Lentiviral gene therapy for the treatment of inherited liver disease

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LENTIVIRAL GENE THERAPY
FOR THE TREATMENT OF INHERITED LIVER DISEASE

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Lentiviral gene therapy
for the treatment of inherited liver disease

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Chapter 1

Introduction

The Liver

The liver plays vital roles in regulation of synthesis, transformation and storage of compounds. In addition, the liver is involved in secretion of many substances as well as regulation of breakdown products. These processes include, amongst others, control of blood glucose, the production of albumin and clotting factors. The liver also excretes bile, which contains breakdown products, such as bilirubin. Bile also contains bile salts that act as detergents and aid in absorption of dietary fats. Cholesterol that reaches the liver via lipoproteins or is synthesized in the liver can be secreted back into blood with VLDL but also excreted into bile.

Fat-insoluble vitamins, such as A, D, E and K, copper and iron are stored in the liver. Clearance of harmful substances, such as ammonia also takes place in the liver. Additionally, this organ is also largely responsible for modification and inactivation of hormones, e.g. inactivation of testosterone and oestrogen, and degradation or modification of drugs and inactivation of toxins.

The liver weighs about 5% of body weight at birth. The liver in adult man constitutes approximately 2.5% of body weight, which means that it weighs approximately 1.5 kg [6]. It is divided into the larger and the smaller lobes, which are further divided into approximately 100.000 lobules. A macroscopic representation of the liver is shown in figure 1 and a part of a liver lobule is depicted in figure 2. The liver receives its blood supply via the hepatic artery (30%), which supplies oxygen rich blood and the hepatic portal vein (70%), which transports nutrients from the intestine.

Hepatocytes represent about 60% of cells in the liver. Because they are larger than the other cells in the liver, they occupy almost 80% of parenchymal volume. In contrast, the liver sinusoidal endothelial cells (LSEC) account for approximately 15%-20% of the total number of cells in the liver, but only 6.5% of volume. Extracellular spaces account for 16-20% of the liver volume, of which about two-thirds represent the sinusoidal lumen and one-third the perisinusoidal tissue space of Disse. These LSECs are fenestrated cells that have a filtration function and are located between the sinusoidal lumen and the space to the basolateral side of the hepatocytes (Space of Disse) [7].

The liver contains cells of the immune system as well, such as T-cells and specialized macrophages, called Kupffer cells (KC). KCs represent about 15% of total liver cells in number [7] and are located on the luminal side of sinusoidal capillaries. The Kupffer cells account for over half of the body's reservoir of macrophages, which helps fighting infections by destroying pathogens that come into contact with them. These pathogens as well as lipopolysaccharides (LPS) are sequestered from the blood by Kupffer cells. Another function of Kupffer cells is the excretion of chemokines and cytokines.

Stellate cells (also called Ito cells) are located in the Space of Disse and store vitamin A. These cells are also involved in development of fibrosis in liver disease. Hepatocytes are polarized cells; the apical domain is called the canalicular membrane. These bile canaliculi are the site of primary bile formation and connect with the smaller branches of the bile duct system, which eventually drain into the gallbladder and the duodenum [149].

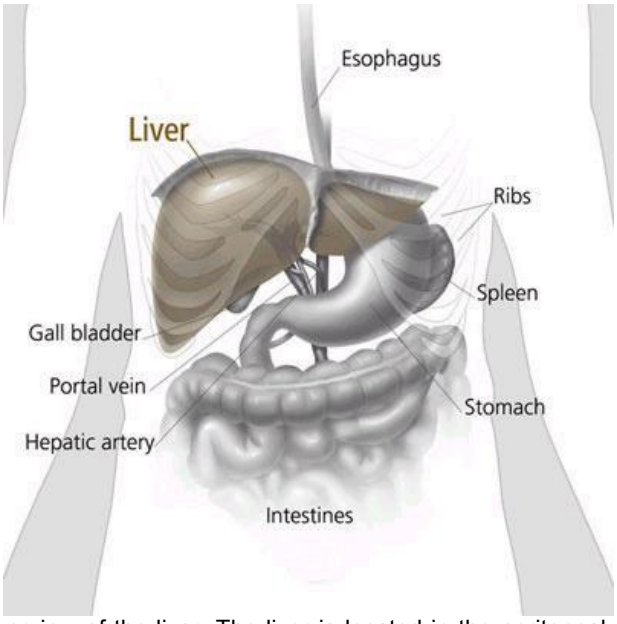

Figure 1. Macroscopic overview of the liver. The liver is located in the peritoneal cavity. It is connected by the portal vein with the intestine and the spleen. It receives oxygen rich blood through the hepatic vein. The liver is connected with the gallbladder via the common bile duct in which the smaller ductules drain their content.

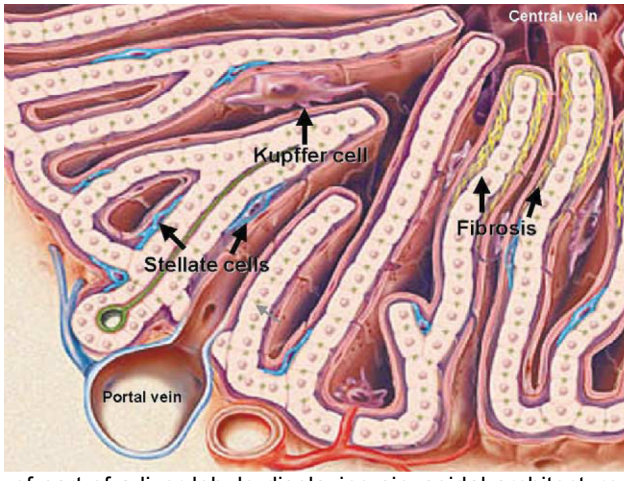

Figure 2. Representation of part of a liver lobule displaying sinusoidal architecture. In normal liver, cords of hepatocytes are surrounded by fenestrated endothelial lining. In the intervening space of Disse are the hepatic stellate cells (blue). Kupffer cells (purple) are typically intrasinusoidal and are shown here as adherent to the endothelial wall. Reprinted from [48] with permission.

Inherited liver diseases

As a result of the many functions that are fulfilled by the liver, there is a large variety of different inherited liver disorders. These diseases can be divided into two types based on their etiology. 1) Directly affecting hepatocyte function, this is often accompanied by liver damage leading to fibrosis and cirrhosis. 2) Affecting the body, but leaving the liver relatively unharmed.

Inborn errors of the first category include α-1-antitrypsin deficiency, Wilson's disease, glycogen storage disease, urea cycle defects and many more. A particularly suitable group of diseases for hepatocyte transplantation and repopulation are patients with progressive familial intrahepatic cholestasis (PFIC) encompassing several different inherited disorders [131,181]. These patients have mutations in a family of transport proteins,

which are the ATP binding cassette (ABC) transporters that require ATP hydrolysis to drive membrane transport.
This group of patients consist of three types of PFIC, namely PFIC1 (*ATP8B1*, FIC1 deficiency/Byler's disease), PFIC2 (*ABCB11*, BSEP deficiency) and PFIC3 (*ABCB4*, MDR3 deficiency). PFIC1 patients have mutations in the *ATP8B1* gene. The function of this protein has not been completely elucidated to date. It has been demonstrated that there is a high percentage of similarity of ATB8B1 to the bovine aminophospholipid flippase that regulates translocation of aminophospholipids (phosphatidylserine, PS, and phosphatidylethanolamine, PE) in plasma membranes. Ujhazy *et al* reported that the energy-dependent translocation of aminophospholipids may indeed be regulated by ATP8B1 protein [183].
A recent publication suggests that the increased sensitivity to the detergent action of hydrophobic bile salts in *Atp8b1*$^{G308V/G308V}$ mutant mice is due to phospholipid randomisation, as shown by increased biliary recovery of cholesterol and ectoenzymes, as well as the presence of phosphatidylserine (PS) in bile [138]. The authors hypothesize that the enhanced extraction of cholesterol by hydrophobic bile salts subsequently may impair the activity of ABCB11 and cause cholestasis. In PFIC2 (BSEP deficiency) patients have a defect in biliary bile salt secretion, caused by mutations in the bile salt export pump, which decreases the transport of bile salt excretion of hepatocytes into bile, leading to accumulation of bile salts.
In PFIC3 (MDR3/ABCB4 deficiency) patients have mutations in the *ABCB4* gene resulting in the absence of ABC transporter ABCB4 (Multidrug Resistance P-glycoprotein 3) [31,33]. This protein is involved in the excretion of phosphatidylcholine into the bile canaliculi resulting in the inactivation of toxic bile salts by formation of mixed micelles. Apparently, these three transporters fulfill crucial roles in bile formation as their absence causes serious liver disease.
Mutations in another ATP-dependent transporter (*ATP7B* gene) may also lead to a severe liver phenotype. Disruption of the *ATP7B* gene causes Wilson disease, which is an autosomal recessive genetic disease resulting from copper toxicity, primarily in liver and extrahepatic sites, such as the brain [15,39,176].
The category of inherited disorders with hepatic pathology also includes metabolic diseases, such as hereditary tyrosinemia type I [57], a recessive liver disease caused by deficiency in fumarylacetoacetate hydrolase (FAH), which converts 4-fumarylacetoacetate into fumarate and acetoacetate, the final step of tyrosine metabolism. Without FAH, metabolic precursors accumulate and induce hepatic and renal toxicity.
Other diseases include inborn errors of ureagenesis. These patients are vulnerable to episodic hyperammonemia, which may result in coma and death. Examples are carbamyl phosphate synthetase, ornithine transcarbamylase or argininosuccinic acid synthetase deficiency. Furthermore, this group of liver diseases also encompasses several glycogen storage diseases [21].
Because defects in the above described diseases may lead to accumulation of toxic compounds or harmful substances in the liver, hepatocytes themselves are being affected. Transplantation of healthy cells may lead to an increase of these cells in the liver, because they render a certain level of resistance to the toxins. All these diseases vary from mild to severe phenotype and as a result the speed of repopulation and the extent of hepatocyte replacement may differ as well. As explained below, these diseases are especially suitable for transplantation with corrected autologous hepatocytes, because repopulation of healthy cells may occur.

Many other metabolic liver disorders do not affect the liver directly, but result in accumulation of toxic compounds or defects elsewhere in the body. In these liver diseases repopulation by transplanted cells will not take place. Examples of this category are the syndrome of Crigler-Najjar and the hemophilias.
Patients with the Crigler-Najjar syndrome do not express bilirubin UDP-glucuronyltransferase (*UGT1A1*)[74,195]. This leads to high levels of unconjugated bilirubin in plasma, which results in jaundice. Patients are generally treated by phototherapy, but this alleviates the symptoms only temporarily and liver transplantation is often necessary, because unconjugated hyperbilirubinemia increases the risk of bilirubin encephalopathy (kernicterus) [1]. For this specific metabolic liver disease the well-established Gunn rat model [157] is available.
In hemophilias there is a decrease of coagulation factors (factor VIII or IX), which are produced in the liver. The most commonly used animal models are based on factor VIII and factor IX deficiency for hemophilia A and hemophilia B respectively.
These coagulation disorders, such as hemophilia A are also well suited for cell replacement therapies or gene therapy, because coagulation factor VIII [187] levels in the blood plasma are therapeutic over a wide range, although unusually high FVIII expression levels may increase thrombotic risk.
All these diseases may require a certain proportion of healthy hepatocyte replacement in the diseased liver to achieve a therapeutic effect. For instance, there is a low therapeutic threshold for hepatocyte replacement of about 5% in murine phenylketonuria [65]. But other diseases, such as defects in lipoproteins can require more than 30% of hepatocytes expressing the normal gene to phenotypically correct the metabolic disorder [18].

Treatment of inherited liver diseases
As mentioned before, there is a broad array of inherited metabolic liver disorders displaying a variety of symptoms; examples include diseases Wilson's disease, α-1-anti-trypsin-deficiency or Crigler-Najjar syndrome. Most of these disorders can only be treated by liver transplantation [35,141,147]. According to the European Liver Transplant Registry (http://www.eltr.org/), of the total of more than 57,000 liver transplantations performed to date in Europe (2003), hepatic associated metabolic disorders represent about 5%. In pediatric patients between the ages of 2 to 15 years relatively more liver transplantations are performed to treat metabolic disorders (27%). The overall 1 year patient survival rate is approximately 83%, and the 10 year survival about 71% [3], with survival of children significantly higher than for adults [72]. Over the years, the number of successful transplantations and the survival of patients has increased considerably [3,14].
Although liver transplantation is a curative treatment option and prolongs life expectancy in these patients, it has major drawbacks [87,108]. It is an invasive procedure, because in patients the whole liver is replaced. Furthermore, in a considerable percentage of the cases retransplantation is required, due to complications [105]. Additionally, the requirement of life-long immunosuppression makes infection by bacterial or fungal organisms one of the most common causes of death [2,71,105]. Immunosuppression may also lead to concomitant kidney failure [122], but improved combinations of immunosuppressive regimens may eventually have less or no toxicity [91,143].
Another drawback is the risk of rejection [2], which may require multiple liver transplantations resulting in high morbidity. Finally, one of the main disadvantages remains the shortage of donor livers resulting in death of patients on the waiting list. Unless these factors are improved in the future, alternatives are required for whole liver transplantation.

Hepatocyte transplantation and repopulation as a potential therapy of inherited liver diseases

Hepatocyte transplantation arose as an alternative for orthotopic transplantation in the 80's. Since then, experimental and clinical applications have been investigated [56,64,128,63]. The liver has the unique ability to grow back to its original size after removal of up to 80% of its mass. This feature can be exploited in some diseases with intrinsic liver damage. Healthy cells can repopulate the liver, because they have a growth advantage over the diseased host liver cells.

Not all livers that become available can be used for orthotopic liver transplantation (OLT), but viable hepatocytes for hepatocyte transplantation can still be harvested from these livers. The isolated hepatocytes from a single liver can be used for multiple recipients; techniques for administration of hepatocytes are relatively simple and minimally invasive with low systemic stress in comparison with OLT. Additionally, these cells can be frozen and stored and subsequently used for transplantation after thawing [30,73]. However, hepatocyte viability decreases after freeze-thaw cycles. Also, hepatocyte transplantation does not require removal of the host liver, which serves as a safety net if hepatocyte transplantation turns out to be ineffective or if the transplanted cells are rejected.

There are many animal models for hepatocyte repopulation of the liver. In a rat model for Wilson disease [199] hepatocyte transplantation and liver repopulation restored biliary copper excretion and lowered liver copper levels with liver histology being completely normalized in Long-Evans Cinnamon rats, as compared to untreated rats [70]. In Mdr2 knockout mice disease was mitigated upon transplantation and subsequent repopulation with corrected hepatocytes [32]. In a mouse model for hereditary tyrosinemia type 1 extensive repopulation of the liver occurs after transplantation of healthy hepatocytes [132].

The development of hepatocyte transplantation has already been translated into clinical application. However, so far hepatocyte transplantation has been performed in diseases without or minor repopulation potential of healthy cells, e.g. for the treatment of Crigler-Najjar syndrome type I [46], in an infant with severe urea cycle disorder [68] and glycogen storage disease type 1a [119]. These patients were all treated with healthy donor hepatocytes.

It has been shown in the mouse model of Hereditary Tyrosinemia Type I, that cultured FAH- hepatocytes could be transduced *ex vivo* with retroviral vectors containing the fumarylacetoacetate hydrolase gene and subsequently repopulated FAH-deficient mice after transplantation [132]. Similarly, in the larger dog model for α-1-antitrypsin deficiency, it was also feasible to *ex vivo* correct autologous hepatocytes by retroviral gene therapy leading to a detectable α-1-antitrypsin expression [82]. In a pilot study five patients with hypercholesterolemia have been treated with low-density lipoprotein (LDL) receptor expressing retrovirus-transduced hepatocytes without morbidity and achieving persistent gene expression lasting for at least four months after gene therapy [60]. Correction of the patient's own cells eliminates the use of donor hepatocytes and reduces the risk of an immune response to the transplanted cells.

In contrast to targeting hepatocytes to the liver by portal vein or intrasplenic administration, hepatocytes can also be transplanted at ectopic sites in the body and correct defects such as hemophilia A in mice [129]. However, survival of ectopic hepatocytes is relatively short. Administration of autologous fibroblasts expressing UDP-glucuronyltransferase can also correct the deficiency of Gunn rats, but these rats developed tumors [162].

Although hepatocyte transplantation may be an alternative, sufficient livers need to be available for isolation, which at present is not enough to serve all patients. As an

alternative to transplantation of primary hepatocytes, an unlimited supply of a hepatocyte cell line is required that can be used for similar transplantation therapies.
To reduce the need of donor livers for isolation of hepatocytes many investigators have tried to culture and expand hepatocytes. Unfortunately, (human) hepatocytes cannot be expanded efficiently *in vitro*, even with addition of growth factors, such as hepatocyte growth factor (HGF) and epidermal growth factor (EGF) [191]. Furthermore, expanded hepatocytes dedifferentiate rapidly. Recently, immortalized hepatocytes have been generated, which could be used to treat liver failure of rats with end-stage cirrhosis [22] and mice with acetaminophen induced acute liver failure [125]. It is remarkable that immortalisation of hepatocytes can lead to maintenance of both hepatocyte specific function and continuous proliferation.

In contrast to mature hepatocytes, human fetal liver cells (HFLC) have the advantage of maintaining differentiation potential and proper function in culture for long periods (up to 4 months) [99]. After transplantation, HFLCs have the capacity to differentiate into mature hepatocytes *in vivo* [103,187]. A comparison between fetal hepatoblasts and adult hepatocytes showed their repopulation potential in rat liver after *ex vivo* transduction with lentiviral vectors [127].
Because HFLCs have these additional properties compared to adult hepatocytes, immortalised human fetal liver cells may be a preferable option to generate an unlimited supply of transplantable cells. Indeed, intrasplenic transplantation of immortalised human fetal hepatocytes has been shown to prolong survival of 90% hepatectomized rats [89,90] and human fetal hepatocytes immortalised by telomerase reconstitution did not affect their differentiation potential *in vivo* [194]. Also, after retroviral vector-mediated expression of simian virus 40 large T antigen to immortalize a primate fetal epithelial liver cell, a bipotent hepatic biliary phenotype was preserved [5]. These bipotent cells could differentiate to both hepatocytes and biliary epithelial cells.
The use of embryonic stem cells [172] has been investigated as an alternative source and treatment option for transplantation in patients with metabolic diseases. It is important to optimise immortalised cell-line culture conditions to preserve their differentiation potential *in vitro* and *in vivo* and preferably maintain multilineage differentiation potential and self-renewing capacity.

An important problem of hepatocyte transplantation at present is the low liver engraftment. This is a serious problem for treatment of liver patients with no destruction of their own liver cells. The low engraftment of hepatocytes in the liver often results in mitigation only, but never a complete correction of the liver disease.

Repopulation models of the liver
Besides the models in which there is an intrinsic repopulation potential of healthy hepatocytes, artificial repopulation models are required to obtain enough healthy hepatocytes to correct disease. There are ample examples of these artificial liver repopulation systems. One such system is the selective repopulation of normal mouse liver by apoptosis resistant Fas/CD95 resistant hepatocytes overexpressing Bcl-2 [109] or Bcl-X_L [112]. Repopulation in this system can be achieved by repetitive administration of the anti-Fas/CD95 antibody Jo-2, which causes apoptosis in the recipient hepatocytes while the donor hepatocytes are resistant through Bcl-2 overexpression. Although Bcl-2 overexpressing cells are not completely resistant to Jo-2 induced apoptosis, the risk that uncontrolled expression of anti-apoptotic genes may lead to tumor development, or may inhibit immune responses to hepatocytes infected with viruses, makes this approach unsuitable for human applications.

The Fah knockout mouse is a model for hereditary tyrosinemia type I [4,58,59], which is a disorder of tyrosine catabolism caused by deficiency of fumarylacetoacetate hydrolase. The compound (2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) blocks tyrosine degradation at the level of 4-hydroxyphenylpyruvate dioxygenase (HPD) and also had been used with success in the clinic [58]. Withdrawal of NTBC causes cell death in the recipient hepatocytes, but not in the healthy donor hepatocytes thereby causing repopulation of the donor cells [134].
In another artificial repopulation model liver-specific overexpression of urokinase plasminogen activator (uPA) causes damage and death of hepatocytes [154]. In this mouse model for hepatocyte repopulation, mice carry the uPA transgene under control of an albumin promoter. These hepatocytes are functionally compromised and uPA negative cells expand in nodular fashion. This model has a strong repopulation potential, as demonstrated by complete reconstitution with xenogeneic rat hepatocytes in immunotolerant Alb-uPA transgenic mice [148]. Although the authors of this paper claimed complete reconstitution, parenchyma in these mice is gradually replaced by clones of cells from the acceptor mouse that have deleted the transgene uPA DNA [154]. Unfortunately, this system cannot be used as a clinical artificial repopulation model, because all endogenous hepatocytes need to overexpress uPA. In both the uPA and Fah model near complete reconstitution may take place compared to the Fas/CD95 resistance or MDR3 deficiency model, which both lead to partial repopulation of the liver only. Other artificial repopulation models that are actually also unfavourable for clinical application are models with mitosis inhibitors. To achieve repopulation in rats and mice administration of retrorsine with or without partial hepatectomy is often used [97,98]. Partial hepatectomy leads to the required proliferative stimulus and only the transplanted cells can proliferate because they did not receive growth restriction. However, mitosis inhibitors may lead to tumor development and hepatectomy is invasive.
Therefore new artificial repopulation systems are required for treatment of patients with inherited liver diseases to increase functional hepatocytes in the liver to a significant proportion. This system should affect only the liver without a stimulus to progress towards uncontrolled cell-cycling, but so far no promising systems have come forward.

Gene therapy for inborn liver errors
As an alternative for orthotopic liver transplantation (OLT) or cell transplantation, gene therapy may be used in the future to treat inherited diseases. The gene therapy approaches that have been explored [18,24,26,83,84,86,95,124,168,178] can be divided in non-viral and viral vector methods. Because viruses have evolved to replicate and survive in their host organisms, elements of viruses may be perfect to introduce genes in cells that need correction. There are three major viral-based vector systems that have been extensively studied for applications in liver directed gene therapy: adenovirus, adeno-associated virus and retroviruses. All these systems have their pros and cons (Table 1) and will be discussed in detail below.

Table 1: Viral vectors. Overview of pros and cons of the four large viral gene therapy groups for treatment of inherited liver diseases.

	Advantages	Disadvantages
Adenovirus	Large cloning capacity (up to 37 kb). High levels of transduction of the liver. AAV vectors are stable.	Acute toxicity and highly immunogenic, but reduced for helper-dependent adenovirus. Short term expression.
AAV	Immunogenicity possible, but often present. Long term expression. Wildtype virus infects humans, but is not associated with illness. Can deliver genes to slow- or non-dividing cells.	Limited cloning capacity (±5 kb). Low integration efficiency, resides mostly episomal.
Retrovirus	Can deliver up to 7.5 kb inserted genetic material. Long term expression possible, stable expression. Generally, no viral immune response, but depends of pseudotype.	Potential risk of insertional mutagenesis, integrates preferentially into promoter regions. Integration in dividing cells only.
Lentivirus	Can deliver genes to non-dividing cells and terminally differentiated cells. Can deliver up to 9 kb inserted genetic material. Long term and stable expression. Generally, no viral immune response, but depends of pseudotype.	Potential risk of insertional mutagenesis, integrates preferentially into genes.

Adenoviral vector system

Human adenovirus serotypes are widespread in the population. Infection of these viruses is often asymptomatic, but can result in respiratory disease in children, conjunctivitis and gastroenteritis, leading to diarrhea [42].

The adenoviral vector systems have a large transgene capacity, but do not integrate into the host genome. A major problem of these vectors is that there is a high probability that the immune system is activated by components of the viral vector itself.

Another disadvantage of adenoviral vectors in treating inherited liver diseases is that lifelong expression of the therapeutic gene is required. In many studies that used adenoviral vectors the gene expression declined slowly, but remained for more than 90% in periods of up to 1 year in mice [36] or dogs [85]. In mouse and dog models for hemophilia A, intravenous injection of high capacity adenoviral vectors expressing B-domain-deleted human or canine factor VIII from liver specific promoters resulted in therapeutic FVIII levels, but in mice expression declined because of neutralizing antibodies and a cellular immune response. In dog, no antibodies to canine FVIII could be detected [25]. On the other hand, there are also studies with long-term correction, for instance in mice treated with an adenoviral vector leading to canine FVIII expression for more than a year [49].

Another study showed that co-expression of CTLA4Ig with BUGT in an adenoviral vector permitted long-term, repeatable transgene expression in the Gunn rat [179]. In an animal model for hereditary tyrosinemia type I (HT1), mice lacking fumarylacetoacetate hydrolase (FAH) were injected with first-generation adenoviral vector expressing the human FAH gene and survived at least 2-9 months. Non-treated FAH mutant control mice died within 6 weeks [133]. Unfortunately, the strong selective advantage of FAH-expressing cells in a FAH-deficient liver led to the development of carcinomas, which are not associated with the viral treatment itself.

The development of helper dependent adenoviral vectors or gutless vectors often provides longer gene expression. These vectors are devoid of all viral coding sequences and provide an increased cloning capacity (up to 37 kb).

Lifelong correction with a single dose of helper-dependent adenoviral vector was sufficient to achieve stable correction of Crigler-Najjar (CN) disease type 1 in rats for more than two years [180]. In low-density lipoprotein receptor-deficient mice, an animal model of familial hypercholesterolemia, helper-dependent adenoviral vector expressing the very low-density lipoprotein receptor produced long-term lowering of plasma cholesterol and prevented development of atherosclerosis in LDLR-deficient mice [10,130] [126]. Large-scale production of helper-dependent adenoviral vectors is difficult and

contamination with helper virus remains a problem to be solved, although Palmer and Ng showed that they could obtain high titers (more than 1 x 10^{13}) and low helper virus contamination after CsCl purification (0.02-0.01%) [135].
Experiments in rats and mice are often non-toxic. However, animal models closer to humans often display toxicity after adenoviral vector administration caused by viral antigens that trigger an immune response [201]. This vector-induced toxicity of high dose recombinant adenoviral vectors remains a problem also in vector naïve and pre-immunized mice [188]. This has been shown in a hemophilia A model in which low dose adenoviral vector administration gave no toxicity, but higher vector doses caused transient hepatotoxicity and thrombocytopenia [16]. In contrast, in a dog model of hemophilia B phenotypic correction was achieved without any detectable symptoms of toxicity [37], and indeed also with a helper dependent adenoviral vector in a similar study [19].
In animal models that are more closely related to humans, high doses of adenoviral vector injected into a baboon resulted in acute symptoms, decreased platelet counts, and increased liver enzymes, and became life threatening at 48 hrs after injection. A baboon treated with a lower dose developed no symptoms [117].
In another study in baboons, long-term expression of the human α_1-antitrypsin (hAAT) gene was observed with the use of gutless vectors and not with regular adenoviral vectors. Re-administration of another serotype could re-establish corrected hAAT values [116]. Injection of a high dose in a baboon, which resulted in a 100% transduction efficiency of hepatocytes, was again lethal. A lower dose only resulted in mild hepatotoxicity [20].
Viral capsid proteins in first-generation adenovirus-expressing lacZ triggered systemic cytokine syndrome in nonhuman primates [159], which was mediated by dendritic cells and macrophages [204]. These toxicity issues should be addressed, before these vectors can be taken into the clinic.
Patients have already been subjected to adenoviral vectors and the first 17 subjects of a clinical trial of adenoviral gene therapy for ornithine transcarbalase (OTC) deficiency showed no clinical signs of jaundice after vector administration, but some adverse effects included fevers, transient rise in hepatic transaminases and thrombocytopenia [145]. The death of a patient in a clinical trial of adenoviral gene therapy for ornithine transcarbamylase (OTC) deficiency [144,145] pointed out that increased sensitivity to viral infections and systemic inflammation in individuals, necessitates alternative gene therapy systems that are less immunogenic.

