 transplanted uPA/SCID mice.

nohistochemical detection of human hepatocyte-specific markers. An average rating of 70% successful engraftment was obtained (11 positive of 16 transplanted mice). We evaluated the level of liver repopulation by quantitative imaging on liver sections of animals at week 12 after transplantation by using both GFP and histochemical staining of hα1-AT. Human cell clusters ranged from 8.9% to 16.1% (mean, 13.5% $\pm$ 9.1%) of total area of liver sections.

The human origin of GFP-expressing hepatocytes was confirmed by immunohistochemical staining with anti-hα1-AT (Fig. 5B) and anti-human hepatocyte antibodies (data not shown). The absence of GFP-negative clusters of human hepatocytes indicates the stability of the transgene expression in mouse liver up to 12 weeks after transplantation.

To confirm the function and viability of human transplanted hepatocytes, we looked for the presence of human specific secreted proteins in the serum of the animals. The concentrations of human albumin and human α1-AT increased with time, thus confirming the expansion of human hepatocytes, which reached a plateau level 4 to 6 weeks after transplantation and persisted for several months thereafter (Fig. 5C and D).

A similar repopulation efficiency and serum concentration of human albumin and α1-AT were found in a control group of 10 uPA/SCID mice transplanted with nontransduced human hepatocytes, indicating that the transduction process does not modify cell physiology (data not shown).

Discussion

Hepatocyte transplantation can be considered as an alternative approach to orthotopic liver transplantation. Moreover, the autotransplantation of genetically engineered hepatocytes avoids immune rejection and side effects of lifelong immunosuppressive treatment. As opposed to direct *in vivo* administration, such an *ex vivo* gene transfer (1) allows a specific and exclusive expression of the transgene in selected target cells, avoiding the accidental transduction of undesired tissues by systemic dissemination, and (2) requires vector amounts compatible with standard laboratory scale vector preparations. However, only a few examples of liver-directed *ex vivo* gene transfer in humans or large animal models have been reported to date.[8,21] These studies clearly showed the potential of this approach. However, several technical difficulties, mainly related to the production and transduction of primary hepatocytes, have limited its use in clinical trials. Recent improvements in oncoretroviral vector titers enabled efficient gene transfer in plated simian hepatocytes using multiple rounds of transduction before reintroduction into the animal.[22]

The development of lentiviral vectors seems to have introduced a new major breakthrough in both *in vivo* and

ex vivo liver gene therapy.[23,24] We show herewith that, using a lentiviral vector, we can bypass the critical steps of the hepatocyte *ex vivo* transduction procedure, namely, the plating and further deleterious trypsin dissociation of cultured hepatocytes. We obtained a highly efficient gene transfer using limited amounts of a lentiviral vector in a very limited period of time in both rat and human hepatocytes that are still in suspension. Moreover, this transduction protocol does not alter hepatocyte viability.

The presence of a DNA FLAP structure in TRIP vectors was crucial to this efficient gene delivery.[25,26] Deletion of the DNA FLAP caused 8- to 20-fold reduction in transduction efficiency that was not compensated for by increasing the vector concentration. Finally, using the medium recovered from vector-producing cells, we propose a simple protocol of hepatocyte transduction, avoiding ultracentrifugation steps. Indeed, we noted a higher transduction efficiency using a nonconcentrated vector preparation. This observation can be explained by partial damage to vector particles during the ultracentrifugation and resuspension steps.

During the preparation of this report, Nguyen et al. showed a 50% to 60% transduction efficiency using a gene transfer protocol of nonadherent human hepatocytes in a medium containing vitamin E.[27] This technique has not been addressed in the present study because, in our hands, transduction efficiency and hepatocyte viability close to 100% were achieved without antioxidants. The significant improvement in transduction efficiency between these recent results and those presented here could be explained by differences in the gene transfer protocol such as transduction volume or continuous rolling agitation, allowing a more rapid and more efficient transduction with a reduced amount of vector. Furthermore, we showed that primary human hepatocytes, isolated from adult donors, transduced without plating were able to colonize and repopulate large areas of uPA/SCID mouse livers (Fig. 5A and B). Transduced human cells maintained a high level of expression of the transgene and a differentiated phenotype, as confirmed by the stable detection of GFP in the liver and albumin and α1-AT in mouse sera and liver for several months (Fig. 5C and D). These data strongly support the hypothesis that differentiated hepatocytes isolated from adult donors can indeed be used for liver-directed *ex vivo* gene therapy and, under appropriate conditions, are able to replicate and repopulate a recipient liver, expanding the number of cells carrying the transgene and maintaining a functional phenotype. The presented results suggest that the global expansion of human transplanted hepatocytes is not due to some side effects of the transduction process, as confirmed by the analogous engrafting and repopulation potential of nontransduced human hepatocytes transplanted in uPA/SCID mice. Moreover, previous studies performed in uPA immunodeficient mice described an equivalent repopulation efficiency (up to 15%) by transplanting adult human hepatocytes.[28] Nevertheless, the safety issue concerning the use of integrative vectors has to be seriously taken into account. It is noteworthy that viral promoter and enhancer sequences in lentiviral vectors used in this study have been deleted (ΔU3 versions). This modification abolishes transactivation of cellular proto-oncogenes by the vector LTR as recently observed in the SCID X1 French trial (M. Cavazzana, personal communication, March 2003). Retroviral vector safety can be further increased by the cotransfer of suicide genes such as herpes thymidine kinase, enabling elimination of transduced cells by treatment with ganciclovir. Insertional mutagenesis and frequency of potentially tumorigenic events after lentiviral-mediated gene transfer remains to be directly addressed.

Previous studies have shown that allogenic hepatocyte transplantation could improve the clinical status of patients harboring an inherited liver disorder such as ornithine carbamoyltransferase deficiency, α1-AT deficiency, glycogen storage disease type IA, and Crigler-Najjar syndrome.[1-3,29] In the patient with Crigler-Najjar syndrome, transplanted hepatocytes, although representing only 2% to 5% of total liver mass, were able to express sufficient levels of uridine diphosphoglucoronate glucoronosyltransferase activity to reduce the need for phototherapy.[3] We can therefore hypothesize that, using our *ex vivo* approach with a lentiviral vector expressing the therapeutic gene at high levels, a limited number of a patient's hepatocytes overexpressing the therapeutic gene may be sufficient to restore a functional phenotype in an autotransplantation protocol. In addition, some acquired liver diseases, such as viral hepatitis C, can be targeted by *ex vivo* liver-directed gene therapy. This could be achieved by transplanting hepatocytes transduced with vectors encoding virus-specific interfering RNAs or ribozymes. These cells, protected by the viral infection, could proliferate and colonize the host liver, taking advantage of the clearing of infected hepatocytes by the host immune system.

In conclusion, we provide here an efficient, simple, and very rapid protocol for *ex vivo* gene transfer in human hepatocytes with a FLAP lentiviral vector. This protocol allows us to circumvent some of the major obstacles previously encountered in *ex vivo* liver gene therapy clinical trials, among which are the requirement to plate and culture hepatocytes during several days, the huge quantities of viral particles needed, and the high proportion of hepatocyte mortality after dis-

sociation from culture plates. This approach could be applied not only to pathologies involving liver function deficiencies but also, because of the unique secretory characteristics and strategic position of the liver, to deficiencies in secreted factors such as hemophilia[23] or to lysosomal enzyme deficiencies such as glycogen storage disease type II (Pompe's disease),[30,31] Fabry's disease,[32] or Tay-Sachs disease.[33]

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