Adeno-associated virus vectors
Adeno-associated virus (AAV) is a parvovirus with a single stranded linear genome [151]. Compared to adenoviral vectors and lentiviral vectors, these viral vectors are relatively small and are therefore not suitable for correction of inherited diseases caused by genetic mutations in large genes.
Some reports have used AAV-vectors as an *ex vivo* approach to transduce liver progenitor cells [167], although in general these vectors have a poor transduction efficiency *ex vivo* and direct injection is required [151]. For some types of AAV, antibodies can be frequently found in human serum samples [52]. The use of other vector serotypes may overcome this immunity. On the other hand, readministration of AAV vectors in muscle-directed gene therapy was not hampered by neutralizing antibodies [41]. An advantage of this viral vector system is that none of the AAV serotypes have been shown to be pathogenic. It was generally thought that integration of wildtype provirus genomes, which often takes place at a common site on human chromosome 19 (19q13-qter) [93,94,153] would provide a safe delivery in the genome, but modified AAV vectors turned out to

integrate at random [152,200]. Despite this random integration, no evidence of tumorigenesis of AAV vectors was observed in an elaborate study performed in mice [11].

AAV vectors have been used extensively in animal models. Sustained expression was observed after single intraportal vein injection of rAAV vector encoding canine factor IX (cFIX) [192,193]. In two other studies, AAV-vectors injected intramuscularly or into the liver also corrected factor IX levels in canine and murine models with no associated toxicity [67,118,166]. Several serotypes of AAV encoding a liver-specific promoter and human blood coagulation factor IX gene injected in mice led to curative levels for patients with hemophilia [55].
In models for glycogen storage disease type Ia AAV delivery of glucose-6-phosphatase-α resulted in sustained expression and improvement of liver histology and metabolic abnormalities [9,53]. In glycogen storage disease type II (Pompe disease), which is a lysosomal storage disease, reduced glycogen content of muscle was obtained by adeno-associated virus vectors expressing α-glucosidase [27,173,174] and this mitigated the disease process.
Therapeutic levels of α-galactosidase were obtained by administration of AAV2 vectors in a mouse model for Fabry disease (X-linked lysosomal storage disease) with a significantly reduced immune response to the transgene if a liver restricted enhancer/promoter was used [207] in contrast to an ubiquitous promoter.
This viral vector system can also be applied in larger animal models and humans. Administration of several AAV serotypes showed different efficiency in nonhuman primates, but it was also shown that pre-existing immunity, gender and immunity to the transgene are important host factors that influence AAV serotype gene transfer [51]. Preclinical evaluations in macaques, with rAAV vectors injected in the hepatic artery or the portal vein, gave 4% and 8% of normal physiological levels of hFIX [121].
Recently, in a clinical trial to treat hemophilia B patients with AAV2 vectors, expression at therapeutic levels was achieved at the highest dose tested, but after about 8 weeks a gradual decline in factor IX was found as a result of an immune response to transduced hepatocytes [104].

Retroviruses
Retroviral vectors are vectors based on murine retroviruses, such as Murine Moloney Leukemia virus (MMLV) or more complex ones such as the lentiviruses. These vectors can integrate in the genome, which may lead to long-term expression of the transgene. The main problem with vectors based on MMLV is that they cannot deliver genes to non-dividing cells, such hepatocytes and neuronal cells.
Long-term expression of coagulation factor VIII by retroviral vectors based on Moloney leukaemia virus resulted in correction of the bleeding disorder in FVIII-deficient mice, but in the majority of the mice inhibitors to FVIII could be detected [187]. Hereditary Tyrosinemia Type I could also be treated with *ex vivo* transduction of hepatocytes followed by transplantation and repopulation [132]. MLV-based retroviral vectors were used for long-term expression of β-galactosidase in dogs with mucopolysaccharidosis VII [197] and therapeutic levels of canine factor IX for hemophilia B in mice and dogs [196].
As mentioned above in a clinical trial to treat familial hypercholesterolemia autologous hepatocytes were successfully corrected with recombinant retroviruses carrying the LDL receptor [60,61].
By using retroviral vectors in Gunn rats, long-term reduction of serum bilirubin levels were obtained [175]. However, retroviruses only integrate in dividing cells and stimulants of

cell-division, such as hepatocyte growth factor, triiodothyronine or partial hepatectomy have to be used [110] to obtain high hepatocyte transduction efficiency.

HIV based vectors

Human Immunodeficiency Virus (HIV) is part of the lentivirus family. The lipid-enveloped particles comprise a homodimer of linear, positive-sense, single stranded RNA genomes of about 9.7 kb [44]. As all retroviruses, the virion RNA genome needs to be converted into double-stranded linear DNA by reverse transcription. This process takes place in the cytoplasm. The resultant viral DNA is then integrated into the host genome to produce the provirus [42,169].

The major advantage of lentiviral vectors as compared to simple retroviral vectors, is that they can integrate in non-dividing cells [114,146]. To increase safety, flexibility and efficiency of the vector system, cis- and trans-activating domains were separated on different plasmids [114]. These lentiviral vectors are often referred to as third generation lentiviral vectors [34]. In this vector system, the *gag* gene encoding a polyprotein that forms the inner virion structures matrix, capsid and nucleoprotein and the *pol* gene encoding the viral enzymes protease, reverse transcriptase, RNase H, and integrase are located on a single plasmid and the envelope protein on another one.

The regulatory protein Tat is not required anymore, because the constitutive promoters upstream of the vector transcript take care of producing the messenger [34]. The Rev protein regulates the nuclear export of the vector transcript. To decrease the chance of recombination, the *rev* gene has also been removed from the packaging construct. This resulted in the necessity of Rev complementation in trans for the expression of gag and pol [34] on a third plasmid.

The four accessory proteins Vif, Vpr, Vpu and Nef, which are specific for lentiviruses and are involved in HIV pathogenesis, are omitted from third generation lentiviral vectors. In most third-generation lentiviral vectors, posttranslational regulatory elements are included in the transfer vector to increase transcription of transgenes, such as the Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (PRE) [208] or human hepatitis B virus PRE [161]. These vectors also contain central polypurine tract (cPPT), which increase efficient transport into the nucleus leading to incorporation into the host cell genome [186].

Self-inactivating vectors were developed to make the viral therapeutics safer [113,209]. In these constructs the promoter and enhancer elements from the U3 region of the long terminal repeat sequence (LTR) were deleted to remove LTR-directed transcription. The generation of self-inactivating vectors was based on similar modifications in retroviral vector systems. In the 5'LTR the complete U3 region has been replaced by the Rous sarcoma virus (RSV) promoter to produce the lentiviral vector transcript. This makes it also feasible to use internal promoters in the lentiviral vector for tissue specific expression. A part of the U3 region of the 3' LTR has been deleted as well, i.e. 400bp sequence including the TATA box. The packaging constructs that are expressed in *trans* do not contain any LTR sequences, which makes certain that recombination cannot lead to reconstitution of complete wildtype LTRs. All these modifications made sure that less than 20% of the HIV-1 genome is present in the viral construct for functional gene therapy vectors.

In most cases, human embryonic kidney 293T cells are used for production of lentiviral vectors by transient transfection, but other cell-types that have high transfection efficiency may be used as well. Additionally, stable cell lines expressing the required trans-elements can be generated, either inducible or not [38], to increase consistency of production. A schematic representation of plasmids for the production of lentiviral vectors is depicted in figure 3.

Other lentiviral vector systems based on bovine [106], simian [54,190] or feline lentiviruses are being tested and under further development in addition to the lentiviral vector systems based on HIV. Another general problem of retroviruses is that they integrate randomly with a strong preference for actively transcribed regions in the genome [28,107,160]. Themis *et al* showed that lentiviral vectors based on equine infectious anaemia virus could induce tumors in the liver if injected *in utero* [177], but in this model HIV-based vectors did not. In a more recently published paper, low genotoxicity of HIV vectors has been demonstrated in a tumor-prone mouse model [115]. This genotoxicity may not lead to uncontrolled cell division in patients with liver disease, because hepatocytes hardly proliferate under normal conditions *in vivo* and it is therefore unlikely that viral vectors integrate near or disrupt genes that are involved in cell-cycle.

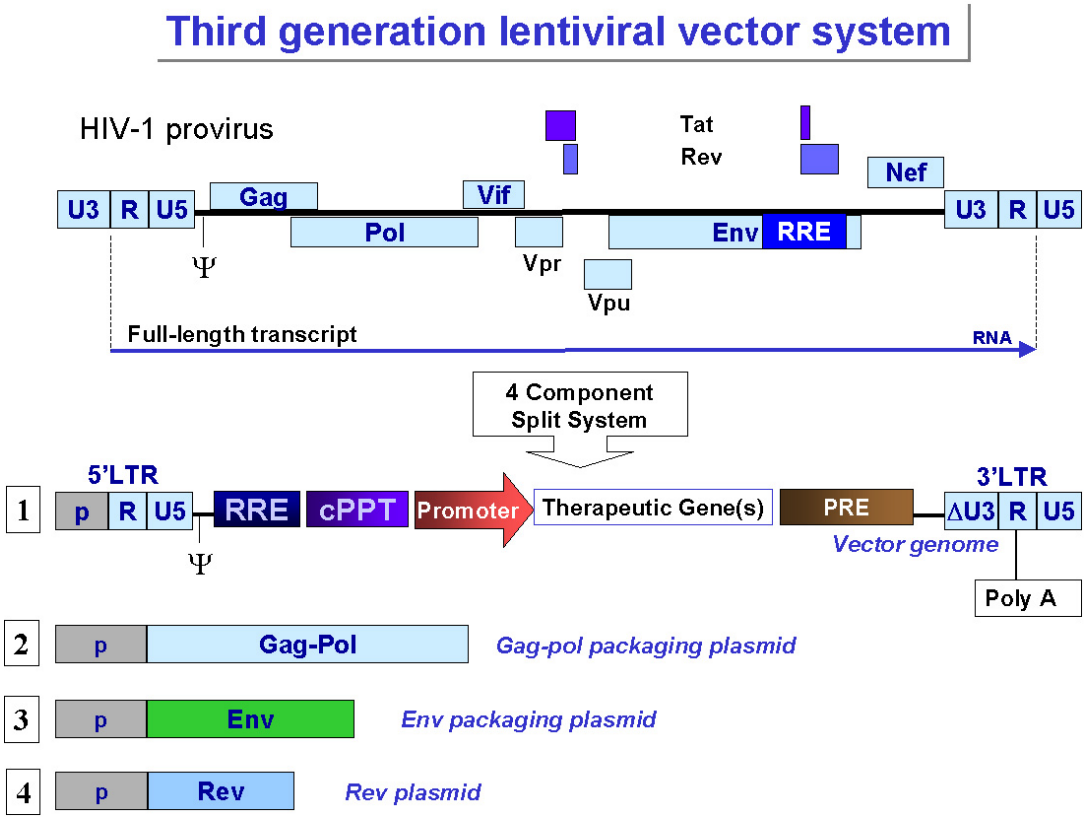

Figure 3. Third generation lentiviral vector four plasmid system. The wildtype HIV provirus is depicted in the upper section. For the lentiviral transfer vector, the majority of the genes of wildtype HIV have been removed from the transfer vector (1). Sequences that have been removed include the regulatory gene *Tat* and the accessory genes *vif*, *vpr*, *vpu* and *nef*. The accessory proteins are involved in pathogenesis of HIV infection and not necessary for efficient viral vector production. The LTR sequences necessary for integration remain in the transfer vector. Additionally, the packaging signal Ψ is required for efficient inclusion into the viral particle, the Rev Responsive Element (RRE) for transport for stable messenger from the nucleus and the central polypurine tract (cPPT) to increase the transport into the nucleus after reverse transcription. Promoters of interest are inserted downstream of the cPPT region followed by the therapeutic gene. Downstream of the transgene cassette, a posttranslational regulatory element (PRE) increases the number of viral transcripts, but also of the therapeutic transcript. Our lentiviral vector contains the hepatitis B PRE, but more regularly the Woodchuck PRE element has been used. The vector is self-inactivating, because the U3 region of the 5' LTR has been removed and replaced by the RSV promoter, which is not included in the viral transcript. Additionally, part of the U3 region in the 3'LTR has also been removed (ΔU3). Both these modifications are necessary for the so-called self-inactivating vectors (SIN). To terminate transcription, the R region in the 3'LTR contains the HIV poly A signal.
The packaging plasmids, (2) gag-pol, containing the *gag* structural proteins for matrix and core and *pol* consisting of reverse transcriptase, integrase and protease (3) the envelope protein and (4) Rev are expressed in *trans*.

Lentiviral vector liver directed gene therapy

Lentiviral vectors have been used in various animal models to treat metabolic liver diseases. Many different routes of administration have been used. Injection into the portal vein may target the liver, because then the viral vectors encounter hepatic cells first. Systemic delivery in adult mice by tail vein injection of lentiviral vectors expressing the marker green fluorescent protein (GFP) could lead to high levels (up to 30%) of transduction of parenchymal and non-parenchymal cells of the liver [43,45,76]. Vascular injection of HIV-derived lentiviral vector encoding human factor VIII or human factor IX (hFIX) also led to therapeutic levels of the protein [43,45,136,182]. The injection into either the portal vein or the tail vein of mice could produce potentially therapeutic serum levels of hFIX protein [120], but the portal vein administration produced the highest levels of hFIX [182]. In FVIII knockout mice, correction was achieved at levels lower than 5% of normal by intraperitoneal infusion of LV vector containing human B-domain-deleted factor VIII [92]. Another circulatory route of administration may be via the hepatic vein.

We have shown that injection through the common bile duct can also lead to transduction of hepatocytes (chapter 2, van Til *et al*).

Age dependent administration and effect on immune response

Early treatment of patients with metabolic diseases may be required from an ethical point of view, because if treatment is possible from an early stage it should be started as soon as possible to increase the quality of life. Gene transfer at young age has several other advantages. It has been shown by Park *et al* [137], that efficacy of hepatic gene transfer decreased with age. The opinion of the authors was that this was caused by the reduction in the number and size of the fenestrae of the endothelial cells in older animals. However, we show that the number and size does not hamper lentiviral hepatocyte transduction (chapter 2 and [185]). Other factors in older mice may therefore hamper efficient lentiviral transduction of hepatocytes. Applying routine biochemical assays may help identify genetic disorders at an early stage [69] and may allow early treatment.

The immunological reaction to a particular transgene may be solved as well if treatment is started *in utero* or in neonates, before complete maturation of the immune system has been achieved.

Indeed, in immunocompetent mice, injection in the neonatal phase is often associated with a reduced immune response to the transgene product or none at all [158], especially if the viral vectors are injected directly into the circulation [202].

Several groups have shown that injection of high doses of lentiviral vectors in neonatal rats can completely correct the disease long-term, e.g. in an animal model of Crigler-Najjar type 1 [123] by injection of lentiviral vectors into the temporal vein. In a similar study, oncoretroviral vectors had similar effect [12]. Also, neonatal FVIII deficient mice and dogs treated with retroviral vectors resulted in normalised plasma canine FVIII [198]. No FVIII inhibitors were produced in neonatal transduction in contrast to adult transduction. At an earlier stage of development, injection in rat fetuses at embryonic day 19 with lentiviral vectors expressing bilirubin UDP-glucuronyltransferase (UGT1A1) reduced serum bilirubin levels about 50% for more than a year [163].

In an animal model for the lysosomal storage disease mucopolysaccharidosis I (MPS I), α-L-iduronidase (IDUA) expression was restored by injection of retroviral vectors in neonates and complete correction was achieved of clinical manifestations associated with deficiency [101]. Intravenous neonatal lentiviral gene delivery into IDUA-deficient mice resulted in near normalization of the phenotypic manifestations of the disease [88]. In contrast, we [164] have shown that neonatal injection, and even *in utero* treatment, can still

lead to generation of immune response against the transgene UGT1A1, as shown by the development of anti-UGT antibodies. Unfortunately, many investigators only investigate the therapeutic effect, and do not look at antibody development. For the human situation this may be very important and these examples therefore pinpoint the importance of early intervention, the vector design and the route of administration, which can lead to successful treatment of inherited liver diseases.
On the other hand, treatment can still be successful, if intervention is started later in life. In juvenile Gunn rats treated with an lentiviral vector with a liver specific promoter (e.g. albumin promoter) bilirubin levels were normalised [184] and in 5-month-old hypercholesterolemic rabbits, third generation lentiviral vectors with liver-specific promoter expressing low-density lipoprotein receptor lowered cholesterol levels significantly up to 2 years for 34% [81].

Liver targeting by changing viral vector tropism
The endogenous wildtype HIV-envelope protein has a tropism for macrophages and T-lymphocytes [42]. Replacement of the HIV envelope by heterologous envelope proteins may change tropism for the cell-type of interest. It may also address another problem of the HIV envelope protein, i.e. the poor ability to be concentrated through centrifugation. The most often used envelope protein is the G-glycoprotein of the vesicular stomatitis virus (VSV), which enables transduction of a wide variety of cells [139] and makes concentration by centrifugation feasible [8].
Many other envelope proteins have been tested for stability and incorporation in retroviral vectors. One idea was to find envelope proteins less toxic than VSV-G to generate stable producer cell lines. Retroviral vectors could be pseudotyped with Ross River Virus (RRV), Semliki Forest virus or Sindbis virus envelope proteins. These envelope proteins are derived from viruses, which can replicate in many hosts and the incorporation of these envelope proteins results in a broad tropism [165,171]. Semliki Forest Virus envelope proteins incorporate less efficient into lentivirus vectors than RRV envelope protein and infectious titers of RRV pseudotype are again lower than VSV-G [77,78,170], but can be concentrated. The use of Jaagsiekte sheep retrovirus envelope led to detectable virus, but was also much lower than VSV-G [100].
In vivo gene therapy of feline immunodeficiency virus (FIV) vectors also efficiently incorporated RRV into FIV virions and systemic delivery predominantly transduced liver of recipient mice [79]. The RRV/FIV transduction efficiency of the liver was 20-fold higher than VSV-G FIV with a reduction of cytotoxicity [79].
GP64 envelope glycoprotein from baculovirus *Autographica californica* multinuclear polydrosis virus can also efficiently pseudotype lentiviral vectors [96]. Portal vein injection could efficiently deliver gp64-pseudotyped vectors *in vivo* with comparable transgene expression to VSV-G-pseudotyped vectors while tropism of gp64 was more restricted than VSV-G *in vitro*, with especially poor ability to transduce hematopoietic cell types, including dendritic cells [156]. Use of this envelope protein may also reduce cytotoxicity [156]. FIV vectors were efficiently pseudotyped with gp64 and showed hepatocyte tropism in a hemophilia A mice model [80].
In another study, fetuses were injected with lentiviral vectors pseudotyped with VSV-G, Mokola virus, or with Ebola virus envelope glycoproteins, but out of these three VSV-G pseudotyped virus vectors transduced the liver most efficiently [102]. Lymphocytic choriomeningitis virus glycoprotein pseudotyped lentivirus did transduce a broad range of cell types, e.g. human hepatoma cell line. It could also be concentrated and was not cell-toxic [13].

A problem with the majority of the envelope proteins used for pseudotyping lentiviral vectors are the low titers that are obtained, while the viral particles cannot be concentrated to high titers.

Manipulation of envelope proteins, i.e. changing the intracellular or extracellular domains may lead to more stable proteins or increased incorporation. It has been shown before that lentivirus vectors can be pseudotyped with feline endogenous virus RD114 envelope glycoproteins. Chimeric glycoproteins, with the transmembrane and extracellular domains fused to cytoplasmic domain derived from the amphotropic Moloney murine leukaemia virus 4070A glycoprotein resulted in 15 fold higher titer than unmodified RD114 glycoprotein [203]. The above mentioned GP64 glycoprotein has also been fused to Decay Accelerating Factor (DAF) and efficiently presents this moiety for binding to its cognate receptor [62].
Unfortunately, it is difficult to obtain a 100% cell-type specific tropism, but this remains an important issue as reduced transduction of antigen-presenting cells is crucial to prevent transgene-induced immune responses.

Hepatocyte specific expression and immune response against the transgene
As mentioned above, one of the main problems in gene therapy may be the activation of the immune system and subsequent clearance of cells expressing the therapeutic gene.
First of all, the route of administration may affect the development of an immune response [142]. Therapeutic levels of human FIX were detected in the majority of immunocompetent hemophilia B mice following intravenous administration of rAAV vector without the development of anti-hFIX antibodies; in contrast, hFIX protein was not detected in 14 immunocompetent mice following intramuscular administration of the virus which led to production of neutralizing antibodies [120]. On the other hand, intramuscular injection in hemophilia B mice failed to activate FIX-specific cytotoxic T-lymphocytes [40]. Development of an immune response may therefore also be dependent on the route of administration. Other factors may also influence the immune response. The applied vector dose and the expression level of the transgene often influence factor IX-specific T and B cell responses in muscle-directed gene therapy [66]. For some inherited liver diseases though, high expression of the therapeutic gene is required and to obtain that a high dose is injected. The development of an immune response is often a dose response effect, because low dose often does not lead to immune reactivity. The use of some AAV vectors, that expressed erythropoietin (EPO) even led to autoimmune anemia in macaques [50], even though the monkeys already expressed EPO before treatment.

The different viral vectors can elicit various immune responses. Recombinant adenovirus vectors activate T-cells by direct transduction of dendritic cells and by cross-presentation of the transgene product, while rAAV gene transfer only activates T cells by the latter mechanism [155]. In adult animals, immune responses to the transgene are often observed if the therapeutic vehicle is injected into the circulation [43,186], because antigen presenting cells are transduced [186].
Other groups also showed that cell types involved in regulating immune responses can efficiently be transduced by lentiviral vectors, e.g. macrophages, splenic lymphocytes [150] and primary unstimulated T-cells [29,206]. The lentiviral gene transfer into primary mouse and human B-lymphocytes is pseudotype dependent [75]. The transduction of these cells may be deleterious as they contribute to the immune response.

Ex vivo transduction of hepatocytes followed by transplantation may reduce the development of immune responses [23], because the transgene expression will be limited to hepatocytes. This can also be reached by *in vivo* transduction with vectors that contain hepatocyte-specific promoters. Brown *et al* showed that prevention of transgene expression in antigen presenting cells indeed enables stable gene transfer in the liver [17]. Earlier publications also showed that evasion of immune responses was obtained in hepatocyte-restricted expression of human acid α-glucosidase in glycogen storage disease type II in mice [47]. In AAV mediated expression of coagulation factor IX by a chimeric promoter, hepatocyte-specific gene transfer evaded the development of antibodies [111]. Although cell type specific promoters can reduce the immune response against the transgene, constitutive promoters can induce immune tolerance as well. Immune tolerance to coagulation factor IX was achieved with the use of the elongation factor 1α (EF1α) promoter, because there was no decrease in transgene expression, although antibodies against the transgene were detected.
The purity of the viral vector preparation may also have its effect on the generation of an immune response. In animals injected with retrovirus-mediated gene transfer, development of an immune response was lowest if viral supernatant was perfused after complete vascular exclusion of the organ and if the viral vector preparation had been purified on an affinity column [140].
In addition to all this, pre-existing immunity to viral vectors may be a future problem in gene therapy. It has been clearly shown that administration of adenoviral vectors in a context of pre-existing immunity can lead to vector induced-toxicity [189].In addition, adeno-associated virus serotype 2 delivered transgenes can be imparted by genetic predisposition to autoimmunity [205].

Aim of this thesis
The aim of this thesis is the development of gene and cell-therapies for treatment of inherited liver diseases.
In gene therapy the transduction of liver by lentiviral vectors is low. Because the majority of the cells that are transduced in the liver are non-parenchymal cells, such as Kupffer cells and liver sinusoidal endothelial cells (LSEC), one of our aims was to develop methods that improve lentivirus transduction of hepatocytes, i.e. by affecting different cell types in the liver. It was thought that the fenestrae of the LSEC, which give access to the Space of Disse may hamper hepatocyte transduction, because of size restrictions. We showed in chapter 2 that removal of LSEC layer did not improve hepatocyte transduction. Another cell-type that might affect targeting of lentivirus to hepatocytes is the Kupffer cell. In other studies with adenoviral vectors and retroviral vectors hepatocyte transduction could be improved, which was in agreement with what we observed. Kupffer cell depletion by treatment with gadoliniumchloride did improve lentiviral transduction of hepatocytes (chapter 2).
Improving lentiviral hepatocyte transduction may also be achieved by changing the viral vector itself. HIV lentiviral vectors are enveloped particles, which contain envelope glycoproteins that bind to the cell surface of the target cell. Alteration of the glycoproteins into one that only bind to receptors present on hepatocytes should lead to increased transduction efficiency of the preferred cell-type and a lower required viral dose. We tried to use the Sendai envelope protein, instead of the commonly used VSV-G envelope protein, because the Sendai envelope protein has been described to specifically bind to the asialoglycoprotein receptor on hepatocytes. To obtain stable lentiviral particles, part of the Sendai envelope was fused to GP64. Lentivirus pseudotyped Sendai/GP64 particles showed a relatively higher transduction of HepG2 cells as compared to HeLa cells. Additionally, after *in vivo* administration in mice less Kupffer cells were transduced

compared to GP64 injected lentiviral particles. Unfortunately, there was no increased transduction of hepatocytes. These results are described in chapter 3.
Crigler-Najjar patients have high serum bilirubin levels due to low or no bilirubin-UDP-glucuronyl transferase activity. The high serum bilirubin levels may eventually lead to kernicterus and death. Gene therapy may provide a future alternative to the current phototherapy and liver transplantation to treat these patients completely.
In chapter 4 *in utero* and neonatal injection of BUGT-expressing lentiviral vectors resulted in long-term decreased serum bilirubin levels. Administration of proteins during early development is often considered to avoid the immune response directed against the transgene. In chapter 4 we show that if lentiviral vectors are injected in the peritoneum an antibody response can still be elicited to the transgene. Although an immune response was elicited the correction was long-term in these rats. However, these results may still have consequences for the treatment of human patients.
Another severe inherited liver disease is Progressive Familial Intrahepatic Cholestasis type 3 (PFIC3). These patients have no expression of ABCB4 protein, which is a phosphatidylcholine (PC) translocases. The translocation of PC in healthy patients results in neutralization of bile salts. Because PFIC3 patients lack the expression of ABCB4 they develop cholestasis. Gene therapy may also provide an alternative treatment option in the future to treat these patients. We show in chapter 5 that expression of ABCB4 leads to reduction in the production of infectious lentiviral particles. We hypothesize that this is due to a change in lipid composition of the lentiviral particle. This has implications for lentiviral gene therapy of PFIC3, because high dose is required to obtain remission of clinical symptoms.
Not all primary isolated cell-types can be easily transduced by lentiviral vectors, without increasing the number of copies per cell substantially. In chapter 6 we describe a novel autologous selection marker for mammalian cells. This selection marker is based on resistance of cells against α-amanitin by over-expression of a mutated largest subunit of polymerase II. Finally, in chapter 7 we made a clone of human fetal liver cells with over-expression of hTERT that could be grown for more than 120 population doublings and still maintained the ability to differentiate to hepatocytes, if transplanted in mice. HFLC derived cell-lines such as these may therefore be used as an unlimited source for transplantation into patients with inherited liver diseases.

References

1. The Metabolic and Molecular Bases of Inherited Disease. Scrivner, CR, Beaudet, AL, Sly, WA, and Valle, D. 8th. 2001. New York, NY, McGraw-Hill.
2. **Adam, R., V. Cailliez, P. Majno, V. Karam, P. McMaster, R. Y. Caine, J. O'Grady, R. Pichlmayr, P. Neuhaus, J. B. Otte, K. Hoeckerstedt, and H. Bismuth**. 2000. Normalised intrinsic mortality risk in liver transplantation: European Liver Transplant Registry study. Lancet **356**:621-627.
3. **Adam, R., P. McMaster, J. G. O'Grady, D. Castaing, J. L. Klempnauer, N. Jamieson, P. Neuhaus, J. Lerut, M. Salizzoni, S. Pollard, F. Muhlbacher, X. Rogiers, J. C. Garcia Valdecasas, J. Berenguer, D. Jaeck, and G. E. Moreno**. 2003. Evolution of liver transplantation in Europe: report of the European Liver Transplant Registry. Liver Transpl. **9**:1231-1243.
4. **al Dhalimy, M., K. Overturf, M. Finegold, and M. Grompe**. 2002. Long-term therapy with NTBC and tyrosine-restricted diet in a murine model of hereditary tyrosinemia type I. Mol. Genet. Metab **75**:38-45.
5. **Allain, J. E., I. Dagher, D. Mahieu-Caputo, N. Loux, M. Andreoletti, K. Westerman, P. Briand, D. Franco, P. Leboulch, and A. Weber**. 2002. Immortalization of a primate bipotent epithelial liver stem cell. Proc. Natl. Acad. Sci. U. S. A **99**:3639-3644.
6. **Arias, I., W. Jakoby, H. Popper, D. Schachter, and D. Shafritz**. 1988. The liver: biology and pathobiology. Raven Press Ltd, New York.
7. **Arii, S. and M. Imamura**. 2000. Physiological role of sinusoidal endothelial cells and Kupffer cells and their implication in the pathogenesis of liver injury. J. Hepatobiliary. Pancreat. Surg. **7**:40-48.
8. **Bartz, S. R. and M. A. Vodicka**. 1997. Production of high-titer human immunodeficiency virus type 1 pseudotyped with vesicular stomatitis virus glycoprotein. Methods **12**:337-342.
9. **Beaty, R. M., M. Jackson, D. Peterson, A. Bird, T. Brown, D. K. Benjamin, Jr., T. Juopperi, P. Kishnani, A. Boney, Y. T. Chen, and D. D. Koeberl**. 2002. Delivery of glucose-6-phosphatase in a canine model for glycogen storage disease, type Ia, with adeno-associated virus (AAV) vectors. Gene Ther. **9**:1015-1022.
10. **Belalcazar, L. M., A. Merched, B. Carr, K. Oka, K. H. Chen, L. Pastore, A. Beaudet, and L. Chan**. 2003. Long-term stable expression of human apolipoprotein A-I mediated by helper-dependent adenovirus gene transfer inhibits atherosclerosis progression and remodels atherosclerotic plaques in a mouse model of familial hypercholesterolemia. Circulation **107**:2726-2732.
11. **Bell, P., L. Wang, C. Lebherz, D. B. Flieder, M. S. Bove, D. Wu, G. P. Gao, J. M. Wilson, and N. A. Wivel**. 2005. No evidence for tumorigenesis of AAV vectors in a large-scale study in mice. Mol. Ther. **12**:299-306.
12. **Bellodi-Privato, M., D. Aubert, V. Pichard, A. Myara, F. Trivin, and N. Ferry**. 2005. Successful gene therapy of the Gunn rat by in vivo neonatal hepatic gene transfer using murine oncoretroviral vectors. Hepatology **42**:431-438.
13. **Beyer, W. R., M. Westphal, W. Ostertag, and D. von Laer**. 2002. Oncoretrovirus and lentivirus vectors pseudotyped with lymphocytic choriomeningitis virus glycoprotein: generation, concentration, and broad host range. J. Virol. **76**:1488-1495.
14. **Bismuth, H., D. Castaing, B. G. Ericzon, J. B. Otte, K. Rolles, B. Ringe, and M. Sloof**. 1987. Hepatic transplantation in Europe. First Report of the European Liver Transplant Registry. Lancet **2**:674-676.
15. **Brewer, G. J. and F. K. Askari**. 2005. Wilson's disease: clinical management and therapy. J. Hepatol. **42 Suppl**:S13-S21.
16. **Brown, B. D., C. X. Shi, S. Powell, D. Hurlbut, F. L. Graham, and D. Lillicrap**. 2004. Helper-dependent adenoviral vectors mediate therapeutic factor VIII expression for several months with minimal accompanying toxicity in a canine model of severe hemophilia A. Blood **103**:804-810.
17. **Brown, B. D., M. A. Venneri, A. Zingale, L. S. Sergi, and L. Naldini**. 2006. Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. Nat. Med. **12**:585-591.
18. **Brunetti-Pierri, N. and B. Lee**. 2005. Gene therapy for inborn errors of liver metabolism. Mol. Genet. Metab **86**:13-24.
19. **Brunetti-Pierri, N., T. C. Nichols, S. McCorquodale, E. Merricks, D. J. Palmer, A. L. Beaudet, and P. Ng**. 2005. Sustained phenotypic correction of canine hemophilia B after systemic administration of helper-dependent adenoviral vector. Hum. Gene Ther. **16**:811-820.
20. **Brunetti-Pierri, N., D. J. Palmer, A. L. Beaudet, K. D. Carey, M. Finegold, and P. Ng**. 2004. Acute toxicity after high-dose systemic injection of helper-dependent adenoviral vectors into nonhuman primates. Hum. Gene Ther. **15**:35-46.
21. **Burwinkel, B., A. J. Maichele, O. Aagenaes, H. D. Bakker, A. Lerner, Y. S. Shin, J. A. Strachan, and M. W. Kilimann**. 1997. Autosomal glycogenosis of liver and muscle due to phosphorylase kinase deficiency is caused by mutations in the phosphorylase kinase beta subunit (PHKB). Hum. Mol. Genet. **6**:1109-1115.
22. **Cai, J., M. Ito, H. Nagata, K. A. Westerman, D. Lafleur, J. R. Chowdhury, P. Leboulch, and I. J. Fox**. 2002. Treatment of liver failure in rats with end-stage cirrhosis by transplantation of immortalized hepatocytes. Hepatology **36**:386-394.
23. **Chen, X. G., H. Z. Zhu, J. L. Gong, F. Li, and J. L. Xue**. 2004. Efficient delivery of human clotting factor IX after injection of lentiviral vectors in utero. Acta Pharmacol. Sin. **25**:789-793.
24. **Chuah, M. K., D. Collen, and T. VandenDriessche**. 1998. Gene therapy for hemophilia: hopes and hurdles. Crit Rev. Oncol. Hematol. **28**:153-171.
25. **Chuah, M. K., G. Schiedner, L. Thorrez, B. Brown, M. Johnston, V. Gillijns, S. Hertel, N. Van Rooijen, D. Lillicrap, D. Collen, T. VandenDriessche, and S. Kochanek**. 2003. Therapeutic factor VIII levels and negligible toxicity in mouse and dog models of hemophilia A following gene therapy with high-capacity adenoviral vectors. Blood **101**:1734-1743.

26. **Coutelle, C., M. Themis, S. N. Waddington, S. M. Buckley, L. G. Gregory, M. S. Nivsarkar, A. L. David, D. Peebles, B. Weisz, and C. Rodeck**. 2005. Gene therapy progress and prospects: fetal gene therapy--first proofs of concept--some adverse effects. Gene Ther. **12**:1601-1607.
27. **Cresawn, K. O., T. J. Fraites, C. Wasserfall, M. Atkinson, M. Lewis, S. Porvasnik, C. Liu, C. Mah, and B. J. Byrne**. 2005. Impact of humoral immune response on distribution and efficacy of recombinant adeno-associated virus-derived acid alpha-glucosidase in a model of glycogen storage disease type II. Hum. Gene Ther. **16**:68-80.
28. **Crise, B., Y. Li, C. Yuan, D. R. Morcock, D. Whitby, D. J. Munroe, L. O. Arthur, and X. Wu**. 2005. Simian immunodeficiency virus integration preference is similar to that of human immunodeficiency virus type 1. J. Virol. **79**:12199-12204.
29. **Cui, Y., J. Golob, E. Kelleher, Z. Ye, D. Pardoll, and L. Cheng**. 2002. Targeting transgene expression to antigen-presenting cells derived from lentivirus-transduced engrafting human hematopoietic stem/progenitor cells. Blood **99**:399-408.
30. **David, P., E. Alexandre, M. P. Chenard-Neu, M. Audet, P. Wolf, D. Jaeck, A. Azimzadeh, and L. Richert**. 2000. Engraftment and function of freshly isolated and cryopreserved Sprague Dawley rat hepatocytes after intrasplenic transplantation in analbuminemic rats. Transplant. Proc. **32**:2796-2797.
31. **de Vree, J. M., E. Jacquemin, E. Sturm, D. Cresteil, P. J. Bosma, J. Aten, J. F. Deleuze, M. Desrochers, M. Burdelski, O. Bernard, R. P. Oude Elferink, and M. Hadchouel**. 1998. Mutations in the MDR3 gene cause progressive familial intrahepatic cholestasis. Proc. Natl. Acad. Sci. U. S. A **95**:282-287.
32. **de Vree, J. M., R. Ottenhoff, P. J. Bosma, A. J. Smith, J. Aten, and R. P. Oude Elferink**. 2000. Correction of liver disease by hepatocyte transplantation in a mouse model of progressive familial intrahepatic cholestasis. Gastroenterology **119**:1720-1730.
33. **Deleuze, J. F., E. Jacquemin, C. Dubuisson, D. Cresteil, M. Dumont, S. Erlinger, O. Bernard, and M. Hadchouel**. 1996. Defect of multidrug-resistance 3 gene expression in a subtype of progressive familial intrahepatic cholestasis. Hepatology **23**:904-908.
34. **Dull, T., R. Zufferey, M. Kelly, R. J. Mandel, M. Nguyen, D. Trono, and L. Naldini**. 1998. A third-generation lentivirus vector with a conditional packaging system. J. Virol. **72**:8463-8471.
35. **Eghtesad, B., N. Nezakatgoo, L. C. Geraci, N. Jabbour, W. D. Irish, W. Marsh, J. J. Fung, and J. Rakela**. 1999. Liver transplantation for Wilson's disease: a single-center experience. Liver Transpl. Surg. **5**:467-474.
36. **Ehrhardt, A. and M. A. Kay**. 2002. A new adenoviral helper-dependent vector results in long-term therapeutic levels of human coagulation factor IX at low doses in vivo. Blood **99**:3923-3930.
37. **Ehrhardt, A., H. Xu, A. M. Dillow, D. A. Bellinger, T. C. Nichols, and M. A. Kay**. 2003. A gene-deleted adenoviral vector results in phenotypic correction of canine hemophilia B without liver toxicity or thrombocytopenia. Blood **102**:2403-2411.
38. **Farson, D., R. Witt, R. McGuinness, T. Dull, M. Kelly, J. Song, R. Radeke, A. Bukovsky, A. Consiglio, and L. Naldini**. 2001. A new-generation stable inducible packaging cell line for lentiviral vectors. Hum. Gene Ther. **12**:981-997.
39. **Ferenci, P.** 2004. Pathophysiology and clinical features of Wilson disease. Metab Brain Dis. **19**:229-239.
40. **Fields, P. A., D. W. Kowalczyk, V. R. Arruda, E. Armstrong, M. L. McCleland, J. N. Hagstrom, K. J. Pasi, H. C. Ertl, R. W. Herzog, and K. A. High**. 2000. Role of vector in activation of T cell subsets in immune responses against the secreted transgene product factor IX. Mol. Ther. **1**:225-235.
41. **Fisher, K. J., K. Jooss, J. Alston, Y. Yang, S. E. Haecker, K. High, R. Pathak, S. E. Raper, and J. M. Wilson**. 1997. Recombinant adeno-associated virus for muscle directed gene therapy. Nat. Med. **3**:306-312.
42. Flint.S.J., L.W.Enquist, V.R.Racaniello, and A.M.Skalka. Principles of Virology: Molecular Biology, Pathogenesis, and Control of Animal Viruses. 2004.
43. **Follenzi, A., M. Battaglia, A. Lombardo, A. Annoni, M. G. Roncarolo, and L. Naldini**. 2004. Targeting lentiviral vector expression to hepatocytes limits transgene-specific immune response and establishes long-term expression of human antihemophilic factor IX in mice. Blood **103**:3700-3709.
44. **Follenzi, A. and L. Naldini**. 2002. HIV-based vectors. Preparation and use. Methods Mol. Med. **69**:259-274.
45. **Follenzi, A., G. Sabatino, A. Lombardo, C. Boccaccio, and L. Naldini**. 2002. Efficient gene delivery and targeted expression to hepatocytes in vivo by improved lentiviral vectors. Hum. Gene Ther. **13**:243-260.
46. **Fox, I. J., J. R. Chowdhury, S. S. Kaufman, T. C. Goertzen, N. R. Chowdhury, P. I. Warkentin, K. Dorko, B. V. Sauter, and S. C. Strom**. 1998. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. N. Engl. J. Med. **338**:1422-1426.
47. **Franco, L. M., B. Sun, X. Yang, A. Bird, H. Zhang, A. Schneider, T. Brown, S. P. Young, T. M. Clay, A. Amalfitano, Y. T. Chen, and D. D. Koeberl**. 2005. Evasion of immune responses to introduced human acid alpha-glucosidase by liver-restricted expression in glycogen storage disease type II. Mol. Ther. **12**:876-884.
48. **Friedman, S. L. and Arthur M.J.P.** 2002. Reversibility of Liver Fibrosis and Cirrhosis, p. 192-203. Science in Medicine.
49. **Gallo-Penn, A. M., P. S. Shirley, J. L. Andrews, D. B. Kayda, A. M. Pinkstaff, M. Kaloss, S. Tinlin, C. Cameron, C. Notley, C. Hough, D. Lillicrap, M. Kaleko, and S. Connelly**. 1999. In vivo evaluation of an adenoviral vector encoding canine factor VIII: high-level, sustained expression in hemophiliac mice. Hum. Gene Ther. **10**:1791-1802.
50. **Gao, G., C. Lebherz, D. J. Weiner, R. Grant, R. Calcedo, B. McCullough, A. Bagg, Y. Zhang, and J. M. Wilson**. 2004. Erythropoietin gene therapy leads to autoimmune anemia in macaques. Blood **103**:3300-3302.
51. **Gao, G., Y. Lu, R. Calcedo, R. L. Grant, P. Bell, L. Wang, J. Figueredo, M. Lock, and J. M. Wilson**. 2006. Biology of AAV serotype vectors in liver-directed gene transfer to nonhuman primates. Mol. Ther. **13**:77-87.
52. **Georg-Fries, B., S. Biederlack, J. Wolf, and H. H. zur**. 1984. Analysis of proteins, helper dependence, and seroepidemiology of a new human parvovirus. Virology **134**:64-71.

53. **Ghosh, A., M. Allamarvdasht, C. J. Pan, M. S. Sun, B. C. Mansfield, B. J. Byrne, and J. Y. Chou**. 2005. Long-term correction of murine glycogen storage disease type Ia by recombinant adeno-associated virus-1-mediated gene transfer. Gene Ther.
54. **Gilbert, J. R. and F. Wong-Staal**. 2001. HIV-2 and SIV vector systems. Somat. Cell Mol. Genet. **26**:83-98.
55. **Grimm, D., S. Zhou, H. Nakai, C. E. Thomas, T. A. Storm, S. Fuess, T. Matsushita, J. Allen, R. Surosky, M. Lochrie, L. Meuse, A. McClelland, P. Colosi, and M. A. Kay**. 2003. Preclinical in vivo evaluation of pseudotyped adeno-associated virus vectors for liver gene therapy. Blood **102**:2412-2419.
56. **Grompe, M.** 2001. Liver repopulation for the treatment of metabolic diseases. J. Inherit. Metab Dis. **24**:231-244.
57. **Grompe, M.** 2001. The pathophysiology and treatment of hereditary tyrosinemia type 1. Semin. Liver Dis. **21**:563-571.
58. **Grompe, M., M. al Dhalimy, M. Finegold, C. N. Ou, T. Burlingame, N. G. Kennaway, and P. Soriano**. 1993. Loss of fumarylacetoacetate hydrolase is responsible for the neonatal hepatic dysfunction phenotype of lethal albino mice. Genes Dev. **7**:2298-2307.
59. **Grompe, M., S. Lindstedt, M. al Dhalimy, N. G. Kennaway, J. Papaconstantinou, C. A. Torres-Ramos, C. N. Ou, and M. Finegold**. 1995. Pharmacological correction of neonatal lethal hepatic dysfunction in a murine model of hereditary tyrosinaemia type I. Nat. Genet. **10**:453-460.
60. **Grossman, M., D. J. Rader, D. W. Muller, D. M. Kolansky, K. Kozarsky, B. J. Clark, III, E. A. Stein, P. J. Lupien, H. B. Brewer, Jr., S. E. Raper, and .** 1995. A pilot study of ex vivo gene therapy for homozygous familial hypercholesterolaemia. Nat. Med. **1**:1148-1154.
61. **Grossman, M., S. E. Raper, K. Kozarsky, E. A. Stein, J. F. Engelhardt, D. Muller, P. J. Lupien, and J. M. Wilson**. 1994. Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia. Nat. Genet. **6**:335-341.
62. **Guibinga, G. H. and T. Friedmann**. 2005. Baculovirus GP64-pseudotyped HIV-based lentivirus vectors are stabilized against complement inactivation by codisplay of decay accelerating factor (DAF) or of a GP64-DAF fusion protein. Mol. Ther. **11**:645-651.
63. **Gupta, S. and J. R. Chowdhury**. 2002. Therapeutic potential of hepatocyte transplantation. Semin. Cell Dev. Biol. **13**:439-446.
64. **Gupta, S., G. R. Gorla, and A. N. Irani**. 1999. Hepatocyte transplantation: emerging insights into mechanisms of liver repopulation and their relevance to potential therapies. J. Hepatol. **30**:162-170.
65. **Hamman, K., H. Clark, E. Montini, M. al Dhalimy, M. Grompe, M. Finegold, and C. O. Harding**. 2005. Low therapeutic threshold for hepatocyte replacement in murine phenylketonuria. Mol. Ther. **12**:337-344.
66. **Herzog, R. W., P. A. Fields, V. R. Arruda, J. O. Brubaker, E. Armstrong, D. McClintock, D. A. Bellinger, L. B. Couto, T. C. Nichols, and K. A. High**. 2002. Influence of vector dose on factor IX-specific T and B cell responses in muscle-directed gene therapy. Hum. Gene Ther. **13**:1281-1291.
67. **Herzog, R. W., E. Y. Yang, L. B. Couto, J. N. Hagstrom, D. Elwell, P. A. Fields, M. Burton, D. A. Bellinger, M. S. Read, K. M. Brinkhous, G. M. Podsakoff, T. C. Nichols, G. J. Kurtzman, and K. A. High**. 1999. Long-term correction of canine hemophilia B by gene transfer of blood coagulation factor IX mediated by adeno-associated viral vector. Nat. Med. **5**:56-63.
68. **Horslen, S. P., T. C. McCowan, T. C. Goertzen, P. I. Warkentin, H. B. Cai, S. C. Strom, and I. J. Fox**. 2003. Isolated hepatocyte transplantation in an infant with a severe urea cycle disorder. Pediatrics **111**:1262-1267.
69. **Iorio, R., A. Sepe, A. Giannattasio, F. Cirillo, and A. Vegnente**. 2005. Hypertransaminasemia in childhood as a marker of genetic liver disorders. J. Gastroenterol. **40**:820-826.
70. **Irani, A. N., H. Malhi, S. Slehria, G. R. Gorla, I. Volenberg, M. L. Schilsky, and S. Gupta**. 2001. Correction of liver disease following transplantation of normal rat hepatocytes into Long-Evans Cinnamon rats modeling Wilson's disease. Mol. Ther. **3**:302-309.
71. **Jain, A., G. Mazariegos, R. Kashyap, B. Kosmach-Park, T. E. Starzl, J. Fung, and J. Reyes**. 2002. Pediatric liver transplantation. A single center experience spanning 20 years. Transplantation **73**:941-947.
72. **Jain, A., J. Reyes, R. Kashyap, S. F. Dodson, A. J. Demetris, K. Ruppert, K. Abu-Elmagd, W. Marsh, J. Madariaga, G. Mazariegos, D. Geller, C. A. Bonham, T. Gayowski, T. Cacciarelli, P. Fontes, T. E. Starzl, and J. J. Fung**. 2000. Long-term survival after liver transplantation in 4,000 consecutive patients at a single center. Ann. Surg. **232**:490-500.
73. **Jamal, H. Z., T. C. Weglarz, and E. P. Sandgren**. 2000. Cryopreserved mouse hepatocytes retain regenerative capacity in vivo. Gastroenterology **118**:390-394.
74. **Jansen, P. L.** 1999. Diagnosis and management of Crigler-Najjar syndrome. Eur. J. Pediatr. **158 Suppl 2**:S89-S94.
75. **Janssens, W., M. K. Chuah, L. Naldini, A. Follenzi, D. Collen, J. M. Saint-Remy, and T. VandenDriessche**. 2003. Efficiency of onco-retroviral and lentiviral gene transfer into primary mouse and human B-lymphocytes is pseudotype dependent. Hum. Gene Ther. **14**:263-276.
76. **Kafri, T., U. Blomer, D. A. Peterson, F. H. Gage, and I. M. Verma**. 1997. Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. Nat. Genet. **17**:314-317.
77. **Kahl, C. A., J. Marsh, J. Fyffe, D. A. Sanders, and K. Cornetta**. 2004. Human immunodeficiency virus type 1-derived lentivirus vectors pseudotyped with envelope glycoproteins derived from Ross River virus and Semliki Forest virus. J. Virol. **78**:1421-1430.
78. **Kahl, C. A., K. Pollok, L. S. Haneline, and K. Cornetta**. 2005. Lentiviral vectors pseudotyped with glycoproteins from Ross River and vesicular stomatitis viruses: variable transduction related to cell type and culture conditions. Mol. Ther. **11**:470-482.
79. **Kang, Y., C. S. Stein, J. A. Heth, P. L. Sinn, A. K. Penisten, P. D. Staber, K. L. Ratliff, H. Shen, C. K. Barker, I. Martins, C. M. Sharkey, D. A. Sanders, P. B. McCray, Jr., and B. L. Davidson**. 2002. In vivo gene transfer using a nonprimate lentiviral vector pseudotyped with Ross River Virus glycoproteins. J. Virol. **76**:9378-9388.

80. **Kang, Y., L. Xie, D. T. Tran, C. S. Stein, M. Hickey, B. L. Davidson, and P. B. McCray, Jr.** 2005. Persistent expression of factor VIII in vivo following nonprimate lentiviral gene transfer. Blood **106**:1552-1558.
81. **Kankkonen, H. M., E. Vahakangas, R. A. Marr, T. Pakkanen, A. Laurema, P. Leppanen, J. Jalkanen, I. M. Verma, and S. Yla-Herttuala**. 2004. Long-term lowering of plasma cholesterol levels in LDL-receptor-deficient WHHL rabbits by gene therapy. Mol. Ther. **9**:548-556.
82. **Kay, M. A., P. Baley, S. Rothenberg, F. Leland, L. Fleming, K. P. Ponder, T. Liu, M. Finegold, G. Darlington, W. Pokorny, and .** 1992. Expression of human alpha 1-antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes. Proc. Natl. Acad. Sci. U. S. A **89**:89-93.
83. **Kay, M. A., J. C. Glorioso, and L. Naldini**. 2001. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nat. Med. **7**:33-40.
84. **Kay, M. A. and K. High**. 1999. Gene therapy for the hemophilias. Proc. Natl. Acad. Sci. U. S. A **96**:9973-9975.
85. **Kay, M. A., C. N. Landen, S. R. Rothenberg, L. A. Taylor, F. Leland, S. Wiehle, B. Fang, D. Bellinger, M. Finegold, A. R. Thompson, and .** 1994. In vivo hepatic gene therapy: complete albeit transient correction of factor IX deficiency in hemophilia B dogs. Proc. Natl. Acad. Sci. U. S. A **91**:2353-2357.
86. **Kay, M. A., D. Liu, and P. M. Hoogerbrugge**. 1997. Gene therapy. Proc. Natl. Acad. Sci. U. S. A **94**:12744-12746.
87. **Kelly, D. A.** 2003. Strategies for optimizing immunosuppression in adolescent transplant recipients: a focus on liver transplantation. Paediatr. Drugs **5**:177-183.
88. **Kobayashi, H., D. Carbonaro, K. Pepper, D. Petersen, S. Ge, H. Jackson, H. Shimada, R. Moats, and D. B. Kohn**. 2005. Neonatal gene therapy of MPS I mice by intravenous injection of a lentiviral vector. Mol. Ther. **11**:776-789.
89. **Kobayashi, N., T. Fujiwara, K. A. Westerman, Y. Inoue, M. Sakaguchi, H. Noguchi, M. Miyazaki, J. Cai, N. Tanaka, I. J. Fox, and P. Leboulch**. 2000. Prevention of acute liver failure in rats with reversibly immortalized human hepatocytes. Science **287**:1258-1262.
90. **Kobayashi, N., M. Miyazaki, K. Fukaya, H. Noguchi, N. Tanaka, and M. Namba**. 2000. Intrasplenic transplantation of immortalized human fetal hepatocytes prolongs the survival of 90% hepatectomized rats. Transplant. Proc. **32**:2365-2367.
91. **Koch, R. O., I. W. Graziadei, F. Schulz, K. Nachbaur, A. Konigsrainer, R. Margreiter, and W. Vogel**. 2004. Long-term efficacy and safety of mycophenolate mofetil in liver transplant recipients with calcineurin inhibitor-induced renal dysfunction. Transpl. Int. **17**:518-524.
92. **Kootstra, N. A., R. Matsumura, and I. M. Verma**. 2003. Efficient production of human FVIII in hemophilic mice using lentiviral vectors. Mol. Ther. **7**:623-631.
93. **Kotin, R. M., J. C. Menninger, D. C. Ward, and K. I. Berns**. 1991. Mapping and direct visualization of a region-specific viral DNA integration site on chromosome 19q13-qter. Genomics **10**:831-834.
94. **Kotin, R. M., M. Siniscalco, R. J. Samulski, X. D. Zhu, L. Hunter, C. A. Laughlin, S. McLaughlin, N. Muzyczka, M. Rocchi, and K. I. Berns**. 1990. Site-specific integration by adeno-associated virus. Proc. Natl. Acad. Sci. U. S. A **87**:2211-2215.
95. **Kren, B. T., N. R. Chowdhury, J. R. Chowdhury, and C. J. Steer**. 2002. Gene therapy as an alternative to liver transplantation. Liver Transpl. **8**:1089-1108.
96. **Kumar, M., B. P. Bradow, and J. Zimmerberg**. 2003. Large-scale production of pseudotyped lentiviral vectors using baculovirus GP64. Hum. Gene Ther. **14**:67-77.
97. **Laconi, E., R. Oren, D. K. Mukhopadhyay, E. Hurston, S. Laconi, P. Pani, M. D. Dabeva, and D. A. Shafritz**. 1998. Long-term, near-total liver replacement by transplantation of isolated hepatocytes in rats treated with retrorsine. Am. J. Pathol. **153**:319-329.
98. **Laconi, S., S. Pillai, P. P. Porcu, D. A. Shafritz, P. Pani, and E. Laconi**. 2001. Massive liver replacement by transplanted hepatocytes in the absence of exogenous growth stimuli in rats treated with retrorsine. Am. J. Pathol. **158**:771-777.
99. **Lazaro, C. A., E. J. Croager, C. Mitchell, J. S. Campbell, C. Yu, J. Foraker, J. A. Rhim, G. C. Yeoh, and N. Fausto**. 2003. Establishment, characterization, and long-term maintenance of cultures of human fetal hepatocytes. Hepatology **38**:1095-1106.
100. **Liu, S. L., C. L. Halbert, and A. D. Miller**. 2004. Jaagsiekte sheep retrovirus envelope efficiently pseudotypes human immunodeficiency virus type 1-based lentiviral vectors. J. Virol. **78**:2642-2647.
101. **Liu, Y., L. Xu, A. K. Hennig, A. Kovacs, A. Fu, S. Chung, D. Lee, B. Wang, R. S. Herati, O. J. Mosinger, S. R. Cai, and P. K. Parker**. 2005. Liver-directed neonatal gene therapy prevents cardiac, bone, ear, and eye disease in mucopolysaccharidosis I mice. Mol. Ther. **11**:35-47.
102. **MacKenzie, T. C., G. P. Kobinger, N. A. Kootstra, A. Radu, M. Sena-Esteves, S. Bouchard, J. M. Wilson, I. M. Verma, and A. W. Flake**. 2002. Efficient transduction of liver and muscle after in utero injection of lentiviral vectors with different pseudotypes. Mol. Ther **6**:349-358.
103. **Malhi, H., A. N. Irani, S. Gagandeep, and S. Gupta**. 2002. Isolation of human progenitor liver epithelial cells with extensive replication capacity and differentiation into mature hepatocytes. J. Cell Sci. **115**:2679-2688.
104. **Manno, C. S., V. R. Arruda, G. F. Pierce, B. Glader, M. Ragni, J. Rasko, M. C. Ozelo, K. Hoots, P. Blatt, B. Konkle, M. Dake, R. Kaye, M. Razavi, A. Zajko, J. Zehnder, H. Nakai, A. Chew, D. Leonard, J. F. Wright, R. R. Lessard, J. M. Sommer, M. Tigges, D. Sabatino, A. Luk, H. Jiang, F. Mingozzi, L. Couto, H. C. Ertl, K. A. High, and M. A. Kay**. 2006. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat. Med. **12**:342-347.
105. **Martin, S. R., P. Atkison, R. Anand, and A. S. Lindblad**. 2004. Studies of Pediatric Liver Transplantation 2002: patient and graft survival and rejection in pediatric recipients of a first liver transplant in the United States and Canada. Pediatr. Transplant. **8**:273-283.
106. **Matukonis, M., M. Li, R. P. Molina, B. Paszkiet, M. Kaleko, and T. Luo**. 2002. Development of second- and third-generation bovine immunodeficiency virus-based gene transfer systems. Hum. Gene Ther. **13**:1293-1303.

107. **Maxfield, L. F., C. D. Fraize, and J. M. Coffin**. 2005. Relationship between retroviral DNA-integration-site selection and host cell transcription. Proc. Natl. Acad. Sci. U. S. A **102**:1436-1441.
108. **McDiarmid, S. V.** 2000. Liver transplantation. The pediatric challenge. Clin. Liver Dis. **4**:879-927.
109. **Mignon, A., J. E. Guidotti, C. Mitchell, M. Fabre, A. Wernet, C. A. De La, O. Soubrane, H. Gilgenkrantz, and A. Kahn**. 1998. Selective repopulation of normal mouse liver by Fas/CD95-resistant hepatocytes. Nat. Med. **4**:1185-1188.
110. **Minami, H., K. Tada, N. R. Chowdhury, J. R. Chowdhury, and M. Onji**. 2000. Enhancement of retrovirus-mediated gene transfer to rat liver in vivo by infusion of hepatocyte growth factor and triiodothyronine. J. Hepatol. **33**:183-188.
111. **Mingozzi, F., Y. L. Liu, E. Dobrzynski, A. Kaufhold, J. H. Liu, Y. Wang, V. R. Arruda, K. A. High, and R. W. Herzog**. 2003. Induction of immune tolerance to coagulation factor IX antigen by in vivo hepatic gene transfer. J. Clin. Invest **111**:1347-1356.
112. **Mitchell, C., V. O. Mallet, J. E. Guidotti, C. Goulenok, A. Kahn, and H. Gilgenkrantz**. 2002. Liver repopulation by Bcl-x(L) transgenic hepatocytes. Am. J. Pathol. **160**:31-35.
113. **Miyoshi, H., U. Blomer, M. Takahashi, F. H. Gage, and I. M. Verma**. 1998. Development of a self-inactivating lentivirus vector. J. Virol. **72**:8150-8157.
114. **Mochizuki, H., J. P. Schwartz, K. Tanaka, R. O. Brady, and J. Reiser**. 1998. High-titer human immunodeficiency virus type 1-based vector systems for gene delivery into nondividing cells. J. Virol. **72**:8873-8883.
115. **Montini, E., D. Cesana, M. Schmidt, F. Sanvito, M. Ponzoni, C. Bartholomae, L. S. Sergi, F. Benedicenti, A. Ambrosi, C. Di Serio, C. Doglioni, C. von Kalle, and L. Naldini**. 2006. Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. Nat. Biotechnol. **24**:687-696.
116. **Morral, N., W. O'Neal, K. Rice, M. Leland, J. Kaplan, P. A. Piedra, H. Zhou, R. J. Parks, R. Velji, E. Aguilar-Cordova, S. Wadsworth, F. L. Graham, S. Kochanek, K. D. Carey, and A. L. Beaudet**. 1999. Administration of helper-dependent adenoviral vectors and sequential delivery of different vector serotype for long-term liver-directed gene transfer in baboons. Proc. Natl. Acad. Sci. U. S. A **96**:12816-12821.
117. **Morral, N., W. K. O'Neal, K. Rice, M. M. Leland, P. A. Piedra, E. Aguilar-Cordova, K. D. Carey, A. L. Beaudet, and C. Langston**. 2002. Lethal toxicity, severe endothelial injury, and a threshold effect with high doses of an adenoviral vector in baboons. Hum. Gene Ther. **13**:143-154.
118. **Mount, J. D., R. W. Herzog, D. M. Tillson, S. A. Goodman, N. Robinson, M. L. McCleland, D. Bellinger, T. C. Nichols, V. R. Arruda, C. D. Lothrop, Jr., and K. A. High**. 2002. Sustained phenotypic correction of hemophilia B dogs with a factor IX null mutation by liver-directed gene therapy. Blood **99**:2670-2676.
119. **Muraca, M., G. Gerunda, D. Neri, M. T. Vilei, A. Granato, P. Feltracco, M. Meroni, G. Giron, and A. B. Burlina**. 2002. Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. Lancet **359**:317-318.
120. **Nathwani, A. C., A. Davidoff, H. Hanawa, J. F. Zhou, E. F. Vanin, and A. W. Nienhuis**. 2001. Factors influencing in vivo transduction by recombinant adeno-associated viral vectors expressing the human factor IX cDNA. Blood **97**:1258-1265.
121. **Nathwani, A. C., A. M. Davidoff, H. Hanawa, Y. Hu, F. A. Hoffer, A. Nikanorov, C. Slaughter, C. Y. Ng, J. Zhou, J. N. Lozier, T. D. Mandrell, E. F. Vanin, and A. W. Nienhuis**. 2002. Sustained high-level expression of human factor IX (hFIX) after liver-targeted delivery of recombinant adeno-associated virus encoding the hFIX gene in rhesus macaques. Blood **100**:1662-1669.
122. **Neau-Cransac, M., D. Morel, P. H. Bernard, P. Merville, P. Revel, L. Potaux, and J. Saric**. 2002. Renal failure after liver transplantation: outcome after calcineurin inhibitor withdrawal. Clin. Transplant. **16**:368-373.
123. **Nguyen, T. H., M. Bellodi-Privato, D. Aubert, V. Pichard, A. Myara, D. Trono, and N. Ferry**. 2005. Therapeutic Lentivirus-Mediated Neonatal in Vivo Gene Therapy in Hyperbilirubinemic Gunn Rats. Mol. Ther.
124. **Nguyen, T. H. and N. Ferry**. 2004. Liver gene therapy: advances and hurdles. Gene Ther. **11 Suppl 1**:S76-S84.
125. **Nguyen, T. H., G. Mai, P. Villiger, J. Oberholzer, P. Salmon, P. Morel, L. Buhler, and D. Trono**. 2005. Treatment of acetaminophen-induced acute liver failure in the mouse with conditionally immortalized human hepatocytes. J. Hepatol. **43**:1031-1037.
126. **Nomura, S., A. Merched, E. Nour, C. Dieker, K. Oka, and L. Chan**. 2004. Low-density lipoprotein receptor gene therapy using helper-dependent adenovirus produces long-term protection against atherosclerosis in a mouse model of familial hypercholesterolemia. Gene Ther. **11**:1540-1548.
127. **Oertel, M., R. Rosencrantz, Y. Q. Chen, P. N. Thota, J. S. Sandhu, M. D. Dabeva, A. L. Pacchia, M. E. Adelson, J. P. Dougherty, and D. A. Shafritz**. 2003. Repopulation of rat liver by fetal hepatoblasts and adult hepatocytes transduced ex vivo with lentiviral vectors. Hepatology **37**:994-1005.
128. **Ohashi, K., F. Park, and M. A. Kay**. 2001. Hepatocyte transplantation: clinical and experimental application. J. Mol. Med. **79**:617-630.
129. **Ohashi, K., J. M. Waugh, M. D. Dake, T. Yokoyama, H. Kuge, Y. Nakajima, M. Yamanouchi, H. Naka, A. Yoshioka, and M. A. Kay**. 2005. Liver tissue engineering at extrahepatic sites in mice as a potential new therapy for genetic liver diseases. Hepatology **41**:132-140.
130. **Oka, K., L. Pastore, I. H. Kim, A. Merched, S. Nomura, H. J. Lee, M. Merched-Sauvage, C. Arden-Riley, B. Lee, M. Finegold, A. Beaudet, and L. Chan**. 2001. Long-term stable correction of low-density lipoprotein receptor-deficient mice with a helper-dependent adenoviral vector expressing the very low-density lipoprotein receptor. Circulation **103**:1274-1281.
131. **Oude Elferink, R. P.** 2005. Manipulation of biliary lipids by gene therapy: potential consequences for patients with progressive familial intrahepatic cholestasis. Curr. Drug Targets. Immune. Endocr. Metabol. Disord. **5**:199-202.

132. **Overturf, K., M. al Dhalimy, K. Manning, C. N. Ou, M. Finegold, and M. Grompe**. 1998. Ex vivo hepatic gene therapy of a mouse model of Hereditary Tyrosinemia Type I. Hum. Gene Ther. **9**:295-304.
133. **Overturf, K., M. al Dhalimy, C. N. Ou, M. Finegold, R. Tanguay, A. Lieber, M. Kay, and M. Grompe**. 1997. Adenovirus-mediated gene therapy in a mouse model of hereditary tyrosinemia type I. Hum. Gene Ther. **8**:513-521.
134. **Overturf, K., M. al Dhalimy, R. Tanguay, M. Brantly, C. N. Ou, M. Finegold, and M. Grompe**. 1996. Hepatocytes corrected by gene therapy are selected in vivo in a murine model of hereditary tyrosinaemia type I. Nat. Genet. **12**:266-273.
135. **Palmer, D. and P. Ng**. 2003. Improved system for helper-dependent adenoviral vector production. Mol. Ther. **8**:846-852.
136. **Park, F., K. Ohashi, and M. A. Kay**. 2000. Therapeutic levels of human factor VIII and IX using HIV-1-based lentiviral vectors in mouse liver. Blood **96**:1173-1176.
137. **Park, F., K. Ohashi, and M. A. Kay**. 2003. The effect of age on hepatic gene transfer with self-inactivating lentiviral vectors in vivo. Mol. Ther. **8**:314-323.
138. **Paulusma, C. C., A. Groen, C. Kunne, K. S. Ho-Mok, A. L. Spijkerboer, d. W. Rudi, F. J. Hoek, H. Vreeling, K. A. Hoeben, J. van Marle, L. Pawlikowska, L. N. Bull, A. F. Hofmann, A. S. Knisely, and R. P. Oude Elferink**. 2006. Atp8b1 deficiency in mice reduces resistance of the canalicular membrane to hydrophobic bile salts and impairs bile salt transport. Hepatology **44**:195-204.
139. **Pfeifer, A., T. Kessler, M. Yang, E. Baranov, N. Kootstra, D. A. Cheresh, R. M. Hoffman, and I. M. Verma**. 2001. Transduction of liver cells by lentiviral vectors: analysis in living animals by fluorescence imaging. Mol. Ther. **3**:319-322.
140. **Podevin, G., E. Otta, J. M. Nguyen, V. Pichard, D. Aubert, P. Moullier, and N. Ferry**. 2004. Factors influencing immune response after in vivo retrovirus-mediated gene transfer to the liver. J. Gene Med. **6**:16-21.
141. **Pratschke, J., T. Steinmuller, W. O. Bechstein, R. Neuhaus, S. G. Tullius, S. Jonas, G. Schumacher, W. Luck, M. Becker, and P. Neuhaus**. 1998. Orthotopic liver transplantation for hepatic associated metabolic disorders. Clin. Transplant. **12**:228-232.
142. **Puppi, J., C. Guillonneau, V. Pichard, M. Bellodi-Privato, M. C. Cuturi, I. Anegon, and N. Ferry**. 2004. Long term transgene expression by hepatocytes transduced with retroviral vectors requires induction of immune tolerance to the transgene. J. Hepatol. **41**:222-228.
143. **Raimondo, M. L., L. Dagher, G. V. Papatheodoridis, N. Rolando, D. W. Patch, B. R. Davidson, K. Rolles, and A. K. Burroughs**. 2003. Long-term mycophenolate mofetil monotherapy in combination with calcineurin inhibitors for chronic renal dysfunction after liver transplantation. Transplantation **75**:186-190.
144. **Raper, S. E., N. Chirmule, F. S. Lee, N. A. Wivel, A. Bagg, G. P. Gao, J. M. Wilson, and M. L. Batshaw**. 2003. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol. Genet. Metab **80**:148-158.
145. **Raper, S. E., M. Yudkoff, N. Chirmule, G. P. Gao, F. Nunes, Z. J. Haskal, E. E. Furth, K. J. Propert, M. B. Robinson, S. Magosin, H. Simoes, L. Speicher, J. Hughes, J. Tazelaar, N. A. Wivel, J. M. Wilson, and M. L. Batshaw**. 2002. A pilot study of in vivo liver-directed gene transfer with an adenoviral vector in partial ornithine transcarbamylase deficiency. Hum. Gene Ther. **13**:163-175.
146. **Reiser, J., G. Harmison, S. Kluepfel-Stahl, R. O. Brady, S. Karlsson, and M. Schubert**. 1996. Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. Proc. Natl. Acad. Sci. U. S. A **93**:15266-15271.
147. **Rela, M., P. Muiesan, N. D. Heaton, M. Corbally, H. Hajj, A. P. Mowat, R. Williams, and K. C. Tan**. 1995. Orthotopic liver transplantation for hepatic-based metabolic disorders. Transpl. Int. **8**:41-44.
148. **Rhim, J. A., E. P. Sandgren, R. D. Palmiter, and R. L. Brinster**. 1995. Complete reconstitution of mouse liver with xenogeneic hepatocytes. Proc. Natl. Acad. Sci. U. S. A **92**:4942-4946.
149. **Roskams, T. A., N. D. Theise, C. Balabaud, G. Bhagat, P. S. Bhathal, P. Bioulac-Sage, E. M. Brunt, J. M. Crawford, H. A. Crosby, V. Desmet, M. J. Finegold, S. A. Geller, A. S. Gouw, P. Hytiroglou, A. S. Knisely, M. Kojiro, J. H. Lefkowitch, Y. Nakanuma, J. K. Olynyk, Y. N. Park, B. Portmann, R. Saxena, P. J. Scheuer, A. J. Strain, S. N. Thung, I. R. Wanless, and A. B. West**. 2004. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. Hepatology **39**:1739-1745.
150. **Rossi, G. R., M. R. Mautino, and R. A. Morgan**. 2003. High-efficiency lentiviral vector-mediated gene transfer into murine macrophages and activated splenic B lymphocytes. Hum. Gene Ther. **14**:385-391.
151. **Russell, D. W. and M. A. Kay**. 1999. Adeno-associated virus vectors and hematology. Blood **94**:864-874.
152. **Russell, D. W., A. D. Miller, and I. E. Alexander**. 1994. Adeno-associated virus vectors preferentially transduce cells in S phase. Proc. Natl. Acad. Sci. U. S. A **91**:8915-8919.
153. **Samulski, R. J., X. Zhu, X. Xiao, J. D. Brook, D. E. Housman, N. Epstein, and L. A. Hunter**. 1991. Targeted integration of adeno-associated virus (AAV) into human chromosome 19. EMBO J. **10**:3941-3950.
154. **Sandgren, E. P., R. D. Palmiter, J. L. Heckel, C. C. Daugherty, R. L. Brinster, and J. L. Degen**. 1991. Complete hepatic regeneration after somatic deletion of an albumin-plasminogen activator transgene. Cell **66**:245-256.
155. **Sarukhan, A., S. Camugli, B. Gjata, H. von Boehmer, O. Danos, and K. Jooss**. 2001. Successful interference with cellular immune responses to immunogenic proteins encoded by recombinant viral vectors. J. Virol. **75**:269-277.
156. **Schauber, C. A., M. J. Tuerk, C. D. Pacheco, P. A. Escarpe, and G. Veres**. 2004. Lentiviral vectors pseudotyped with baculovirus gp64 efficiently transduce mouse cells in vivo and show tropism restriction against hematopoietic cell types in vitro. Gene Ther **11**:266-275.
157. **SCHMID, R., J. AXELROD, L. HAMMAKER, and R. L. SWARM**. 1958. Congenital jaundice in rats, due to a defect in glucuronide formation. J. Clin. Invest **37**:1123-1130.

158. **Schneider, H., C. Muhle, A. M. Douar, S. Waddington, Q. J. Jiang, M. K. von der, C. Coutelle, and W. Rascher**. 2002. Sustained delivery of therapeutic concentrations of human clotting factor IX--a comparison of adenoviral and AAV vectors administered in utero. J. Gene Med. **4**:46-53.
159. **Schnell, M. A., Y. Zhang, J. Tazelaar, G. P. Gao, Q. C. Yu, R. Qian, S. J. Chen, A. N. Varnavski, C. LeClair, S. E. Raper, and J. M. Wilson**. 2001. Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. Mol. Ther. **3**:708-722.
160. **Schroder, A. R., P. Shinn, H. Chen, C. Berry, J. R. Ecker, and F. Bushman**. 2002. HIV-1 integration in the human genome favors active genes and local hotspots. Cell **110**:521-529.
161. **Seppen, J., M. Rijnberg, M. P. Cooreman, and R. P. Oude Elferink**. 2002. Lentiviral vectors for efficient transduction of isolated primary quiescent hepatocytes. J. Hepatol. **36**:459-465.
162. **Seppen, J., K. Tada, R. Ottenhoff, K. Sengupta, N. R. Chowdhury, J. R. Chowdhury, P. J. Bosma, and R. P. Oude Elferink**. 1997. Transplantation of Gunn rats with autologous fibroblasts expressing bilirubin UDP-glucuronosyltransferase: correction of genetic deficiency and tumor formation. Hum. Gene Ther. **8**:27-36.
163. **Seppen, J., R. R. van der, N. Looije, N. P. van Til, W. H. Lamers, and R. P. Oude Elferink**. 2003. Long-term correction of bilirubin UDPglucuronyltransferase deficiency in rats by in utero lentiviral gene transfer. Mol. Ther. **8**:593-599.
164. **Seppen, J., N. P. van Til, R. R. van der, J. K. Hiralall, C. Kunne, and R. P. Elferink**. 2005. Immune response to lentiviral bilirubin UDP-glucuronosyltransferase gene transfer in fetal and neonatal rats. Gene Ther.
165. **Sharkey, C. M., C. L. North, R. J. Kuhn, and D. A. Sanders**. 2001. Ross River virus glycoprotein-pseudotyped retroviruses and stable cell lines for their production. J. Virol. **75**:2653-2659.
166. **Snyder, R. O., C. Miao, L. Meuse, J. Tubb, B. A. Donahue, H. F. Lin, D. W. Stafford, S. Patel, A. R. Thompson, T. Nichols, M. S. Read, D. A. Bellinger, K. M. Brinkhous, and M. A. Kay**. 1999. Correction of hemophilia B in canine and murine models using recombinant adeno-associated viral vectors. Nat. Med. **5**:64-70.
167. **Song, S., R. P. Witek, Y. Lu, Y. K. Choi, D. Zheng, M. Jorgensen, C. Li, T. R. Flotte, and B. E. Petersen**. 2004. Ex vivo transduced liver progenitor cells as a platform for gene therapy in mice. Hepatology **40**:918-924.
168. **Soriano, H. E. and M. Grompe**. 2002. Gene therapy and pediatric liver disease. J. Pediatr. Gastroenterol. Nutr. **35 Suppl 1**:S51-S54.
169. **Srinivasakumar, N.** 2001. HIV-1 vector systems. Somat. Cell Mol. Genet. **26**:51-81.
170. **Strang, B. L., Y. Takeuchi, T. Relander, J. Richter, R. Bailey, D. A. Sanders, M. K. Collins, and Y. Ikeda**. 2005. Human immunodeficiency virus type 1 vectors with alphavirus envelope glycoproteins produced from stable packaging cells. J. Virol. **79**:1765-1771.
171. **Strauss, J. H. and E. G. Strauss**. 1994. The alphaviruses: gene expression, replication, and evolution. Microbiol. Rev. **58**:491-562.
172. **Strick-Marchand, H. and M. C. Weiss**. 2003. Embryonic liver cells and permanent lines as models for hepatocyte and bile duct cell differentiation. Mech. Dev. **120**:89-98.
173. **Sun, B., H. Zhang, L. M. Franco, T. Brown, A. Bird, A. Schneider, and D. D. Koeberl**. 2005. Correction of glycogen storage disease type II by an adeno-associated virus vector containing a muscle-specific promoter. Mol. Ther. **11**:889-898.
174. **Sun, B., H. Zhang, L. M. Franco, S. P. Young, A. Schneider, A. Bird, A. Amalfitano, Y. T. Chen, and D. D. Koeberl**. 2005. Efficacy of an adeno-associated virus 8-pseudotyped vector in glycogen storage disease type II. Mol. Ther. **11**:57-65.
175. **Tada, K., N. R. Chowdhury, D. Neufeld, P. J. Bosma, M. Heard, V. R. Prasad, and J. R. Chowdhury**. 1998. Long-term reduction of serum bilirubin levels in Gunn rats by retroviral gene transfer in vivo. Liver Transpl. Surg. **4**:78-88.
176. **Terada, K., M. L. Schilsky, N. Miura, and T. Sugiyama**. 1998. ATP7B (WND) protein. Int. J. Biochem. Cell Biol. **30**:1063-1067.
177. **Themis, M., S. N. Waddington, M. Schmidt, C. von Kalle, Y. Wang, F. Al Allaf, L. G. Gregory, M. Nivsarkar, M. Themis, M. V. Holder, S. M. Buckley, N. Dighe, A. T. Ruthe, A. Mistry, B. Bigger, A. Rahim, T. H. Nguyen, D. Trono, A. J. Thrasher, and C. Coutelle**. 2005. Oncogenesis following delivery of a nonprimate lentiviral gene therapy vector to fetal and neonatal mice. Mol. Ther. **12**:763-771.
178. **Thomas, C. E., A. Ehrhardt, and M. A. Kay**. 2003. Progress and problems with the use of viral vectors for gene therapy. Nat. Rev. Genet. **4**:346-358.
179. **Thummala, N. R., S. S. Ghosh, S. W. Lee, B. Reddy, A. Davidson, M. S. Horwitz, J. R. Chowdhury, and N. R. Chowdhury**. 2002. A non-immunogenic adenoviral vector, coexpressing CTLA4Ig and bilirubin-uridine-diphosphoglucuronateglucuronosyltransferase permits long-term, repeatable transgene expression in the Gunn rat model of Crigler-Najjar syndrome. Gene Ther. **9**:981-990.
180. **Toietta, G., V. P. Mane, W. S. Norona, M. J. Finegold, P. Ng, A. F. McDonagh, A. L. Beaudet, and B. Lee**. 2005. Lifelong elimination of hyperbilirubinemia in the Gunn rat with a single injection of helper-dependent adenoviral vector. Proc. Natl. Acad. Sci. U. S. A **102**:3930-3935.
181. **Trauner, M., P. J. Meier, and J. L. Boyer**. 1998. Molecular pathogenesis of cholestasis. N. Engl. J. Med. **339**:1217-1227.
182. **Tsui, L. V., M. Kelly, N. Zayek, V. Rojas, K. Ho, Y. Ge, M. Moskalenko, J. Mondesire, J. Davis, M. V. Roey, T. Dull, and J. G. McArthur**. 2002. Production of human clotting Factor IX without toxicity in mice after vascular delivery of a lentiviral vector. Nat. Biotechnol. **20**:53-57.
183. **Ujhazy, P., D. Ortiz, S. Misra, S. Li, J. Moseley, H. Jones, and I. M. Arias**. 2001. Familial intrahepatic cholestasis 1: studies of localization and function. Hepatology **34**:768-775.
184. **van der Wegen, P., R. Louwen, A. M. Imam, R. M. Buijs-Offerman, M. Sinaasappel, F. Grosveld, and B. J. Scholte**. 2006. Successful treatment of UGT1A1 deficiency in a rat model of Crigler-Najjar disease by intravenous administration of a liver-specific lentiviral vector. Mol. Ther **13**:374-381.

185. **van Til, N. P., D. M. Markusic, R. R. van der, C. Kunne, J. K. Hiralall, H. Vreeling, W. M. Frederiks, R. P. Oude-Elferink, and J. Seppen**. 2005. Kupffer cells and not liver sinusoidal endothelial cells prevent lentiviral transduction of hepatocytes. Mol. Ther. **11**:26-34.
186. **VandenDriessche, T., L. Thorrez, L. Naldini, A. Follenzi, L. Moons, Z. Berneman, D. Collen, and M. K. Chuah**. 2002. Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells in vivo. Blood **100**:813-822.
187. **VandenDriessche, T., V. Vanslembrouck, I. Goovaerts, H. Zwinnen, M. L. Vanderhaeghen, D. Collen, and M. K. Chuah**. 1999. Long-term expression of human coagulation factor VIII and correction of hemophilia A after in vivo retroviral gene transfer in factor VIII-deficient mice. Proc. Natl. Acad. Sci. U. S. A **96**:10379-10384.
188. **Varnavski, A. N., R. Calcedo, M. Bove, G. Gao, and J. M. Wilson**. 2005. Evaluation of toxicity from high-dose systemic administration of recombinant adenovirus vector in vector-naive and pre-immunized mice. Gene Ther. **12**:427-436.
189. **Varnavski, A. N., Y. Zhang, M. Schnell, J. Tazelaar, J. P. Louboutin, Q. C. Yu, A. Bagg, G. P. Gao, and J. M. Wilson**. 2002. Preexisting immunity to adenovirus in rhesus monkeys fails to prevent vector-induced toxicity. J. Virol. **76**:5711-5719.
190. **Wagner, R., M. Graf, K. Bieler, H. Wolf, T. Grunwald, P. Foley, and K. Uberla**. 2000. Rev-independent expression of synthetic gag-pol genes of human immunodeficiency virus type 1 and simian immunodeficiency virus: implications for the safety of lentiviral vectors. Hum. Gene Ther. **11**:2403-2413.
191. **Walldorf, J., H. Aurich, H. Cai, D. Runge, B. Christ, S. C. Strom, and W. E. Fleig**. 2004. Expanding hepatocytes in vitro before cell transplantation: donor age-dependent proliferative capacity of cultured human hepatocytes. Scand. J. Gastroenterol. **39**:584-593.
192. **Wang, L., R. Calcedo, T. C. Nichols, D. A. Bellinger, A. Dillow, I. M. Verma, and J. M. Wilson**. 2005. Sustained correction of disease in naive and AAV2-pretreated hemophilia B dogs: AAV2/8-mediated, liver-directed gene therapy. Blood **105**:3079-3086.
193. **Wang, L., T. C. Nichols, M. S. Read, D. A. Bellinger, and I. M. Verma**. 2000. Sustained expression of therapeutic level of factor IX in hemophilia B dogs by AAV-mediated gene therapy in liver. Mol. Ther. **1**:154-158.
194. **Wege, H., H. T. Le, M. S. Chui, L. Liu, J. Wu, R. Giri, H. Malhi, B. S. Sappal, V. Kumaran, S. Gupta, and M. A. Zern**. 2003. Telomerase reconstitution immortalizes human fetal hepatocytes without disrupting their differentiation potential. Gastroenterology **124**:432-444.
195. **Wells, P. G., P. I. Mackenzie, J. R. Chowdhury, C. Guillemette, P. A. Gregory, Y. Ishii, A. J. Hansen, F. K. Kessler, P. M. Kim, N. R. Chowdhury, and J. K. Ritter**. 2004. Glucuronidation and the UDP-glucuronosyltransferases in health and disease. Drug Metab Dispos. **32**:281-290.
196. **Xu, L., C. Gao, M. S. Sands, S. R. Cai, T. C. Nichols, D. A. Bellinger, R. A. Raymer, S. McCorquodale, and K. P. Ponder**. 2003. Neonatal or hepatocyte growth factor-potentiated adult gene therapy with a retroviral vector results in therapeutic levels of canine factor IX for hemophilia B. Blood **101**:3924-3932.
197. **Xu, L., M. E. Haskins, J. R. Melniczek, C. Gao, M. A. Weil, T. M. O'Malley, P. A. O'Donnell, H. Mazrier, N. M. Ellinwood, J. Zweigle, J. H. Wolfe, and K. P. Ponder**. 2002. Transduction of hepatocytes after neonatal delivery of a Moloney murine leukemia virus based retroviral vector results in long-term expression of beta-glucuronidase in mucopolysaccharidosis VII dogs. Mol. Ther. **5**:141-153.
198. **Xu, L., T. C. Nichols, R. Sarkar, S. McCorquodale, D. A. Bellinger, and K. P. Ponder**. 2005. Absence of a desmopressin response after therapeutic expression of factor VIII in hemophilia A dogs with liver-directed neonatal gene therapy. Proc. Natl. Acad. Sci. U. S. A **102**:6080-6085.
199. **Yamaguchi, Y., M. E. Heiny, N. Shimizu, T. Aoki, and J. D. Gitlin**. 1994. Expression of the Wilson disease gene is deficient in the Long-Evans Cinnamon rat. Biochem. J. **301 (Pt 1)**:1-4.
200. **Yang, C. C., X. Xiao, X. Zhu, D. C. Ansardi, N. D. Epstein, M. R. Frey, A. G. Matera, and R. J. Samulski**. 1997. Cellular recombination pathways and viral terminal repeat hairpin structures are sufficient for adeno-associated virus integration in vivo and in vitro. J. Virol. **71**:9231-9247.
201. **Yang, Y., H. C. Ertl, and J. M. Wilson**. 1994. MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. Immunity. **1**:433-442.
202. **Yoshimitsu, M., T. Sato, K. Tao, J. S. Walia, V. I. Rasaiah, G. T. Sleep, G. J. Murray, A. G. Poeppl, J. Underwood, L. West, R. O. Brady, and J. A. Medin**. 2004. Bioluminescent imaging of a marking transgene and correction of Fabry mice by neonatal injection of recombinant lentiviral vectors. Proc. Natl. Acad. Sci. U. S. A **101**:16909-16914.
203. **Zhang, X. Y., V. F. La Russa, and J. Reiser**. 2004. Transduction of bone-marrow-derived mesenchymal stem cells by using lentivirus vectors pseudotyped with modified RD114 envelope glycoproteins. J. Virol. **78**:1219-1229.
204. **Zhang, Y., N. Chirmule, G. P. Gao, R. Qian, M. Croyle, B. Joshi, J. Tazelaar, and J. M. Wilson**. 2001. Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. Mol. Ther. **3**:697-707.
205. **Zhang, Y. C., M. Powers, C. Wasserfall, T. Brusko, S. Song, T. Flotte, R. O. Snyder, M. Potter, M. Scott-Jorgensen, M. Campbell-Thompson, J. M. Crawford, H. S. Nick, A. Agarwal, T. M. Ellis, and M. A. Atkinson**. 2004. Immunity to adeno-associated virus serotype 2 delivered transgenes imparted by genetic predisposition to autoimmunity. Gene Ther. **11**:233-240.
206. **Zhou, X., Y. Cui, X. Huang, Z. Yu, A. M. Thomas, Z. Ye, D. M. Pardoll, E. M. Jaffee, and L. Cheng**. 2003. Lentivirus-mediated gene transfer and expression in established human tumor antigen-specific cytotoxic T cells and primary unstimulated T cells. Hum. Gene Ther. **14**:1089-1105.
207. **Ziegler, R. J., S. M. Lonning, D. Armentano, C. Li, D. W. Souza, M. Cherry, C. Ford, C. M. Barbon, R. J. Desnick, G. Gao, J. M. Wilson, R. Peluso, S. Godwin, B. J. Carter, R. J. Gregory, S. C. Wadsworth, and S. H. Cheng**. 2004. AAV2 vector harboring a liver-restricted promoter facilitates sustained expression of

therapeutic levels of alpha-galactosidase A and the induction of immune tolerance in Fabry mice. Mol. Ther. **9**:231-240.

208. **Zufferey, R., J. E. Donello, D. Trono, and T. J. Hope**. 1999. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. J. Virol. **73**:2886-2892.
209. **Zufferey, R., T. Dull, R. J. Mandel, A. Bukovsky, D. Quiroz, L. Naldini, and D. Trono**. 1998. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J. Virol. **72**:9873-9880.

Chapter 2

Kupffer cells and not liver sinusoidal endothelial cells prevent lentiviral transduction of hepatocytes.

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Kupffer Cells and Not Liver Sinusoidal Endothelial Cells Prevent Lentiviral Transduction of Hepatocytes

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Lentiviral vectors can stably transduce dividing and nondividing cells *in vivo* and are best suited to long-term correction of inherited liver diseases. Intraportal administration of lentiviral vectors expressing green fluorescent protein (Lenti-GFP) in mice resulted in a higher transduction of nonparenchymal cells than hepatocytes (7.32 ± 3.66% vs 0.22 ± 0.08%, respectively). Therefore, various treatments were explored to increase lentiviral transduction of hepatocytes. Lenti-GFP was injected into the common bile duct, which led to transduction of biliary epithelium and hepatocytes at low efficiency. Transient removal of the sinusoidal endothelial cell layer by cyclophosphamide to increase accessibility to hepatocytes did not improve hepatocyte transduction (0.42 ± 0.36%). Inhibition of Kupffer cell function by gadolinium chloride led to a significant decrease in GFP-positive nonparenchymal cells (2.15 ± 3.14%) and a sevenfold increase in GFP-positive hepatocytes compared to nonpretreated mice (1.48 ± 2.01%). These findings suggest that sinusoidal endothelial cells do not significantly limit lentiviral transduction of hepatocytes, while Kupffer cells sequester lentiviral particles thereby preventing hepatocyte transduction. Therefore, the use of agents that inhibit Kupffer cell function may be important for lentiviral vector treatment of liver disease.

Key Words: lentiviral vectors, gene transfer, GFP, liver, hepatocytes, Kupffer cells, sinusoidal endothelial cells

INTRODUCTION

Lentiviral vectors are able to deliver genes into a wide variety of both dividing and nondividing cell types *in vivo,* such as muscle, retina, neurons, pancreatic, and liver cells [1–5]. This property makes them an attractive tool for *in vivo* treatment of genetic liver disorders. Unfortunately, when lentiviral vectors are injected into the circulation, the majority of cells that are transduced in the liver are of nonparenchymal origin, notably endothelial cells and Kupffer cells [6–9]. Since many inherited liver diseases have an impaired hepatocyte function, improved lentiviral particle delivery to hepatocytes is a requirement.

Recently it has been shown that, *in vitro,* primary hepatocytes are efficiently transduced by lentiviral vectors [10–12]. However, *in vivo* transduction is much less efficient. We therefore investigated if there is a barrier that prevents efficient hepatocyte transduction *in vivo*. Several factors may cause preferential transduction of nonparenchymal cells.

The sinusoidal endothelium forms a barrier between the blood and the hepatocytes and may hamper passage of viral particles to the hepatocytes. Cyclophosphamide is an alkylating agent that has been used in cancer therapy [13] and also inhibits tumor growth by damaging the tumor vasculature [14]. Endothelial cells are relatively susceptible to cyclophosphamide-induced toxicity [15] and the drug has been shown to disrupt the endothelial cell layer in the sinusoids of the liver [16,17].

The liver is able to clear viruses such as vesicular stomatitis virus [18], simian immunodeficiency virus [19], and adenoviruses [20] efficiently from the bloodstream. In the liver, specialized macrophages called Kupffer cells are located inside the sinusoids and play

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an important role in the clearance of these viruses. Vesicular stomatitis virus G-protein (VSV-G)-pseudotyped lentiviral vectors may also be efficiently cleared from the circulation by Kupffer cells. It has been shown that agents such as gadolinium chloride ($GdCl_3$) can block phagocytosis and eliminate macrophages transiently [21] and can increase adenoviral transduction [22]. Selective depletion of these cells led to a higher and prolonged transgene expression after transduction with adenoviral vectors [23].

In addition to the ability of Kupffer cells to scavenge viral particles, Kupffer cells are also transduced themselves by VSV-G-pseudotyped lentiviral vectors [7,9].

The most common routes of administration to target lentiviral vectors to the liver are the portal vein and tail vein [9,24–27]. Adenoviral vector administration has been performed via the common bile duct in rats and resulted in efficient transgene expression in hepatocytes, but not in biliary epithelial cells [28]. In addition, it may be possible to administer lentiviral vectors via the common bile duct to avoid many of the above-mentioned barriers in sinusoids and to target viral particles exclusively to the liver. A drawback of this approach is the potential toxic effect of bile on the lipid-enveloped lentiviral particles.

The objective of this study was to improve lentiviral transduction of hepatocytes by exploring an alternate route of administration, by disruption of the sinusoidal endothelial cell layer, and by depletion of Kupffer cells. We show that by intraportal vein delivery of lentiviral vectors the majority of transduced liver cells are of nonparenchymal origin. Administration of lentiviral vectors via the common bile duct led to exclusive transduction of bile duct epithelium and some hepatocytes, but at low efficiency. Mild disruption of liver sinusoidal endothelial cells did not lead to an increase in hepatocyte transduction, while Kupffer cell depletion led to a significant increase in the transduction efficiency of hepatocytes.

RESULTS

Lentiviral Transduction of the Liver by Bile Duct Administration

We infused animals with 0.5×10^8 HeLa transducing units (HTU) into the bile duct, which led to GFP-positive epithelium throughout the biliary tract (Fig. 1A). The total number of GFP-positive cells in these livers (Fig. 1B) was much lower than in the animals injected intraportally. Only a small part of the GFP-positive cells were hepatocytes, most of them were localized in the periportal areas (Fig. 1B). No endothelial cells or Kupffer cells were transduced. No splenocytes were GFP positive (data not shown).

We found that incubation of bile with lentivirus resulted in decreased transduction of HeLa cells (data not shown). The relatively poor transduction of liver cells *in vivo* may be caused by inactivation of the lipid-enveloped lentiviral particles by the strong detergent action of bile, although we chose conditions to minimize the toxicity of bile, such as depletion of bile for 1 h and dissolving the lentiviral preparations in a relatively large volume (300 μl) to dilute residual bile in the biliary tract.

Lentiviral Transduction of Liver by Portal Vein Injection

In mice intraportally injected with 0.5×10^8 HTU, we observed transduction of hepatocytes and nonparenchymal cells (Fig. 2A). The total number of hepatocytes transduced in the liver was $3.16 \pm 1.11/mm^2$ ($n = 7$) 1 week after injection (Table 1). This represents about $0.22 \pm 0.08\%$ of the total hepatocyte population. The number of GFP-positive nonparenchymal cells was more than 20 times higher ($70.27 \pm 35.11/mm^2$, $n = 7$), which is comparable with other studies [6]. GFP-positive splenocytes were observed (data not shown).

Liver Sinusoidal Endothelial Cell Disruption

Animals treated with cyclophosphamide were analyzed for damage of sinusoidal endothelial cells with electron microscopy. Fig. 3 shows the variable degree of endothelial cell disruption in a cyclophosphamide-treated animal compared to a control animal. The endothelial lining is not intact at some places, whereas at other places the space of Disse (the area between the endothelial cells and hepatocytes) is dramatically enlarged. Other than the endothelial cell disruption, the morphology of the liver was normal.

Effect of Cyclophosphamide on Lentiviral Transduction *in Vitro*

Transduction of Hepa 1-6 mouse hepatoma cells was not affected by cyclophosphamide *in vitro* (data not shown). Cells incubated with cyclophosphamide for 24 h and subsequently incubated with lentivirus were transduced as efficiently as untreated cells. Therefore, cyclophosphamide did not seem to have a direct deleterious effect on lentiviral transduction.

Lentiviral Transduction of Liver by Portal Vein Injection after Cyclophosphamide Treatment

Upon treatment of mice with cyclophosphamide, the number of transduced hepatocytes and nonparenchymal cells did not change significantly (6.09 ± 5.19 and $95.12 \pm 71.76/mm^2$, respectively) compared to nonpretreated animals (Table 1). Additionally, there was no significant difference in the percentage of GFP-positive hepatocytes of the total number of GFP-positive cells in cyclophosphamide-treated animals ($5.19 \pm 2.73\%$) compared to nonpretreated animals ($5.27 \pm 2.78\%$, Table 1). GFP-positive splenocytes were observed (data not shown).

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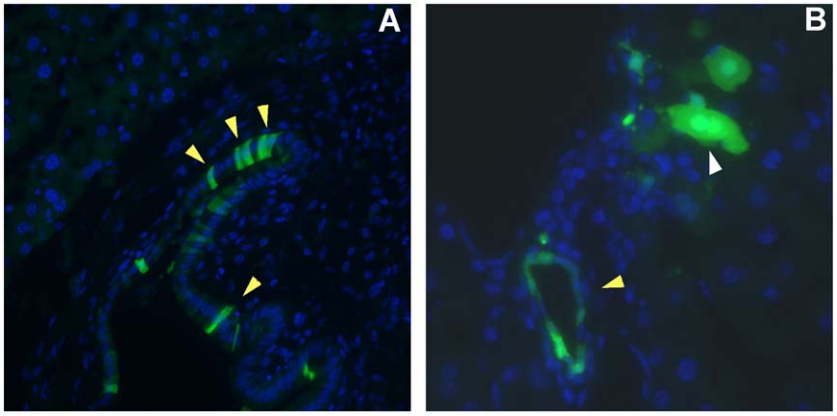

FIG. 1.

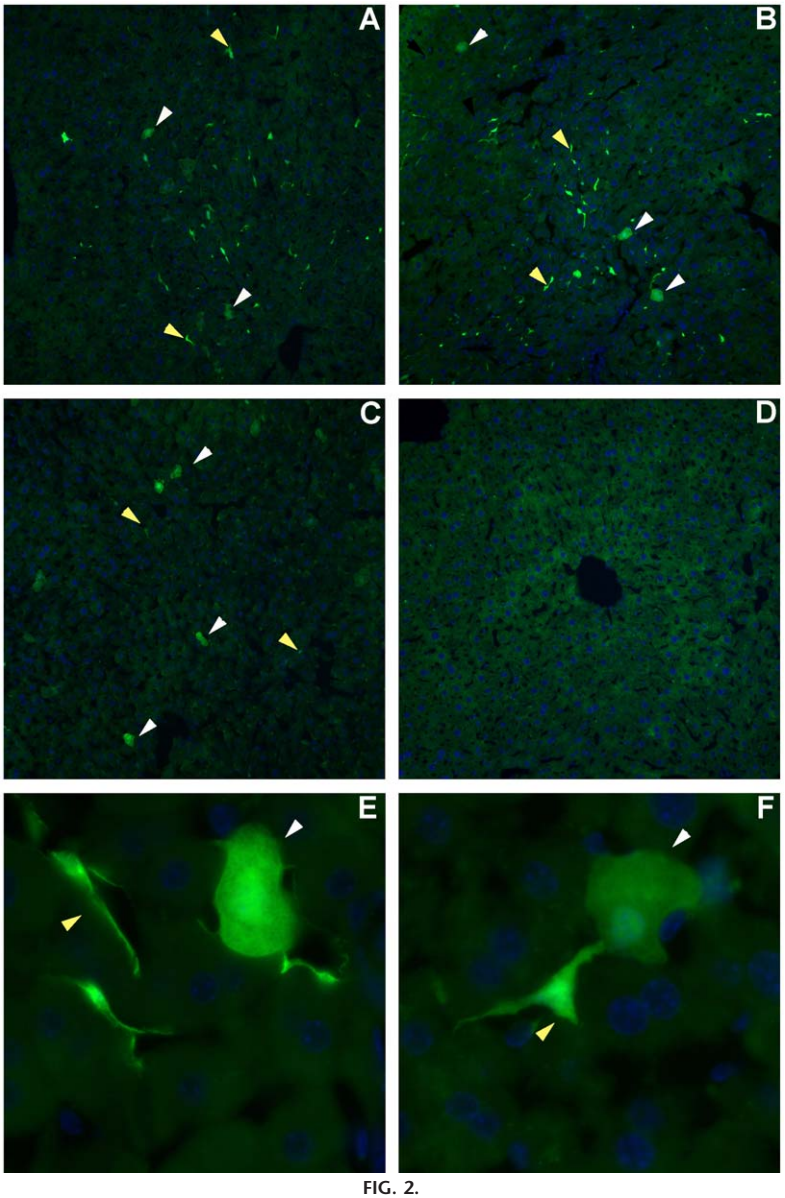

FIG. 2.

TABLE 1: GFP-positive cells in liver sections

	No pretreatment (n = 7)	Cyclophosphamide (n = 5)	$GdCl_3$ (n = 7)
GFP-positive hepatocytes[a]	3.16 ± 1.11	6.09 ± 5.18	21.43 ± 28.85*
GFP-positive nonparenchymal cells[a]	70.27 ± 35.11	95.12 ± 71.76	20.65 ± 30.13*
Percentage GFP-positive hepatocytes[b]	5.27 ± 2.78%	5.19 ± 2.73%	51.01 ± 16.12%**

GFP-positive cells were counted in liver sections of animals injected intraportally with Lenti-GFP. Mean values are presented ± SD.
[a] GFP-positive cells per mm^2.
[b] Percentage of GFP-positive hepatocytes of total number of GFP-positive cells in the liver.
* Significant difference compared to nonpretreated group: $P < 0.05$.
** Significant difference compared to nonpretreated group: $P \leq 0.001$.

Lentiviral Transduction of Liver by Portal Vein Injection after Kupffer Cell Blockage of Phagocytosis

It has been shown that the majority of lentivirally transduced cells in the liver may be Kupffer cells [7]. We also found that most of the GFP-positive nonparenchymal cells were positive for the Kupffer cell marker F4/80 (Fig. 4). Therefore we ablated Kupffer cells before lentiviral injection to determine the effect of this treatment on hepatocyte transduction. We performed transient ablation of Kupffer cells by two injections of $GdCl_3$ (10 mg/kg) at 30 and 6 h before transduction [21].

We assessed depletion of Kupffer cells by injection of India ink in a parallel group of mice that was treated identically. In control animals there was a clear accumulation of black pigment in the Kupffer cells localized in the sinusoids, while this was largely absent in $GdCl_3$-treated mice (Fig. 5).

Analysis of liver sections revealed that Kupffer cell depletion increased the number of GFP-positive hepatocytes approximately seven times (21.43 ± 28.85/mm^2, n = 7, $P < 0.05$) compared to animals with no pretreatment (3.16 ± 1.11/mm^2). Kupffer cell depletion reduced the number of nonparenchymal cell transduction by about 70% to 20.65 ± 30.13/mm^2 (n = 7, $P < 0.05$). The percentage of GFP-positive hepatocytes of the total number GFP-positive cells significantly increased from 5.27 ± 2.78% in nonpretreated animals to 51.01 ± 16.12% in $GdCl_3$-treated mice ($P \leq 0.001$, Table 1). We also observed GFP-positive splenocytes (data not shown).

Quantitative PCR of Lentiviral Integrations

We analyzed the total number of viral integrations in animals injected with lentivirus by quantitative PCR on genomic DNA isolated from the anterior right lobe of the liver (Table 2). The genomic integration percentage in nonpretreated animals was 10.54 ± 5.34% and there was no significant difference from cyclophosphamide-treated mice (6.22 ± 3.71%). The number of genomic integrations significantly decreased after $GdCl_3$ treatment (2.40 ± 1.80%, P = 0.003, Table 2). These data confirm our microscopic findings.

Alanine Aminotransferase Activity after Lentiviral Injections

Intraportal injection of lentivirus caused modest and transient increase in serum alanine aminotransferase (ALT) levels (Table 3) in nonpretreated, cyclophosphamide-treated, and $GdCl_3$-treated animals. However, the rise in ALT was not significant in $GdCl_3$-treated animals. At day 7 ALT levels returned almost back to normal.

DISCUSSION

The main aim of liver-directed gene therapy is to transduce sufficient numbers of hepatocytes, because in nearly all disorders this is the cell type that needs to be corrected. Thus far, in most studies that made use of lentiviral vectors, the route of administration was either the portal vein or the tail vein [9,24–26]. In the present study we wished to optimize hepatocyte transduction. For this purpose we used the third-generation lentiviral vector that we have previously described [10]. Given the fact that the percentage of hepatocytes transduced by this and other lentiviral vectors is low, we tested various treatments to increase targeting to the hepatocyte. We chose to use a dose of 0.5 × 10^8 HTU to demonstrate the mechanism without saturation of the reticuloendothelial system.

FIG. 1. GFP expression in the liver after bile duct infusion of lentiviral vectors. Fluorescence microscopy of tissue sections of mice injected with 0.5 × 10^8 HTU lentiviral vectors in the common bile duct. A small number of cells were transduced, consisting of bile duct epithelium (yellow arrowheads) and hepatocytes (white arrowheads). (A) Bile duct. (B) Liver. Both at 20× original magnification.

FIG. 2. GFP expression in the liver after portal vein injection of lentiviral vectors. Fluorescence microscopy of liver sections 1 week after intraportal injection of 0.5 × 10^8 HTU lentiviral vectors. Hepatocytes (white arrows) and nonparenchymal cells (yellow arrows) in the liver of mice were GFP positive. Nuclei were stained with DAPI. (A) No pretreatment. (B) Cyclophosphamide treatment. (C) $GdCl_3$ treatment. (D) Negative control (no lentivirus). All 10× original magnification. (E, F) No pretreatment, 40× original magnification.

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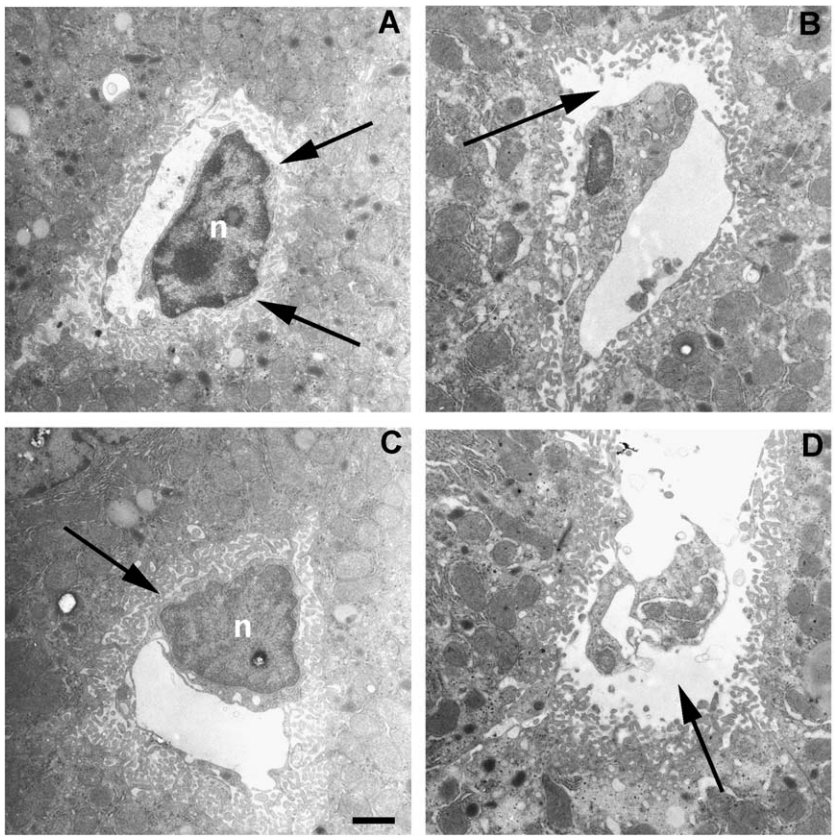

FIG. 3. Electron microscopy of livers of cyclophosphamide-injected animals. (A, C) Control animals. Normal liver morphology: hepatocytes and the fenestrated lining of the sinusoidal endothelial cells are intact. Arrows point to the space of Disse and microvilli of hepatocytes. (B, D) Animals treated with cyclophosphamide show variable degrees of disruption of the endothelial lining. Endothelial cells are detached from the hepatocytes, leading to a widening of the space of Disse. Bar denotes 1 μm.

To reduce Kupffer cell and endothelial cell transduction, we injected virus into the common bile duct, which led to transduction of hepatocytes and also of biliary epithelium. However, overall transduction efficiency by retrograde infusion of lentiviral vector into the biliary tract was low.

Inactivation of lentiviral vectors by bile (data not shown) might play a role in the low transduction efficiency. It could also indicate that the virus did not reach the bile canuliculi, because the majority of GFP-positive hepatocytes were located in portal tracts.

Hence, retrograde perfusion may reduce transduction of Kupffer cells and sinusoidal endothelial cells. In addition, extrahepatic transduction was reduced as well, which was determined by the disappearance of splenocyte transduction compared to animals injected into the portal vein (data not shown), but the efficiency of hepatocyte transduction is too low for therapeutic use.

Viral particles have to travel through the fenestrae of the liver sinusoidal endothelial cells to arrive in the space of Disse, before the particles can enter the hepatocytes. The size of the endothelial fenestrae is variable between 100 and 200 nm [29–32], which may form a physical barrier for HIV-derived lentiviral particles to pass through, because the size of HIV-1 particles has been estimated to be between 120 and 200 nm [33].

Disruption of sinusoidal endothelial cells by cyclophosphamide has been shown to improve grafting of transplanted liver cells [16]. We reasoned that it might similarly improve lentiviral transduction of hepatocytes. However, the effect of disruption of the sinusoidal endothelial lining on hepatocyte transduction was limited. The number of GFP-positive hepatocytes increased, but this was not significant (Table 1) and represented less than 0.5% of the total hepatocyte population. The total number of genomic integrations determined by quantitative PCR only decreased by approximately 30% compared to nonpretreated animals, which confirmed the cell counting (Tables 1 and 2). Our results therefore indicate that the endothelial layer is not a major obstacle to lentiviral hepatocyte transduction.

Kupffer cells are involved in the phagocytosis of foreign particles, such as viruses. $GdCl_3$ has been used in many studies to block Kupffer cell-mediated phagocytosis and deplete these cells transiently [20–22].

Kupffer cells may not only scavenge viruses, but are also highly prone to viral infection [7,9]. Indeed, we

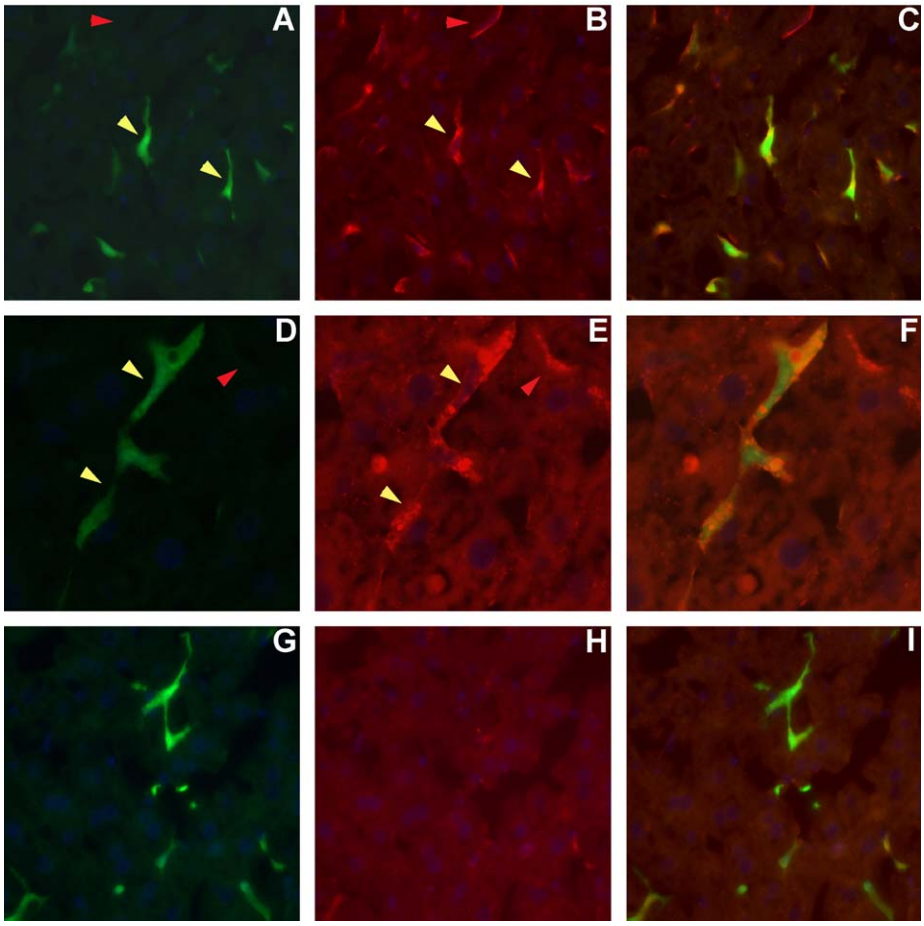

FIG. 4. Kupffer cells and viral transduction after portal vein injection of lentiviral vectors. Demonstration of GFP-positive Kupffer cells by colabeling with the Kupffer cell marker F4/80. Left (A, D, G): GFP expression. Middle (B, E): KC marker expression. (H) Negative control F4/80 staining, secondary antibody only. Right (C, F, I): merge. All 40× original magnification. The majority of GFP-positive cells costained for the KC marker. GFP-positive Kupffer cells (yellow arrowheads). GFP-negative/KC marker-positive cells (red arrowheads).

observed that the majority of GFP-positive nonparenchymal cells were positive for the Kupffer cell marker F4/80.

Injection of $GdCl_3$ led to inactivation of the function of Kupffer cells as shown by histochemical analysis (Fig. 5). Inactivation of Kupffer cells by $GdCl_3$ significantly decreased the transduction percentage of nonparenchymal cells from 7.32 ± 3.66% in nonpretreated mice to 2.15 ± 3.14%, indicating that the majority of nonparenchymal cell transduction was due to Kupffer cell transduction. Simultaneously, the number of transduced hepatocytes increased significantly by a factor of 7, which represents approximately 1.48 ± 2.01% of the total hepatocyte population.

The most straightforward interpretation of these results is that the Kupffer cells scavenge most viral particles before they reach the hepatocytes. Elimination of this scavenger function dramatically increases hepatocyte transduction.

Kupffer cell depletion led to 80% decrease in the number of viral integrations as assessed by quantitative

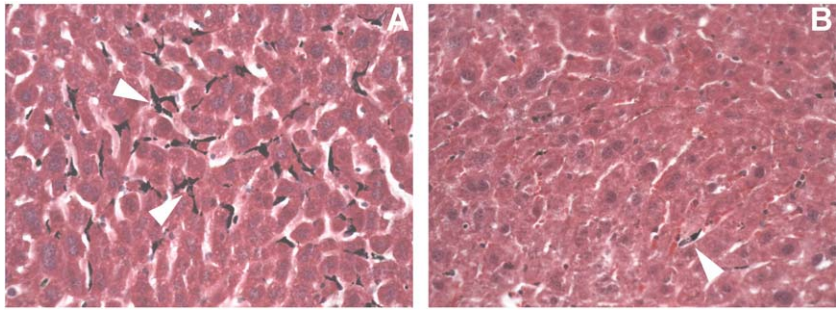

FIG. 5. Kupffer cell depletion by $GdCl_3$ treatment in mice. Histochemistry of ink-injected control and $GdCl_3$-treated mice. In animals treated with $GdCl_3$ the number of Kupffer cells is lower compared to animals without $GdCl_3$. Kupffer cells (white arrowheads). (A) Nonpretreated animal injected with ink. (B) Animal treated with $GdCl_3$ and ink. Both 40× original magnification.

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TABLE 2: Quantitative PCR of genomic DNA

	No pretreatment (n = 8)	Cyclophosphamide (n = 5)	$GdCl_3$ (n = 6)
Proviral integrations	10.54 ± 5.34	6.22 ± 3.71	2.40 ± 1.80*

Number of proviral integrations per 100 genomes in the liver determined by quantitative PCR of genomic DNA.
* Significant difference compared to nonpretreated group: $P = 0.003$.

PCR (Table 2). However, the same treatment reduced the total number of GFP-positive cells by only 40%. Hence, in nonpretreated livers there were more viral integrations than in the Kupffer cell-depleted state. This observation is again in line with the scavenging function of the Kupffer cells. If Kupffer cells function to filter viral particles from blood, they are expected to take up more viral particles per cell and multiple integrations probably occur in these cells. Depletion of the Kupffer cells allows the viral particles to spread over the more numerous hepatocytes.

Kupffer cells are involved in the activation of the innate immune response against adenoviruses, which can lead to early phase hepatotoxicity [22]. The lentiviral vector administrations in our experiments induced mild but significant elevations of ALT in nonpretreated animals and cyclophosphamide-treated animals (Table 3). Our observation that lentivirus administration to Kupffer cell-depleted mice was relatively less toxic than in nonpretreated mice is in line with the reduced liver toxicity after adenovirus administration with $GdCl_3$ treatment [22].

Our results confirm earlier observations that *in vivo* administration of VSV-G-pseudotyped lentivirus can cause a modest but transient hepatotoxicity, with high variability between animals [34], which was also observed in all our treatment groups.

A drawback of the use of lentiviral vectors may be that these vectors could integrate in active cellular genes [35] and activate potential oncogenes. Because the proliferative activity of hepatocytes is approximately about 0.005 to 0.05% *in vivo* [36], the likelihood of tumorigenesis by a single integration is probably very low.

In conclusion, we have shown that Kupffer cells play an important role in limiting lentiviral hepatocyte transduction by sequestration of viral particles. To treat inherited liver diseases in the future, the inclusion of agents that block Kupffer cell activity or even eliminate these cells from the liver prior to gene transfer should be considered.

MATERIALS AND METHODS

Lentiviral vector production. The lentiviral vector prrlcpptpgkgfppressin containing the hepatitis B virus posttranslational regulatory element, central polypurine tract, and phosphoglycerate kinase promoter driving GFP expression was used as described earlier [10].

Lentiviruses were produced as described by transient transfection of 293T cells using a calcium phosphate method, concentrated by ultracentrifugation, and titrated on HeLa cells [5,37]. Cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 0.5 units/ml penicillin, 0.5 mg/ml streptomycin, and 2 mM L-glutamine.

Lentiviral inactivation by bile. Lentiviral particles were incubated with bile for 30 min at 37°C, followed by transduction of HeLa cells in 2 ml DMEM containing 10 μg/μl DEAE-dextran (Pharmacia). After 5 days, the number of transduced cells was determined by flow cytometry.

Animals, viral injections, and processing of tissues. Wild-type male FVB mice ages 8-12 weeks were used in all studies and fed *ad libitum*. All animal experiments were performed in accordance with the Animal Ethical Committee guidelines at the Academic Medical Center of Amsterdam.

Mice were anesthetized with an intraperitoneal injection of FFM mix (2.5 mg Fluanisone/0.105 mg Fentanyl citrate/1.25 mg Midozalam HCl/kg in H_2O, 7 ml/kg). Under deep anesthesia, the peritoneal cavity was opened and the mice were injected intraportally with a volume of 250 μl containing 0.5×10^8 HTU with a 30-gauge needle at day 0. The animals were sutured and received the analgesic Temgesic (20–30 μl, 0.03 mg/ml) subcutaneously following recovery from FFM.

After a period of 7 days, the mice were killed by *in vivo* fixation. Under deep anesthesia, the peritoneal cavity was opened, a ligature was applied around the anterior right lobe of the liver and tightened, and the lobe was removed and snap frozen in liquid nitrogen and stored at −80°C. Subsequently, the animals were perfused intracardially with 20 ml of phosphate-buffered saline (PBS) and 20 ml of 2% formaldehyde in PBS. After perfusion, the liver and spleen were removed and the liver lobes were fixed in a 4% formaldehyde solution in PBS for 4 h at room temperature.

TABLE 3: Analysis of liver toxicity by measurement of plasma ALT levels

	ALT		
	Normal level	Day 1	Day 7
No pretreatment	41.00 ± 1.73	179.86 ± 260.07*	68.00 ± 70.47
Cyclophosphamide	41.75 ± 7.04	188.25 ± 229.75*	85.25 ± 75.17
$GdCl_3$	39.71 ± 8.52	149.67 ± 195.72	106.71 ± 143.91

ALT levels (U/L) in mice intraportally injected with lentivirus with and without cyclophosphamide or $GdCl_3$ treatment. ALT levels were higher in nonpretreated, cyclophosphamide-treated, and $GdCl_3$-treated animals at day 1 after viral injections. The ALT levels were lower at day 7 compared to day 1. Mean values are presented with SD (n = 4–7).
* Significant difference compared to normal levels: $P < 0.05$.

The tissues were transferred to a 30% sucrose solution at 4°C overnight and subsequently snap frozen in liquid nitrogen and stored at −80°C.

Before cryosectioning, tissue was embedded in Tissue-Tek OCT medium (Bayer). Sections (6 μm) were laid on poly-L-lysine-coated glass slides and enclosed in mounting medium ((20 mg 1,4-diazabicyclo[2.2.2]octane (DABCO, Sigma), 0.1 M Tris/HCl, pH 8.0, in glycerol)/ml) containing 4′,6-diamidino-2-phenylindole (DAPI; Sigma).

Determination of plasma alanine aminotransferase activity. Serum ALT levels were taken as a measure of liver toxicity induced by various treatments [38,39]. Blood was collected by orbital puncture 3 days before lentiviral vector injection, 1 day after injection, and 30 min before the animals were killed at day 7. ALT was measured in plasma by routine clinical chemistry.

Mouse bile duct cannulation and lentiviral transduction. The animals were anesthetized as described above. After opening the peritoneal cavity, the common bile duct was clamped downstream of the gallbladder. Two sutures were placed around the gallbladder and an incision was made in the tip of the gallbladder. A catheter was placed in the common bile duct and one ligature was closed to keep the catheter in place. For 1 h bile was depleted and subsequently 300 μl of 0.5×10^8 HTU in PBS was injected in the catheter, which was then closed for 1 h. after viral transduction, the bile flow was restored by removal of the clamp. The catheter was removed and the gallbladder ligature placed at the start of the operation was closed. finally, the abdomen was closed. After 7 days, the animals were killed and organs were harvested.

Cell count and statistics. GFP-positive cells were counted in sections of the left lobe and median lobe with an inverse microscope (Leica DM*RA*2; Leica). An independent person randomly numbered the sections and all counting was performed in a blinded fashion. Per animal GFP-positive hepatocytes were counted in five sections of nonoverlapping cell layers. Images of the sections were captured and surface areas were measured by using Leica FW4000 software to determine the number of hepatocytes per square millimeter. The number of hepatocytes/mm^2 was determined in 80 times enlarged fields. This enabled us to determine the percentage of GFP-positive hepatocytes in the liver.

GFP-positive nonparenchymal cells were counted in a 20 times magnification field and were adjusted to nonparenchymal cells per square millimeter. The transduction percentage of nonparenchymal cells was determined by the assumption that hepatocytes represent 60% of the cells in the liver [40].

Statistical analyses were performed using SPSS10.0 software and significant difference was considered if $P < 0.05$ determined by Mann-Whitney *U* test.

Disruption of the sinusoidal endothelial cell layer. Disruption of sinusoidal endothelium was performed by injecting 200 mg/kg body weight cyclophosphamide in H_2O [16] (Endoxan, Baxter, Utrecht, The Netherlands) intraperitoneally 24 h before lentiviral vector injection.

Effect of cyclophosphamide on lentiviral transduction efficiency in vitro. To determine the effect of cyclophosphamide on lentiviral transduction efficiency Hepa 1–6 mouse hepatoma cells were transduced with lentivirus in a volume of 2 ml medium for 4 h in the presence or absence of cyclophosphamide. We assumed that 0.2 mg/g *in vivo* was equal to 0.2 mg/ml *in vitro*. Cells were incubated with serially diluted cyclophosphamide from 1 to 0 mg/ml. In addition, Hepa 1–6 mouse hepatoma cells were also cultured with cyclophosphamide for 28 h, with subsequent incubation of lentivirus for the last 4 h. Flow cytometry for gfp was performed 5 days later to determine the transduction efficiency.

Kupffer cell blockage of phagocytosis. For Kupffer cell blockage of phagocytosis and partial depletion from the liver, mice were injected intravenously with 10 mg/kg gadolinium chloride (5.65 mg/ml gadolinium chloride hexahydrate containing 4 mg/ml gadolinium chloride; Sigma) per body weight at 30 and 6 h [21] prior to lentiviral vector injection.

To determine Kupffer cell phagocytotic activity and distribution of Kupffer cells in the liver animals were injected intravenously via the tail vein with india ink (0.08 ml per 100 g body weight; Pelikan, Germany) [21]. Thirty minutes after injection, the animals were perfused with PBS and 2% formaldehyde in PBS; then, the liver was removed and incubated overnight in 4% formaldehyde in PBS and transferred to 70% ethanol. Liver tissue was dehydrated with ethanol and embedded in Paraplast Plus (Kendall). Sections (7 μm) were made and stained with hematoxylin and azophloxine.

Immunostaining. *In vivo* formaldehyde-fixed, sucrose-incubated, and subsequently snap-frozen material was used. Sections of 6 μm were made and kept at −20°C before use. Sections were washed in PBS for 15 min and blocked in PBS/Tween 20 (0.05%) with 10% mouse serum for 1 h. Subsequently, primary antibody was incubated for 1 h at room temperature. For Kupffer cell staining, sections were incubated with rat anti-mouse F4/80 antigen (1:10; Serotec). After primary incubation, sections were washed in PBS/Tween 0.05% for 15 min, followed by incubation with Texas red-conjugated goat anti-rat antibody (1:500; Rockland Immunochemicals) for another hour at room temperature. Then the sections were washed for another 15 min in PBS and embedded in mounting medium containing DAPI.

Electron microscopy. To determine the effect of cyclophosphamide on sinusoidal endothelial cells, liver tissue was prepared for electron microscopy as previously described [41]. In short, after treatment with cyclophosphamide as described above, the animal was perfused with PBS, followed by 2% paraformaldehyde in PBS. Small liver pieces (<1 mm^3) were made and fixed in a mixture of 1% (wt/vol) glutaraldehyde and 4% (wt/vol) formaldehyde in 100 mmol/L sodium cacodylate buffer (pH 7.4) and stored at 4°C for further use.

Genomic DNA isolation and quantitative PCR. Genomic DNA was isolated using the DNeasy tissue kit (Qiagen) according to the manufacturer's protocol. Quantitative PCR was performed in a Lightcycler apparatus (Roche) using Lightcycler Faststart DNA Master SYBR Green I (Roche) with 2 mM $MgCl_2$ according to the manufacturer's protocol. The following primer set was used to amplify a 274-bp product: HIV-U3 forward primer, 5′-CTGGAAGGGCTAATTCACTC-3′, and HIV PSI reverse primer, 5′-GGTTTCCCTTTCGCTTTCAG-3′. The primers amplify the integrated provirus only and not the transfer plasmid, thus reducing the risk of contamination. For every reaction 50 ng of genomic DNA was used. Three different PCRs were performed and averaged for every individual sample.

PCR conditions were 95°C for 10 min and then 40 cycles as follows: 95°C for 1 s, 62°C for 7 s, 72°C for 30 s, and 82°C for 1 s. Negative control sample was PCR amplification of animals not injected with lentivirus and amplification of PCR reagents without template. To determine the number of integrations per genome, Hepa 1–6 mouse hepatoma cells (ATCC, CRL-1830) were transduced at a low multiplicity of infection (14.8% GFP positive) to have approximately one integration per transduced cell. These cells were sorted to obtain a 100% GFP-positive population. DNA was extracted and diluted with negative DNA to make a standard curve ranging from 100 to 1% genomic integrations.

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REFERENCES

1. Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L., and Trono, D. (1997). Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat. Biotechnol.* **15:** 871–875.
2. Zufferey, R., *et al.* (1998). Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J. Virol.* **72:** 9873–9880.
3. Kafri, T., Blomer, U., Peterson, D. A., Gage, F. H., and Verma, I. M. (1997). Sustained

doi:10.1016/j.ymthe.2004.09.012

expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat. Genet.* **17:** 314–317.
4. Naldini, L., *et al.* (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272:** 263–267.
5. Seppen, J., van der Rijt, R., Looije, N., van Til, N. P., Lamers, W. H., and Oude Elferink, R. P. (2003). Long-term correction of bilirubin UDPglucuronyltransferase deficiency in rats by in utero lentiviral gene transfer. *Mol. Ther.* **8:** 593–599.
6. Pfeifer, A., *et al.* (2001). Transduction of liver cells by lentiviral vectors: analysis in living animals by fluorescence imaging. *Mol. Ther.* **3:** 319–322.
7. Kang, Y., *et al.* (2002). In vivo gene transfer using a nonprimate lentiviral vector pseudotyped with Ross River virus glycoproteins. *J. Virol.* **76:** 9378–9388.
8. De Palma, M., Venneri, M. A., and Naldini, L. (2003). In vivo targeting of tumor endothelial cells by systemic delivery of lentiviral vectors. *Hum. Gene Ther.* **14:** 1193–1206.
9. Follenzi, A., Sabatino, G., Lombardo, A., Boccaccio, C., and Naldini, L. (2002). Efficient gene delivery and targeted expression to hepatocytes in vivo by improved lentiviral vectors. *Hum. Gene Ther.* **13:** 243–260.
10. 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.
11. Nguyen, T. H., Oberholzer, J., Birraux, J., Majno, P., Morel, P., and Trono, D. (2002). Highly efficient lentiviral vector-mediated transduction of nondividing, fully reimplantable primary hepatocytes. *Mol. Ther.* **6:** 199–209.
12. Giannini, C., *et al.* (2003). A highly efficient, stable, and rapid approach for ex vivo human liver gene therapy via a FLAP lentiviral vector. *Hepatology* **38:** 114–122.
13. Colvin, D. M. (2000). Alkylating agents and platinum antitumor compounds. In *Cancer Medicine* (R. C. Bast, D. W. Kufe, R. E. Pollock, R. R. Weichselbaum, J. F. Holland, and E. Frei, Eds.), BC Decker, Hamilton, ON [Online publication].
14. Browder, T., *et al.* (2000). Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res.* **60:** 1878–1886.
15. DeLeve, L. D. (1996). Cellular target of cyclophosphamide toxicity in the murine liver: role of glutathione and site of metabolic activation. *Hepatology* **24:** 830–837.
16. Malhi, H., *et al.* (2002). Cyclophosphamide disrupts hepatic sinusoidal endothelium and improves transplanted cell engraftment in rat liver. *Hepatology* **36:** 112–121.
17. DeLeve, L. D. (2000). Liver function and hepatotoxicity in cancer. In *Cancer Medicine* (R. C. Bast, D. W. Kufe, R. E. Pollock, R. R. Weichselbaum, J. F. Holland, and E. Frei, Eds.), pp. 2346–2358. BC Decker, Hamilton, ON.
18. Brunner, K. T., Hurez, D., McCluskey, R. T., and Benacerraf, B. (1960). Blood clearance of ^{32}P-labeled vesicular stomatitis and Newcastle disease viruses by the reticuloendothelial system in mice. *J. Immunol.* **85:** 99–105.
19. Zhang, L., Dailey, P. J., Gettie, A., Blanchard, J., and Ho, D. D. (2002). The liver is a major organ for clearing simian immunodeficiency virus in rhesus monkeys. *J. Virol.* **76:** 5271–5273.
20. Alemany, R., Suzuki, K., and Curiel, D. T. (2000). Blood clearance rates of adenovirus type 5 in mice. *J. Gen. Virol.* **81:** 2605–2609.
21. Hardonk, M. J., Dijkhuis, F. W., Hulstaert, C. E., and Koudstaal, J. (1992). Heterogeneity of rat liver and spleen macrophages in gadolinium chloride-induced elimination and repopulation. *J. Leukocyte Biol.* **52:** 296–302.
22. Lieber, A., *et al.* (1997). The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. *J. Virol.* **71:** 8798–8807.
23. Schiedner, G., Hertel, S., Johnston, M., Dries, V., van Rooijen, N., and Kochanek, S. (2003). Selective depletion or blockade of Kupffer cells leads to enhanced and prolonged hepatic transgene expression using high-capacity adenoviral vectors. *Mol. Ther.* **7:** 35–43.
24. Schauber, C. A., Tuerk, M. J., Pacheco, C. D., Escarpe, P. A., and Veres, G. (2004). Lentiviral vectors pseudotyped with baculovirus gp64 efficiently transduce mouse cells in vivo and show tropism restriction against hematopoietic cell types in vitro. *Gene Ther.* **11:** 266–275.
25. Peng, K. W., *et al.* (2001). Organ distribution of gene expression after intravenous infusion of targeted and untargeted lentiviral vectors. *Gene Ther.* **8:** 1456–1463.
26. 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.
27. Pan, D., *et al.* (2002). Biodistribution and toxicity studies of VSVG-pseudotyped lentiviral vector after intravenous administration in mice with the observation of in vivo transduction of bone marrow. *Mol. Ther.* **6:** 19–29.
28. Tominaga, K., *et al.* (2004). Repeated adenoviral administration into the biliary tract can induce repeated expression of the original gene construct in rat livers without immunosuppressive strategies. *Gut* **53:** 1167–1173.
29. Wisse, E., *et al.* (1996). Structure and function of sinusoidal lining cells in the liver. *Toxicol. Pathol.* **24:** 100–111.
30. Steffan, A. M., Gendrault, J. L., and Kirn, A. (1987). Increase in the number of fenestrae in mouse endothelial liver cells by altering the cytoskeleton with cytochalasin B. *Hepatology* **7:** 1230–1238.
31. Braet, F., and Wisse, E. (2002). Structural and functional aspects of liver sinusoidal endothelial cell fenestrae: a review. *Comp. Hepatol.* **1:** 1.
32. Braet, F., Muller, M., Vekemans, K., Wisse, E., and Le Couteur, D. G. (2003). Antimycin A-induced defenestration in rat hepatic sinusoidal endothelial cells. *Hepatology* **38:** 394–402.
33. Briggs, J. A., Wilk, T., Welker, R., Krausslich, H. G., and Fuller, S. D. (2003). Structural organization of authentic, mature HIV-1 virions and cores. *EMBO J.* **22:** 1707–1715.
34. Park, F., Ohashi, K., and Kay, M. A. (2000). Therapeutic levels of human factor VIII and IX using HIV-1-based lentiviral vectors in mouse liver. *Blood* **96:** 1173–1176.
35. Mitchell, R. S., *et al.* (2004). Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS. Biol.* **2:** E234.
36. Leffert, H. L., Koch, K. S., Lad, P. J., Shapiro, I. P., Skelly, H., and de Hemptinne, B. (1988). Hepatocyte regeneration, replication, and differentiation. In *The Liver: Biology and Pathology* (I. M. Arias, W. B. J. Jakoby, H. Popper, D. Schachter, and D. A. Shafritz, Eds.), pp. 833–850. Raven Press, New York.
37. Seppen, J., *et al.* (2000). Apical gene transfer into quiescent human and canine polarized intestinal epithelial cells by lentivirus vectors. *J. Virol.* **74:** 7642–7645.
38. Zhang, J., Huang, W., Chua, S. S., Wei, P., and Moore, D. D. (2002). Modulation of acetaminophen-induced hepatotoxicity by the xenobiotic receptor CAR. *Science* **298:** 422–424.
39. Asamoto, M., Hokaiwado, N., Murasaki, T., and Shirai, T. (2004). Connexin 32 dominant-negative mutant transgenic rats are resistant to hepatic damage by chemicals. *Hepatology* **40:** 205–210.
40. Arias, I. M., Jakoby, W. B. J., Popper, H., Schachter, D., and Shafritz, D. A. (1988). *The Liver: Biology and Pathobiology.* Raven Press, New York.
41. Mook, O. R., Van Marle, J., Vreeling-Sindelarova, H., Jonges, R., Frederiks, W. M., and Van Noorden, C. J. (2003). Visualization of early events in tumor formation of eGFP-transfected rat colon cancer cells in liver. *Hepatology* **38:** 295–304.

Chapter 3

Pseudotyping lentiviral vectors with a hybrid envelope protein strongly reduces liver macrophage gene therapy.

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Abstract

Background In rodents, systemic administration of lentiviral vectors results in poor gene transfer to hepatocytes, with the nonparenchymal liver cells preferentially transduced. Lentiviral vectors often are pseudotyped with envelope proteins capable of gene transfer to a broad range of cell types. Restricted gene transfer to hepatocytes is essential for improving transduction efficiency and long-term transgene expression.
Methods The GP64 protein from the baculovirus *Autographa californica* multiple nuclear polyhedrosisvirus has been successfully used for the surface display of amino terminal fusion proteins in baculovirus. To target gene transfer to hepatocytes, the F2 domain of the hepatocyte specific Sendai Virus fusion protein (SV-F) was fused to the amino terminus of GP64 to make a Sendai-GP64 chimeric envelope protein. Lentiviral vectors were produced with either wild type GP64, Sendai-GP64, or both wild-type GP64 and Sendai-GP64 and tested *in vitro* and *in vivo* for improved hepatocyte gene transfer.
Results The Sendai-GP64 pseudotyped lentiviral vectors showed specific gene transfer to HepG2 hepatoma cells, with no detectable transduction of the HeLa cervical carcinoma cell line. Production of lentiviral vectors with both wild type GP64 and Sendai-GP64 resulted in improved viral titers while retaining affinity for HepG2 cells. *In vivo* administration of Sendai-GP64/GP64 lentiviral vectors resulted in a significant reduction in gene transfer to nonparenchymal liver cells.
Conclusions We demonstrate that it is possible to redirect *in vivo* gene transfer of lentiviral vectors in mouse livers with a chimeric envelope protein. The GP64 envelope protein may be a potential platform for the retargeting lentiviral vectors.

Keywords: Lentiviral vectors, pseudotyping, GP64, SV-F, liver transduction

Introduction

HIV-1 derived lentiviral vectors efficiently transfer genes for stable, long-term gene expression in non-dividing cells [1,5,28]. These properties make lentiviral vectors potentially useful gene transfer vehicles for the correction of inherited disorders of metabolism in the liver. Lentiviral vectors pseudotyped with the envelope glycoprotein from the Vesicular Stomatitis Virus (VSVg) can efficiently transduce primary hepatocytes *in vitro* [18,26]. In contrast, *in vivo* lentiviral vector delivery in rodents results in poor gene transfer to hepatocytes. The nonparenchymal liver cells comprise the major fraction of liver cells transduced [4,9,20]. We have previously shown that the main target of VSVg pseudotyped lentiviral vectors are liver macrophages, the Kupffer cells [29]. Depletion of Kupffer cells leads to a significant increase in the gene transfer to hepatocytes [29].

The viral envelope protein is the primary determinant of cell tropism for lentiviral vectors. VSVg, which is commonly used to pseudotype lentiviral vectors, is capable of transducing a broad range of cells both *in vitro* [8] and *in vivo* [8,14]. Interestingly, the envelope protein from the baculovirus *Autographa californica* multiple nuclear polyhedrosisvirus, GP64, is also able to efficiently pseudotype lentiviral vectors [12]. GP64 pseudotyped lentiviral vectors exhibit comparable tropism and viral titers as that of VSVg, but with reduced cellular toxicity [12]. A further comparison of *in vivo* gene transfer of lentiviral vectors pseudotyped with either VSVg or GP64 showed comparable transduction profiles in murine livers [24]. Even though earlier reports showed that baculovirus vectors displayed a hepatocyte tropism [3,23], pseudotyping lentiviral vectors with GP64 does not appear to enhance hepatocyte gene transfer *in vivo*.

The engineering of retroviral envelope proteins for retargeting represent a challenge as modifications to viral envelope proteins often results in a significant reduction in viral titers [13,22,30]. In baculovirus, amino terminal fusions to the GP64 envelope protein have been used for the surface display of GFP [16], functional single chain antibody fragments [17], *Plasmodium berghei* circumsporozoite protein [31], avidin [21], and gp120 from HIV [2]. Recently, it was demonstrated that Decay Accelerating Factor (DAF) could be successfully fused to the amino terminus of GP64 and incorporated into lentiviral vectors leading to functional display of DAF [6]. In both baculovirus and lentiviral vectors, viral titers were slightly reduced compared to wild type GP64, but were restored to normal levels by co-expression of wild type GP64. This suggests that the GP64 envelope protein may have potential as a platform for the targeting of lentiviral vectors to hepatocytes.

The Sendai Virus Fusion (SV-F) protein utilizes a hepatocyte specific receptor for viral entry [15]. Both murine retroviral [27] and lentiviral vectors [11] could be pseudotyped with the SV-F protein, resulting in hepatocyte specific gene transfer, but viral particles were unstable and viral titers were too low to proceed to *in vivo* studies. We constructed a Sendai-GP64 fusion protein for the pseudotyping of lentiviral vectors to test for improved hepatocyte directed gene transfer.

Materials and Methods:

Construction of plasmid

The Sendai Virus Fusion cDNA was kindly provided by Dr. Allen Portner (St. Jude Children's Research Hospital) in a mammalian expression vector[19]. The GP64 cDNA was kindly provided by Marcel Westenberg, Wageningen University, and subsequently

subcloned into the pcDNA 3.1p mammalian expression vector. An amino terminal truncation of GP64 was made by PCR to remove the native signal peptide (amino acids 1-21) retaining the native GP64 sequence starting at amino acid 25 using the following primers: ClaI GP64F 5'-GATCATCGATGAACGCGCAAATGAAGACGGGT-3' and GP64R 5'-TGCTGGATATCTGCAGAATT-3'. The resulting PCR product was cloned into the pCR2.1 TOPO TA vector (Invitrogen), pCR2.1 AA25GP64 and verified by sequencing. The Sendai-GP64 fusion construct was created by digestion of pCR2.1 AA25PG64 with ClaI and EcoRV to release a 1485 fragment containing the GP64 coding sequence. This fragment was cloned into a ClaI SmaI digest of the pCAG SV-F vector to create an in-frame F2-GP64 fusion cDNA. The resulting Sendai-GP64 fusion plasmid was verified by restriction digest and sequencing.

Lentiviral vector production

Lentiviral vectors with the phosphoglycerate kinase promoter driving eGFP expression were produced as described earlier [26]. Briefly, lentiviral vectors were produced by transient transfection of 293T HEK cells using calcium phosphate precipitation. Production of mixed ratio envelope lentiviral vectors was performed using 7µg as the total amount of envelope plasmid used per plate. The different ratios of 2:1, 9:1, 29:1, and 99:1 represent the relative amounts of Sendai-GP64 to GP64 used for transfection per plate. The 29:1 ratio reflects that 6.8 µg of Sendai-GP64 plasmid and 0.2 µg of GP64 plasmid were used in the transfection mixes for lentiviral vector production. Viral supernatants were concentrated by overnight centrifugation using a Hettich centrifuge (1780g). Viral titers were determined by titration on both HeLa and HepG2 cells. Briefly transductions were performed for four hours in the presence of DEAE Dextran (10 µg/ml).

Cell lines and culturing

HEK293T, HeLa, and HepG2 cells were grown in standard DMEM media supplemented with 10% heat inactivated fetal calf serum (FCS), 2mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37° C in 10% CO_2.

Animals, viral injections, and tissue processing

Wild-type FVB male mice ages 6-8 weeks were used in all studies and were fed *ad libitum* on standard laboratory chow. All animal experiments were performed in accordance with the Animal Ethical Committee guidelines at the Academic Medical Center of Amsterdam.

Mice were anesthetized with an intraperitoneal injection of FFM mix (2.5 mg/ml Fluanisone/0.105 mg Fentanyl citrate/1.25 mg Midozalam HCl/kg in H_2O, 7ml/kg). Under deep anesthesia, the peritoneal cavity was opened and the mice were injected intraportally with the equivalent of 0.5×10^8 HepG2 transducing units (250-500ul) on day 0. The peritoneal cavity was sutured and the animals received the analgesic Temgesic (20-30 µl, 0.03 m mg/ml) subcutaneously following recovering from FFM.

On day 7, the mice were killed by *in vivo* fixation. Under deep anaesthesia, the peritoneal cavity was opened and a ligature was place around the anterior right lobe of the liver, tightened, and the lobe was excised and snap frozen in liquid nitrogen for genomic DNA analysis. Subsequently, the animals were perfused intracardially with 20 ml of phosphate buffered saline (PBS), followed by 20 ml of 2% formaldehyde in PBS. Following perfusion, the liver and spleen were removed and further fixed for 4 hours in 4% formaldehyde in PBS at room temperature. The fixed tissues were then transferred

to 30% sucrose solution and incubated overnight at 4º C, snap frozen in liquid nitrogen and stored at -80º C the following day.

Cryosections were made from both the left and medial lobes. The tissue was embedded in Tissue-Tek OCT (Bayer) and sections (6µm) were applied to poly lysine coated glass slides and enclosed in Vectashield mounting media (Vector Laboratories).

Cell counting and statistics
GFP positive cells were counted in sections made from the left and median lobes using an inverted microscope (Leica DMRA2). All sections/slides were prepared and coded independent of the counter. Per animal, one section from the left lobe and median lobe were counted for GFP positive hepatocytes at (200x). Three fields (200x) per section were counted for GFP positive nonparenchymal liver cells. Images of the counted sections were captured and the surface area of sections was calculated using Leica FW4000 software. The amount of total hepatocytes per mm^2 was estimated by counting amount of hepatocytes present in one field at (400x). Data is reported as number of GFP expressing cells per square millimeter.

Statistical analysis was performed using SPSS 11.0 software using the Mann-Whitney U test. Values were determined to be significantly different with $p < 0.05$.

Genomic DNA isolation and PCR
Genomic DNA was isolated from snap frozen liver and spleen tissue using Dneasy tissue kit (Qiagen) according to manufacturer's instructions. The following primer pairs were used to generate a 274 bp product: HIV-U3 forward primer 5'-CTGGAAGGGCTAATTCACTC-3' and HIV PSI reverse primer 5'-GGTTTCCCTTTCGCTTTCAG-3'. This primer pair is designed to specifically amplify integrated provirus and thus reduce contamination from amplification of the transfer plasmid. Additionally primers directed against GAPDH were used as a template loading control GAPDH forward primer 5'-CAATCACCATCTTCCAGGAG-3' and GAPDH reverse primer 5'-TGCCCACAGCCTTGGCAGC-3'. 100ng of total DNA was used per PCR reaction using the following conditions: 95° C for 5 minutes, followed by 33 cycles of 95° C for 30 seconds, 55° C for 30 seconds, and 72° C for seconds with a fill in at 72° C for 10 minutes. Negative control samples were taken from animals that had not been injected with virus.

Results

Pseudotyping lentiviral vectors with Sendai-GP64
The Sendai Virus Fusion protein (SV-F) is expressed as an inactive precursor protein F_0, which is cleaved by a cellular protease to a F1 and F2 chain [7,25] (Fig. 1). A fragment containing the F2 domain and fusion peptide was fused to the amino terminus of GP64 (Figure 1). The resulting fusion gene of SV-F and GP64, Sendai-GP64, was verified by both restriction fragment analysis and sequencing. Lentiviral vectors expressing GFP were produced with either the GP64 or Sendai-GP64 viral envelope proteins. Viral titers were determined on both HeLa (a human cervical carcinoma cell) and HepG2 (a liver hepatoma cell line) cells. Lentiviral vectors pseudotyped with GP64 can efficiently transduce both HeLa and HepG2 cells, while Sendai-GP64 pseudotyped lentiviral vectors were only able to transduce HepG2 cells. Viral titers determined on HepG2 cells with Sendai-GP64 vectors were approximately 2 orders of magnitude lower than those obtained with GP64 (Table 1).

To investigate expression levels and incorporation into virus particles, 293T cells were transfected with either the expression plasmid for Sendai-GP64 alone or with all the lentiviral vector plasmids. Lysates prepared from the 293T cells transfected with Sendai-GP64 alone and virus supernatant were both analyzed by western blotting. The monoclonal antibody (AcV5) directed against GP64 gave the expected band for the wild type GP64 protein, but did not react with the Sendai-GP64 recombinant protein in both cell lysates and virus supernatant (data not shown). Our GP64 fusion protein does not contain the native GP64 signal peptide and this may lead to differential posttranslational modifications abrogating binding of the AcV5 GP64 antibody. Although we were unable to detect expression of the Sendai-GP64 protein, the altered tropism of lentiviral vectors pseudotyped with Sendai-GP64 strongly suggests that this fusion protein is expressed and incorporated into virus particles.

The viral titers obtained with pseudotyping lentiviral vectors with the Sendai-GP64 envelope alone were too low to scale up for *in vivo* use. Therefore, to attempt to increase viral titers, lentiviral vectors were produced containing both GP64 and Sendai-GP64 envelope proteins. Several different ratios of Sendai-GP64 to GP64 plasmid were used for production of lentiviral vectors. The resulting viral vectors were then titered on both HeLa and HepG2 cells. A clear increase in specificity is observed as the relative amount of Sendai-GP64 is increased, which is also accompanied by a reduction in viral titers (Table 2). Based on the tested ratios of viral envelope proteins, 29:1 was selected as an optimal ratio for both improved specificity and approximately 20 fold higher viral titers as compared to Sendai-GP64 alone (Table 1).

Significant reduction in gene transfer to nonparenchymal liver cells *in vivo* with 29:1 Sendai-GP64/GP64 lentiviral vectors

Male FVB mice 6 to 8 weeks old were injected intraportally with equivalent HepG2 titers of $0.5x10^8$ TU with either GP64 (n=5) or 29:1 Sendai-GP64/GP64 (n=7) pseudotyped lentiviral vectors. One week following viral injections the mice were sacrificed and tissues were fixed *in vivo*. Liver sections were prepared from the left and medial lobes and GFP expression was directly observed using a fluorescence microscope. In the liver sections of mice injected with GP64 lentiviral vectors the majority of transduced liver cells were nonparenchymal cells (Figure 2B), as previously described [24]. Subsequent staining of these sections with the F4/80 antibody directed against a Kupffer cell (liver macrophages) marker confirmed that these cells are indeed transduced (Figures 2D-I). Strikingly, in the liver sections from 29:1 Sendai-GP64/GP64 lentiviral vector injected mice, the ratio of GFP expressing hepatocytes to nonparenchymal liver cells was much higher (Figure 2C). Counting of GFP positive cells in liver sections showed a significant reduction ($p < 0.005$) in the amount of transduced nonparenchymal liver cells in mice injected with 29:1 Sendai-GP64/GP64 lentiviral vectors (Table 3). These data show that the tropism of lentiviral vectors in liver can be altered *in vivo* through modifications to the GP64 envelope protein.

To validate the counting data, PCR amplification specific for integrated lentiviral vectors was performed on genomic DNA isolated from the liver. In the 29:1 Sendai-GP64/GP64 injected animals a weaker band is observed in liver genomic DNA, validating the results from the counting of GFP expressing cells in liver sections (Figure 3 lanes 3 and 4 versus 5 and 6). In contrast, no difference was observed in genomic DNA from spleen, which is in agreement with the GFP expression observed in spleen sections made from mice in each group (data not shown). Since the 29:1 Sendai-GP64/GP64 pseudotyped

lentiviral vectors are not exclusively targeted to hepatocytes (Table 2), it is not unexpected to observe gene transfer to other cell types. Together these data show that the Sendai-GP64 envelope results in an increased affinity for gene transfer to hepatocytes *in vivo.*

Discussion

We report the first use of an amino terminal fusion to GP64 for the detargeting of lentiviral vectors in the liver following *in vivo* administration. The F2 domain of the Sendai virus fusion protein was fused to the amino terminus of GP64 and used to pseudotype lentiviral vectors. Lentiviral vectors pseudotyped with the Sendai-GP64 fusion envelope were no longer able to transduce HeLa cells as compared to wild-type GP64, but were able to transduce HepG2 cells, with approximately 100 fold lower viral titers (Table 1). Viral titers were increased by co-production of lentiviral vectors with both wild type GP64 and Sendai-GP64 (Table 2), in agreement with previous reports of GP64 fusion proteins both in baculoviral [2] and lentiviral vectors [6]. The presence of wild type GP64 may increase incorporation of Sendai-GP64 protein into viral particles through stabilization or may restore fusion function that might be lost due to the addition of the SV-F2 domain [32]. The altered tropism in Sendai-GP64 pseudotyped lentiviral vectors show that the Sendai-GP64 is expressed and incorporated into lentiviral vectors.

In a recent report Kang *et al*. show hepatocyte specific gene transfer using GP64 pseudotyped Feline Immunodeficient Virus (FIV) derived lentiviral vectors [10]. The hepatocyte specific gene transfer was not observed in our GP64 pseudotyped HIV derived lentiviral vectors (Figure 2B and Table 3) and in a previously published comparison of VSVg and GP64 pseudotyped HIV lentiviral vectors [24]. In both cases there is significant gene transfer to nonparenchymal liver cells. Differences in lentiviral vector systems (feline versus human derived vectors), transgene marker, and delivery may in part explain these observed differences. In our hands, decreased nonparenchymal liver cell gene transfer was only observed with lentiviral vectors produced with Sendai-GP64 (Figure 2C and Table 3).

In this study we have shown that it is possible to redirect *in vivo* gene transfer through manipulations to the GP64 envelope protein. Further improvements in GP64 fusion proteins might eventually lead to hybrid envelope proteins with complete retargeting without the need to co-express wild type GP64 envelope protein.

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Table 1: Viral titers (transducing units per ml) of GP64 and Sendai-GP64 pseudotyped lentiviral vectors on HeLa and HepG2 cells

Virus	HeLa	HepG2	Specificity
GP64	$4.2x10^5 \pm 3.1x10^5$	$2.6x10^6 \pm 1.6x10^6$	6.1
Sendai-GP64	UD [a]	$2.7x10^4 \pm 1.3x10^4$	> 27

[a] Undetectable with detection limit of viral titers set to $1x10^3$ TU/ml with flow cytometry. No GFP expressing cells were observed using a fluorescent microscope. Viral titers were determined using unconcentrated lentiviral vectors from at least three different virus preparations.

Table 2. Viral titers (transducing units per ml) of different ratios of Sendai-GP64 to wild type GP64 lentiviral vectors on HeLa or HepG2 cells

Virus	HeLa	HepG2	Specificity
2:1	$2.2x10^5 \pm 1.7x10^5$	$1.6x10^6 \pm 1.2x10^6$	7.4
9:1	$4.5x10^4 \pm 2.2x10^4$	$7.1x10^5 \pm 2.1x10^5$	15.8
29:1	$1.8x10^4 \pm 1.1x10^4$	$5.1x10^5 \pm 2.7x10^5$	28.9
99:1	$2.5.10^3 \pm 1.5x10^3$	$7.7x10^4 \pm 1.6x10^4$	30.7

2:1, 9:1, 29:1, 99:1 refer to the relative amount of Sendai-GP64 to GP64 plasmid used for transfection during virus production. For example for the standard 7µg of envelope plasmid used for virus production 2:1 represents 4.6µg of Sendai-GP64 and 2.4µg GP64 plasmid respectively. Viral titers were determined using unconcentrated lentiviral vectors from at least three different virus preparations.

Table 3 GFP positive cells in liver sections

Lentiviral Vector	Hepatocytes/mm^2	% GFP positive hepatocytes	NPC/mm^2
GP64 (n=5)	9.9 ± 2.7	0.6 ± 0.2	79.3 ± 12.1
29:1 (n=7)	9.5 ± 4.7	0.5 ± 0.3	24.6 ± 19.6 [a]

$0.5x10^8$ HepG2 transducing of each virus were administered by portal vein injection. Frozen sections were prepared from fixed liver, one section from the medial and one from the left lobe were counted per animal. % GFP positive hepatocytes represents the percent of total liver hepatocytes transduced, NPC (nonparenchymal liver cells), 29:1 (Sendai-GP64:GP64).
[a] represents a significant difference ($p < 0.005$) between GP64 and 29:1 Sendai-GP64/GP64 transduced animals

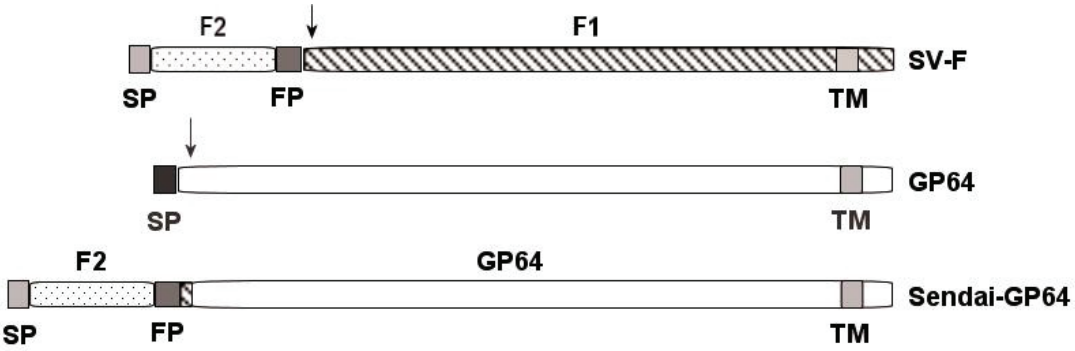

Figure 1. Linear map of the Sendai Virus Fusion (SV-F), GP64, and the Sendai-GP64 glycoproteins with selected domains indicated: SP: Signal Peptide, FP: Fusion peptide, TM: Transmembrane.

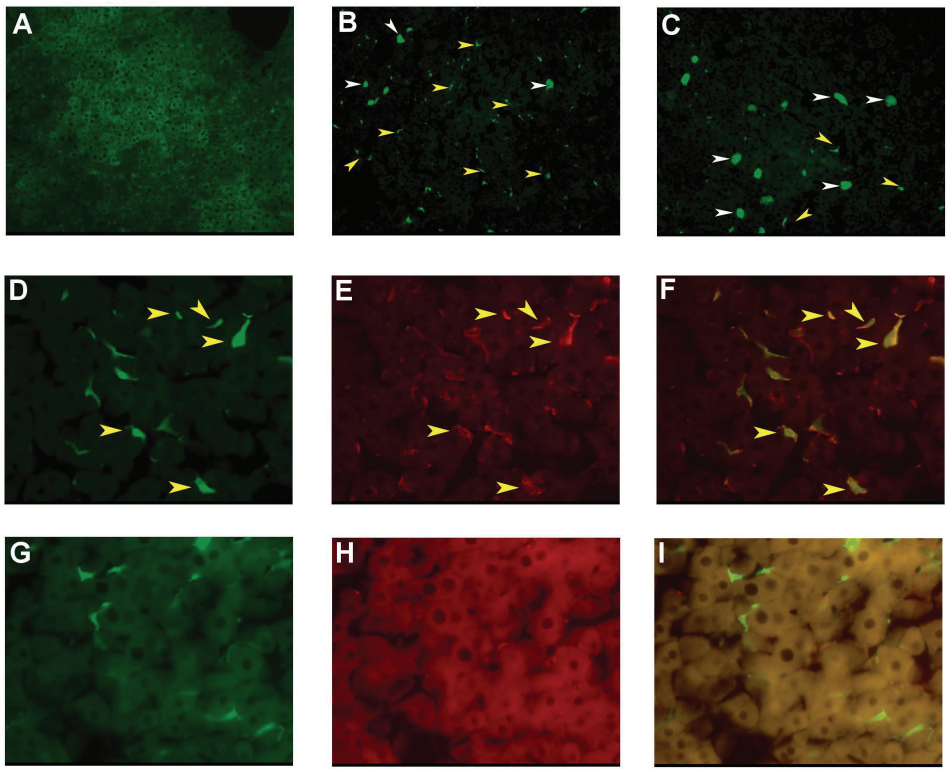

Figure 2. Expression of GFP in the liver following portal vein injection of either GP64 or Sendai-GP64/GP64 (29:1) lentiviral vectors. Representative sections from the livers of (A) control (B) GP64, and (C) 29:1 Sendai-GP64/GP64 transduced mice at 100x magnification. White arrows indicate hepatocytes and yellow arrows indicate nonparenchymal liver cells. Serial liver sections from a GP64 transduced mouse were stained with an antibody directed against a Kupffer cell marker. (D) transduced cells expressing GFP (E) stained with the F4/80 antibody directed against Kupffer cells (F) merge of GFP expressing cells and Kupffer cell staining. Control staining where the primary antibody was not used (G) GFP expressing cells (H) Texas red secondary antibody (I) merge. (D-I images are at 400× magnification).

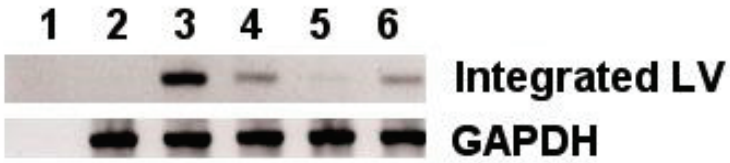

Figure 3. PCR specific for integrated lentiviral vectors and GAPDH performed on genomic DNA isolated from liver. Lane 1 H_2O control, 2 control liver DNA, 3 Mouse 1 (GP64), 4 Mouse 2 (GP64), 5 Mouse 4 (29:1), 6 Mouse 5 (29:1).

References

1. **Ailles, L. E. and L. Naldini**. 2002. HIV-1-derived lentiviral vectors. Curr. Top. Microbiol. Immunol. **261**:31-52.
2. **Boublik, Y., P. Di Bonito, and I. M. Jones**. 1995. Eukaryotic virus display: engineering the major surface glycoprotein of the Autographa californica nuclear polyhedrosis virus (AcNPV) for the presentation of foreign proteins on the virus surface. Biotechnology (N. Y.) **13**:1079-1084.
3. **Boyce, F. M. and N. L. Bucher**. 1996. Baculovirus-mediated gene transfer into mammalian cells. Proc Natl Acad Sci U S A **93**:2348-52.
4. **Follenzi, A., G. Sabatino, A. Lombardo, C. Boccaccio, and L. Naldini**. 2002. Efficient gene delivery and targeted expression to hepatocytes in vivo by improved lentiviral vectors. Hum Gene Ther **13**:243-60.
5. **Galimi, F. and I. M. Verma**. 2002. Opportunities for the use of lentiviral vectors in human gene therapy. Curr. Top. Microbiol. Immunol. **261**:245-254.
6. **Guibinga, G. H. and T. Friedmann**. 2005. Baculovirus GP64-pseudotyped HIV-based lentivirus vectors are stabilized against complement inactivation by codisplay of decay accelerating factor (DAF) or of a GP64-DAF fusion protein. Mol. Ther **11**:645-651.
7. **Homma, M. and M. Ouchi**. 1973. Trypsin action on the growth of Sendai virus in tissue culture cells. 3. Structural difference of Sendai viruses grown in eggs and tissue culture cells. J. Virol. **12**:1457-1465.
8. **Kahl, C. A., K. Pollok, L. S. Haneline, and K. Cornetta**. 2005. Lentiviral vectors pseudotyped with glycoproteins from Ross River and vesicular stomatitis viruses: variable transduction related to cell type and culture conditions. Mol. Ther **11**:470-482.
9. **Kang, Y., C. S. Stein, J. A. Heth, P. L. Sinn, A. K. Penisten, P. D. Staber, K. L. Ratliff, H. Shen, C. K. Barker, I. Martins, C. M. Sharkey, D. A. Sanders, P. B. McCray, Jr., and B. L. Davidson**. 2002. In vivo gene transfer using a nonprimate lentiviral vector pseudotyped with Ross River Virus glycoproteins. J Virol **76**:9378-88.
10. **Kang, Y., L. Xie, D. T. Tran, C. S. Stein, M. Hickey, B. L. Davidson, and P. B. McCray, Jr.** 2005. Persistent expression of factor VIII in vivo following nonprimate lentiviral gene transfer. Blood **106**:1552-1558.
11. **Kowolik, C. M. and J. K. Yee**. 2002. Preferential transduction of human hepatocytes with lentiviral vectors pseudotyped by Sendai virus F protein. Mol Ther **5**:762-9.
12. **Kumar, M., B. P. Bradow, and J. Zimmerberg**. 2003. Large-Scale Production of Pseudotyped Lentiviral Vectors Using Baculovirus GP64. Hum Gene Ther **14**:67-77.
13. **Lavillette, D., S. J. Russell, and F. L. Cosset**. 2001. Retargeting gene delivery using surface-engineered retroviral vector particles. Curr. Opin. Biotechnol. **12**:461-466.
14. **MacKenzie, T. C., G. P. Kobinger, N. A. Kootstra, A. Radu, M. Sena-Esteves, S. Bouchard, J. M. Wilson, I. M. Verma, and A. W. Flake**. 2002. Efficient transduction of liver and muscle after in utero injection of lentiviral vectors with different pseudotypes. Mol Ther **6**:349-58.
15. **Markwell, M. A., A. Portner, and A. L. Schwartz**. 1985. An alternative route of infection for viruses: entry by means of the asialoglycoprotein receptor of a Sendai virus mutant lacking its attachment protein. Proc Natl Acad Sci U S A **82**:978-82.
16. **Mottershead, D., d. L. van, I, C. H. von Bonsdorff, K. Keinanen, and C. Oker-Blom**. 1997. Baculoviral display of the green fluorescent protein and rubella virus envelope proteins. Biochem. Biophys. Res. Commun. **238**:717-722.
17. **Mottershead, D. G., K. Alfthan, K. Ojala, K. Takkinen, and C. Oker-Blom**. 2000. Baculoviral display of functional scFv and synthetic IgG-binding domains. Biochem. Biophys. Res. Commun. **275**:84-90.
18. **Nguyen, T. H., J. Oberholzer, J. Birraux, P. Majno, P. Morel, and D. Trono**. 2002. Highly efficient lentiviral vector-mediated transduction of nondividing, fully reimplantable primary hepatocytes. Mol Ther **6**:199-209.
19. **Niwa, H., K. Yamamura, and J. Miyazaki**. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108**:193-199.
20. **Pfeifer, A., T. Kessler, M. Yang, E. Baranov, N. Kootstra, D. A. Cheresh, R. M. Hoffman, and I. M. Verma**. 2001. Transduction of liver cells by lentiviral vectors: analysis in living animals by fluorescence imaging. Mol. Ther **3**:319-322.
21. **Raty, J. K., K. J. Airenne, A. T. Marttila, V. Marjomaki, V. P. Hytonen, P. Lehtolainen, O. H. Laitinen, A. J. Mahonen, M. S. Kulomaa, and S. Yla-Herttuala**. 2004. Enhanced gene delivery by avidin-displaying baculovirus. Mol. Ther **9**:282-291.
22. **Russell, S. J. and F. L. Cosset**. 1999. Modifying the host range properties of retroviral vectors. J. Gene Med. **1**:300-311.
23. **Sandig, V., C. Hofmann, S. Steinert, G. Jennings, P. Schlag, and M. Strauss**. 1996. Gene transfer into hepatocytes and human liver tissue by baculovirus vectors. Hum. Gene Ther **7**:1937-1945.
24. **Schauber, C. A., M. J. Tuerk, C. D. Pacheco, P. A. Escarpe, and G. Veres**. 2004. Lentiviral vectors pseudotyped with baculovirus gp64 efficiently transduce mouse cells in vivo and show tropism restriction against hematopoietic cell types in vitro. Gene Ther **11**:266-275.
25. **Scheid, A. and P. W. Choppin**. 1974. Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity of proteolytic cleavage of an inactive precursor protein of Sendai virus. Virology **57**:475-490.
26. **Seppen, J., M. Rijnberg, M. P. Cooreman, and R. P. Oude Elferink**. 2002. Lentiviral vectors for efficient transduction of isolated primary quiescent hepatocytes. J Hepatol **36**:459-65.
27. **Spiegel, M., M. Bitzer, A. Schenk, H. Rossmann, W. J. Neubert, U. Seidler, M. Gregor, and U. Lauer**. 1998. Pseudotype formation of Moloney murine leukemia virus with Sendai virus glycoprotein F. J Virol **72**:5296-302.
28. **Trono, D.** 2000. Lentiviral vectors: turning a deadly foe into a therapeutic agent. Gene Ther **7**:20-23.

29. **van Til, N. P., D. M. Markusic, R. R. van der, C. Kunne, J. K. Hiralall, H. Vreeling, W. M. Frederiks, R. P. Oude-Elferink, and J. Seppen**. 2005. Kupffer cells and not liver sinusoidal endothelial cells prevent lentiviral transduction of hepatocytes. Mol. Ther **11**:26-34.
30. **Verhoeyen, E. and F. L. Cosset**. 2004. Surface-engineering of lentiviral vectors. J. Gene Med. **6 Suppl 1**:S83-S94.
31. **Yoshida, S., D. Kondoh, E. Arai, H. Matsuoka, C. Seki, T. Tanaka, M. Okada, and A. Ishii**. 2003. Baculovirus virions displaying Plasmodium berghei circumsporozoite protein protect mice against malaria sporozoite infection. Virology **316**:161-170.
32. **Zhao, Y., L. Zhu, S. Lee, L. Li, E. Chang, N. W. Soong, D. Douer, and W. F. Anderson**. 1999. Identification of the block in targeted retroviral-mediated gene transfer. Proc. Natl. Acad. Sci. U. S. A **96**:4005-4010.

Chapter 4

Immune response to lentiviral bilirubin UDP-glucuronyltransferase gene transfer in fetal and neonatal rats.

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ORIGINAL ARTICLE

Immune response to lentiviral bilirubin UDP-glucuronosyltransferase gene transfer in fetal and neonatal rats

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Gene therapy for inherited disorders might cause an immune response to the therapeutic protein. A solution would be to introduce the gene in the fetal or neonatal period, which should lead to tolerization. Lentiviral vectors mediate long-term gene expression, and are well suited for gene therapy early in development. A model for fetal or neonatal gene therapy is the inherited disorder of bilirubin metabolism, Crigler–Najjar disease (CN). The absence of bilirubin UDP-glucoronyltransferase (UGT1A1) activity in CN patients causes high serum levels of unconjugated bilirubin and brain damage in infancy. CN is attractive for the development of gene therapy because the mutant Gunn rat closely mimics the human disease. Injection of UGT1A1 lentiviral vectors corrected the hyperbilirubinemia for more than a year in rats injected as fetuses and for up to 18 weeks in rats injected the day of birth. UGT1A1 gene transfer was confirmed by the presence of bilirubin glucuronides in bile. All animals injected with UGT1A1 lentiviral vectors developed antibodies to UGT1A1. Animals injected with green fluorescent protein (GFP) lentiviral vectors did not develop antibodies to GFP. Our results indicate that fetal and neonatal gene therapy with immunogenic proteins such as UGT1A1 does not necessarily lead to tolerization.
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Keywords: in utero*; Crigler–Najjar; lentiviral vectors; bilirubin; Gunn rat*

Introduction

A fundamental problem with gene therapy for inherited deficiencies is the potential of an immune response against the novel therapeutic gene product. Correction of deficiency could be short lived because the therapeutic transgene is seen as a foreign antigen by the immune system. This may cause the primed immune system to subsequently eliminate the corrected cells. An immune response to therapeutic transgenes has indeed been observed in a number of gene therapy studies.[1,2] A potential solution to this problem would be to exploit the tolerization that occurs to antigens that are introduced in the fetal and early neonatal period. Tolerization early in development has been well established, the first evidence of this phenomenon was provided by the landmark experiments performed by Billingham *et al*.[3] These studies showed that mice that were injected with heterologous cells shortly after birth, subsequently accepted skin grafts from the donor of the cells. Many experiments since have firmly established the induction of tolerance in the neonatal period.[4] These considerations have led to the development of gene therapy in fetal and neonatal animal models.[5–7] Several studies have established that fetal gene transfer can tolerize mice to foreign therapeutic proteins. Adenoviral and lentiviral clotting factor IX expression vectors were administered to fetal mice. When these mice were challenged with factor IX as adults, the immune response was absent or strongly reduced as compared to untreated mice.[8,9]

Our aim is to develop gene therapy for the severe inherited unconjugated hyperbilirubinemia in patients with Crigler–Najjar disease. Bilirubin is the breakdown product of the heme group of hemoglobin and other heme utilizing enzymes such as cytochrome *P*450s. As bilirubin is hydrophobic, it needs to be glucuronidated by the hepatic enzyme bilirubin UDP glucuronyltransferase (UGT1A1) before it can be excreted into bile.[10,11] Patients with Crigler–Najjar type 1 (CN) have no detectable UGT1A1 activity and therefore have high serum levels of unconjugated bilirubin directly after birth.[12] Unconjugated bilirubin is highly neurotoxic, UGT1A1 deficiency leads to brain damage and death if not treated.[13] The only permanent treatment option for patients with CN is liver transplantation. The Gunn rat is a natural mutant that has no UGT1A1 activity and is therefore a good model for CN[14] and the development of fetal gene therapy for this disease. Tolerization to UGT1A1 is especially important because earlier studies have shown that UGT1A1 gene transfer in adult Gunn rats induces a strong humoral and cellular response to UGT1A1.[15]

Gene therapy for inherited disorders requires long-term expression of the therapeutic transgene. Lentiviral vectors are retroviral vectors based on HIV and are well suited for the development of gene therapy for these

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disorders because they can efficiently transduce a wide variety of cell types *in vivo* and mediate long-term expression.[16–18]

We have previously injected fetal Gunn rats with UGT1A1 lentiviral vectors and showed correction of hyperbilirubinemia.[19] When we injected similar doses of lentiviral vectors in neonatal Gunn rats, we observed a correction of hyperbilirubinemia that slowly decreased over time. This decrease in therapeutic effect was accompanied by the appearance of antibodies against human UGT1A1. When sera from *in utero* injected animals were tested retrospectively, we also found high titers of UGT1A1 antibodies.

Surprisingly, injection of GFP lentiviral vectors into fetal and neonatal animals did not lead to the formation of antibodies to GFP.

Our results therefore indicate that induction of tolerance is not necessarily the outcome of fetal or neonatal gene therapy. Furthermore, our results also suggest that UGT1A1 is a highly immunogenic protein which might explain the presence of UGT1A1 autoantibodies in autoimmune hepatitis.

Results

Correction of hyperbilirubinemia

We have previously demonstrated that administration of UGT1A1 lentiviral vectors to fetal Gunn rats corrected their deficiency. We now report that the difference in serum bilirubin between control and injected animals gradually disappears with loss of significance after 60 weeks (Figure 1a). Injection of UGT1A1 lentiviral vectors in neonatal Gunn rats (Figure 1b) led to a similar correction of hyperbilirubinemia that disappeared more rapidly with loss of significance at 18 weeks.

Demonstration of gene transfer

Bile analysis confirms that neonatal injection of UGT1A1 lentiviral vectors leads to functional expression of UGT1A1 (Figure 2). However, as we have previously shown in fetally injected rats, the overall transduction efficiency was low. Qualitative PCR on chromosomal DNA confirmed the presence of viral integrations in liver, pancreas, spleen and intestine (not shown). All organs contained less than 1% of viral integrations, which precluded quantitative determination by real-time PCR.

Periferal blood mononuclear cells of animals injected with GFP lentiviral vectors contain a low percentage of GFP positive cells (not shown). Cryosections from animals injected neonatally with GFP lentiviral vectors shows that GFP expression in liver and pancreas persist for more than 5 months (Figure 3). Clusters of GFP positive cells were seen in pancreas and liver. Counting of at least three microscopic fields revealed that the total number of positive cells was below 1%, confirming our PCR data. Interestingly, only GFP positive hepatocytes were seen. This is in contrast to the preferential transduction of Kupffer cells by lentiviral vectors injected in the portal vein of adult mice.[20]

Antibodies to UGT1A1

High titers of antibodies to UGT1A1 were detected by ELISA in sera from rats injected in the fetal and neonatal period (Figure 4). The antibodies were present at the first time point sampled, 4 weeks at the time of weaning. Titers increased and reached a plateau that was

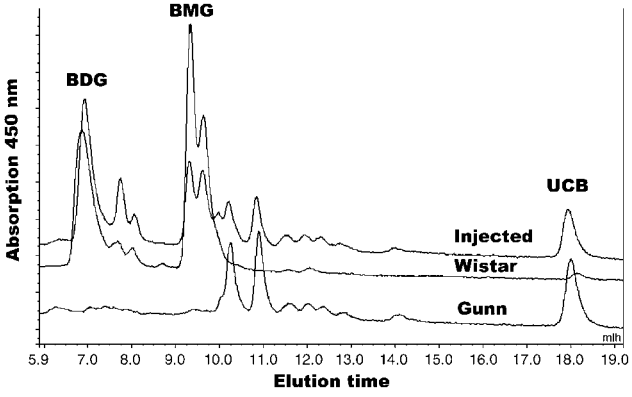

Figure 2 Bile analysis. HPLC chromatograms of bile samples from a normal rat, a Gunn rat injected with UGT1A1 lentiviral vector at the day of birth and an untreated control Gunn rat. BDG, bilirubin di glucuronides. BMG, bilirubin mono glucuronides. UCB, unconjugated bilirubin. Gunn rats have only unconjugated bilirubin and all bilirubin in Wistar rats is conjugated. Injected Gunn rats have an intermediate pattern of bilirubin glucuronidation which confirms functional expression of UGT1A1.

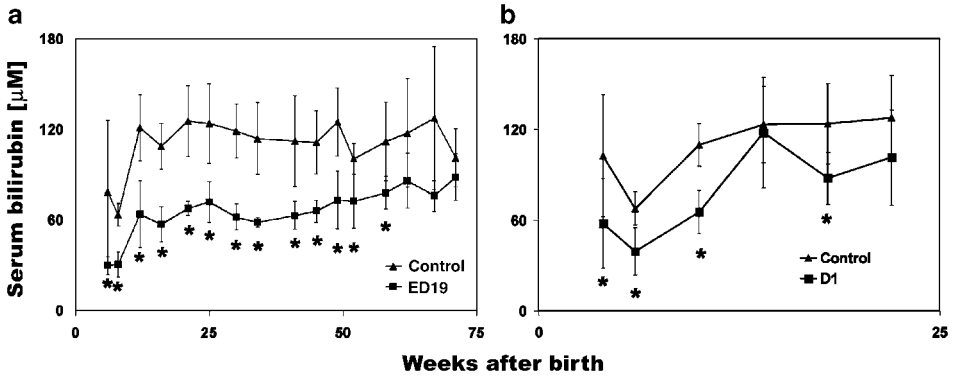

Figure 1 (**a**, **b**) Correction of serum bilirubin levels. (**a**) Injection of lentiviral UGT1A1 vectors at embryonic day 19 (ED19) corrected hyperbilirubinemia significantly for up to 60 weeks ($N=6$). (**b**) Injection at the day of birth (D1) led to a correction for up to 18 weeks ($N=9$) (*$P<0.05$ by Student's *t*-test).

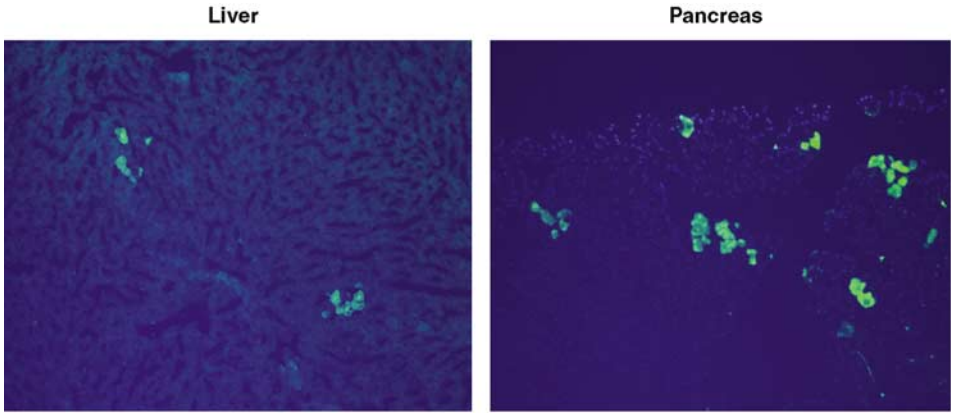

Figure 3 GFP expression in liver and pancreas 5 months after injection of virus. Cryosections from animals injected neonatally with GFP lentiviral vectors show clusters of positive cells in pancreas (right) and liver (left) 5 months after injection. Clusters of GFP positive cells were not more abundant than 1%, confirming our PCR data.

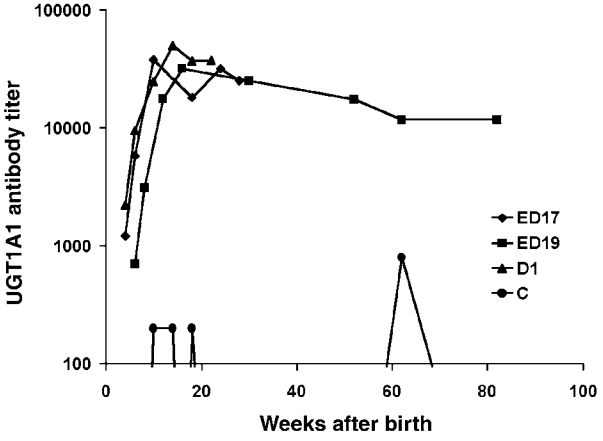

Figure 4 Detection of UGT1A1 antibodies by ELISA. Antibodies to UGT1A1 in rats injected on embryonic day 17 (ED17), 19 (ED19) and the day of birth (D1). No UGT1A1 antibodies were seen in control rats (**c**). Values are averages of two rats.

maintained for up to 2 years in the *in utero* injected animals. A similar response was seen in animals injected at embryonic day 17 and 19. Control animals injected with GFP lentiviral vectors in the fetal and neonatal period did not develop antibodies to GFP as measured by ELISA.

The specificity of the UGT1A1 antibodies was confirmed by Western blotting (Figure 5). A band of 55 kDa was only seen when blots of cell lysates containing UGT1A1 were probed with serum from Gunn rats previously injected with UGT1A1 lentiviral vectors. The sera from UGT1A1 vector injected rats did not react with cell lysates containing GFP. Sera from Gunn rats injected with GFP lentiviral vectors did not contain antibodies reactive with GFP on Western blot, confirming the negative ELISA results.

Discussion

The strong immune response to lentivirally delivered UGT1A1 in fetal and neonatal rats contrasts with numerous observations,[7] including studies in Gunn rats,[21] that gene transfer in fetal and neonatal animals leads to tolerization to the transgene. An immune response to GFP has been observed in adult mice,[22] rats[23] and baboons,[24] which suggests that GFP is an immunogenic protein. We did not observe antibodies to GFP in animals injected *in utero*, or at the day of birth, with GFP vectors. Expression of GFP in liver and pancreas was sustained for more than 5 months in these animals, excluding a cellular immune response to GFP as well. This result was expected since a number of studies have shown that neonatal rats will only mount an immune response to exogenously administered antigens when they are at least 8 days old.[25,26]

A small number of studies have described immune responses in fetuses: fetal baboons could be immunized to hepatitis B virus by injection of an HBV vaccine,[27] an immune response to CMV was observed in prenatal children infected with this virus[28] and low titers of antibodies to beta galactosidase were observed after *in utero* injection of AAV or adenoviral beta galactosidase vectors in mice.[29]

As fetal and neonatal rats do not mount an immune response to GFP but do respond to UGT1A1, a likely explanation of our results is that UGT1A1 is a highly immunogenic protein. Autoimmune hepatitis type 2 (AIH) is a disease of unknown origin which is characterized by the presence of liver kidney microsomal (LKM) antibodies. In 10% of AIH patients, these antibodies are directed against UDP glucuronosyltranferase 1 (UGT1).[30] Little is known about the role of these antibodies and whether they are the primary cause for AIH. The highly immunogenic nature of UGT1A1 could explain why UGT1 autoantibodies are present in patients with autoimmune hepatitis.

In this study the human UGT1A1 gene was used. As the Gunn rat does not express rat UGT1A1 protein,[31] the human and rat isoforms are both foreign to the rat immune system. However, it remains possible that the xenogenic human UGT1A1 protein elicits a stronger immune response than endogenous rat UGT1A1.

The mechanism behind the slow decline in therapeutic efficiency of lentiviral UGT1A1 gene transfer is unclear. We investigated whether the immune response to

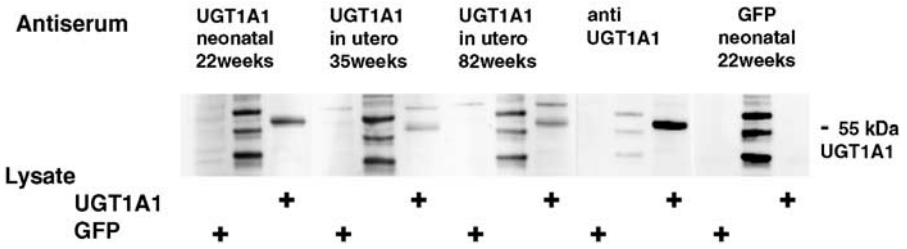

Figure 5 Western blotting shows specificity of UGT1A1 antibodies. Lysates containing UGT1A1 or GFP were probed with serum from rats injected with UGT1A1 or GFP lentiviral vectors. Middle lanes are the molecular mass markers. Sera from UGT1A1 vector injected rats react with UGT1A1 containing lysates only. An antibody to UGT1A1 was included as positive control. GFP antibodies were not detected in sera from rats injected with GFP lentiviral vectors.

UGT1A1 contributed to this decline but were not able to show infiltrating cytotoxic, CD8 positive, T cells in liver and pancreas of animals injected with UGT1A1 vectors in the neonatal period (not shown). However, these results should be considered with some caution. As the transduction efficiency of these organs was low, the amount of infiltrating T cells would likely also be low and may have precluded their detection. UGT1A1 is a resident endoplasmic reticulum membrane protein. A humoral response is not likely to affect expression of such an intracellular protein. However, a small amount of UGT1A1 might be exposed on the plasma membrane.[32] This exposed UGT could be recognized by the circulating antibodies, which would lead to immune elimination of the opsonized cells. This study extends earlier reports that lentiviral gene transfer can be highly immunogenic,[33] possibly because antigen-presenting cells are readily transduced by these vectors.[34] A solution to this problem would be to use tissue-specific promoters and thus prevent expression in antigen-presenting cells.[35] Indeed, a recent paper documents that liver-specific lentiviral UGT1A1 gene transfer in neonatal Gunn rats does not elicit a cytotoxic immune response[36] and mediates long-term correction of UGT1A1 deficiency. However, the presence of UGT1A1 antibodies was not investigated in this study.

Because we do now show that lentiviral UGT1A1 gene transfer is very immunogenic, it is necessary to rigorously test the absence of an immune response to UGT1A1 in animal studies before lentiviral vectors can be used in gene therapy trials for Crigler–Najjar disease.

Materials and methods

Construction and production of lentiviral vectors

An improved third generation self-inactivating lentiviral vector system was the basis of our lentiviral vector constructs and was used as described.[19] To generate PGKUGT1A1, the human UGT1A1 cDNA was cloned as a *Spe*I fragment into the *Xba*I site of the lentiviral transfer vector PGKGFP replacing the GFP gene of this vector. In these vectors, the phosphoglycerate kinase (PGK) promoter is driving expression of the transgene.

Lentiviral vectors were generated by cotransfection of 293T cells and concentrated as described.[19]

Lentiviral vectors were titrated by transducing 10^5 HeLa cells with dilutions of vector stock and determining the percentage of positive cells by flow cytometry. Intracellular flow cytometric detection of UGT1A1 was performed as follows. Transduced HeLa cells were trypsinized and fixed for 30′ at 4°C with 0.25% formaldehyde and permeabilized with 0.2% Tween for 15′ at 37°C. The cells were incubated with a monoclonal antibody WP1[37] that reacts with UGT1A1, and the monoclonal antibody was subsequently detected with FITC conjugated goat anti mouse immunoglobulins (Jackson ImmunoResearch). Cells were washed and fixed with 2% formaldehyde and analyzed on a FACScalibur flow cytometer (Becton Dickinson).

Animal experiments

All animal experiments were approved by the AMC animal ethical committee. Timed pregnancies in Gunn rats were initiated and the day after the copulation was considered embryonic day 1.

Injection of virus in fetal Gunn rats were performed by anesthesis of pregnant Gunn rats by intramuscular injection of KAR mix: 4 ml ketamine (100 mg/ml), 2 ml Rompun (xylazine 20 mg/ml), 1 ml atropine (1 mg/ml), dose: 0.1 ml/100 g bodyweight. The abdomen was opened by an incision of approximately 5 cm and the uterine horns were exposed. The virus was injected, in a volume of 75 μl, with a 27 gauge needle. Injections were aimed at the liver, visible as a large dark spot, and peritoneal cavity. The animals were closed by suturing the abdominal wall and skin and the pregnancy was carried to term in a normal manner. Injection of virus in neonatal Gunn rats was performed within half a day after birth of the pups, injections were aimed at the liver.

Blood was collected by tail vein puncture under gas anesthesia in pediatric heparin tubes. Peripheral blood mononuclear cells were isolated for flow cytometry by lysing the whole blood using FACS lysing solution (Becton Dickinson) and washing twice with PBS. For bile collection rats were anesthesized by intraperitoneal injection of KAR mix as described above and bile was collected by canulation of the bile duct as described.[38]

ELISA for rat antibodies to UGT1A1 and GFP

UGT1A1 and GFP were expressed in 293T cells by calcium phosphate coprecipitation. Expression of UGT1A1 was confirmed by Western blotting and expression of GFP was confirmed by fluorescence microscopy and Western blotting. ELISA plates (Nunc) were coated overnight with 5 μg cellular lysate per well in 50 mM carbonate buffer pH 9.6. The wells were blocked with 1% gelatin in phosphate-buffered saline, washed and incubated with serial dilutions of Gunn

rat plasma. After washing, rat immunoglobins were detected with anti-rat IgG peroxidase (Nordic) and o-phenylenediamine tablets (Sigma).

ELISA's were always performed in duplicate with the same samples applied on UGT1A1 and GFP coated plates. Titers of antibodies were set as the dilution were the absorption of the well was twice the absorption of control wells without rat serum. For the determination of UGT1A1 antibody titers, the titer of the same sample on the GFP coated plate was subtracted to correct for aspecific binding.

Western blotting

Western blots were incubated with rat serum that had been diluted 500 times. The UGT1A1 antibody WP1 served as a positive control.[37] Antibodies were detected with anti mouse peroxidase (Biorad) or anti-rat peroxidase (Nordic) and ECL chemiluminescence reagent (Amersham-Pharmacia). Images were recorded using a lumi imager (Roche).

Analysis of genomic DNA

DNA of organs was isolated and used for PCR as described.[19] To reduce the risk of contamination, a forward primer in the U3 region (ctggaagggctaattcactc) and a reverse primer in the packaging region (ggtttccctttcgctttcag) were selected for amplification. This primer pair only amplifies the integrated provirus and not the transfer vector.

Quantitative PCR was performed on a lightcycler using FastStart DNA master SYBR Green I hot start reaction mix (Roche Applied Science). Primers specific for human UGT1A1 that did not amplify rat UGT1A1 were used. Forward: TTCAGAGGACGTGCAGACAG, reverse: CAAGGTGGCACCTATGAAGC. For calibration, dilutions of human genomic DNA were made in rat genomic DNA. After amplification, samples were retrieved from the light-cycler capilaries and electrophoresed on an agarose gel. Quantification was performed using software provided by the manufacturer. The detection limit of this method was 1%: samples that had a single amplification product on gel and amplification slopes and melting curves identical to the standards always contained more than 1% of human UGT1A1.

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References

1 Nguyen TH, Ferry N. Liver gene therapy: advances and hurdles. *Gene Therapy* 2004; **11**: S76–S84.
2 Wang CH, Liu DW, Tsao YP, Xiao X, Chen SL. Can genes transduced by adeno-associated virus vectors elicit or evade an immune response? *Arch Virol* 2004; **149**: 1–15.
3 Billingham RE, Brent L, Medawar PB. Actively aquired tolerance of foreign cells. *Nature* 1953; **172**: 603–606.
4 Nossal GJ. Cellular mechanisms of immunologic tolerance. *Annu Rev Immunol* 1983; **1**: 33–62.
5 Zanjani ED, Anderson WF. Review: Medicine – Prospects for *in utero* human gene therapy. *Science* 1999; **285**: 2084–2088.
6 Coutelle C, Themis M, Waddington S, Gregory L, Nivsarkar M, Buckley S *et al*. The hopes and fears of *in utero* gene therapy for genetic disease – a review. *Placenta* 2003; **24**: S114–S121.
7 Waddington SN, Kramer MG, Hernandez-Alcoceba R, Buckley SM, Themis M, Coutelle C *et al*. *In utero* gene therapy: current challenges and perspectives. *Mol Ther* 2005; **11**: 661–676.
8 Waddington SN, Buckley SM, Nivsarkar M, Jezzard S, Schneider H, Dahse T *et al*. *In utero* gene transfer of human factor IX to fetal mice can induce postnatal tolerance of the exogenous clotting factor. *Blood* 2003; **101**: 1359–1366.
9 Waddington SN, Nivsarkar MS, Mistry AR, Buckley SM, Kemball-Cook G, Mosley KL *et al*. Permanent phenotypic correction of hemophilia B in immunocompetent mice by prenatal gene therapy. *Blood* 2004; **104**: 2714–2721.
10 Ostrow JD, Mukerjee P, Tiribelli C. Structure and binding of unconjugated bilirubin: relevance for physiological and pathophysiological function. *J Lipid Res* 1994; **35**: 1715–1737.
11 Tukey RH, Strassburg CP. Human UDP-glucuronosyltransferases: metabolism, expression, and disease. *Annu Rev Pharmacol Toxicol* 2000; **40**: 581–616.
12 Jansen PL. Diagnosis and management of Crigler–Najjar syndrome. *Eur J Pediatr* 1999; **158** (Suppl 2): S89–S94.
13 Ostrow JD, Pascolo L, Tiribelli C. Mechanisms of bilirubin neurotoxicity. *Hepatology* 2002; **35**: 1277–1280.
14 Chowdhury JR, Kondapalli R, Chowdhury NR. Gunn rat: a model for inherited deficiency of bilirubin glucuronidation. *Adv Vet Sci Comp Med* 1993; **37**: 149–173.
15 Aubert D, Menoret S, Chiari E, Pichard V, Durand S, Tesson L *et al*. Cytotoxic immune response blunts long-term transgene expression after efficient retroviral-mediated hepatic gene transfer in rat. *Mol Ther* 2002; **5**: 388–396.
16 Ailles LE, Naldini L. HIV-1-derived lentiviral vectors. *Curr Top Microbiol Immunol* 2002; **261**: 31–52.
17 Galimi F, Verma IM. Opportunities for the use of lentiviral vectors in human gene therapy. *Curr Top Microbiol Immunol* 2002; **261**: 245–254.
18 Trono D. Lentiviral vectors: turning a deadly foe into a therapeutic agent. *Gene Therapy* 2000; **7**: 20–23.
19 Seppen J, van der RR, Looije N, van Til NP, Lamers WH, Oude Elferink RP. Long-term correction of bilirubin UDPglucuronyltransferase deficiency in rats by *in utero* lentiviral gene transfer. *Mol Ther* 2003; **8**: 593–599.
20 van Til NP, Markusic DM, van der Rijt R, Kunne C, Hiralall JK, Vreeling H *et al*. Kupffer cells and not liver sinusoidal endothelial cells prevent lentiviral transduction of hepatocytes. *Mol Ther* 2005; **11**: 26–34.
21 Takahashi M, Ilan Y, Chowdhury NR, Guida J, Horwitz M, Chowdhury JR. Long term correction of bilirubin-UDP-glucuronosyltransferase deficiency in Gunn rats by administration of a recombinant adenovirus during the neonatal period. *J Biol Chem* 1996; **271**: 26536–26542.
22 Stripecke R, Villacres MD, Skelton DC, Satake N, Halene S, Kohn DB. Immune response to green fluorescent protein: implications for gene therapy. *Gene Therapy* 1999; **6**: 1305–1312.
23 Inoue H, Ohsawa I, Murakami T, Kimura A, Hakamata Y, Sato Y *et al*. Development of new inbred transgenic strains of rats with LacZ or GFP. *Biochem Biophys Res Commun* 2005; **329**: 288–295.
24 Morris JC, Conerly M, Thomasson B, Storek J, Riddell SR, Kiem HP. Induction of cytotoxic T-lymphocyte responses to enhanced green and yellow fluorescent proteins after myeloablative conditioning. *Blood* 2004; **103**: 492–499.
25 Kimura S, Eldridge JH, Michalek SM, Morisaki I, Hamada S, McGhee JR. Immunoregulation in the rat: ontogeny of B cell responses to types 1, 2, and T-dependent antigens. *J Immunol* 1985; **134**: 2839–2846.
26 Ladics GS, Smith C, Bunn TL, Dietert RR, Anderson PK, Wiescinski CM *et al*. Characterization of an approach to

developmental immunotoxicology assessment in the rat using SRBC as the antigen. *Toxicol Methods* 2000; **10**: 283–311.
27 Watts AM, Stanley JR, Shearer MH, Hefty PS, Kennedy RC. Fetal immunization of baboons induces a fetal-specific antibody response. *Nat Med* 1999; **5**: 427–430.
28 Marchant A, Appay V, Van Der SM, Dulphy N, Liesnard C, Kidd M *et al*. Mature CD8(+) T lymphocyte response to viral infection during fetal life. *J Clin Invest* 2003; **111**: 1747–1755.
29 Jerebtsova M, Batshaw ML, Ye X. Humoral immune response to recombinant adenovirus and adeno-associated virus after *in utero* administration of viral vectors in mice. *Pediatr Res* 2002; **52**: 95–104.
30 Manns MP, Obermayer-Straub P. Cytochromes *P*450 and uridine triphosphate-glucuronosyltransferases: model autoantigens to study drug-induced, virus-induced, and autoimmune liver disease. *Hepatology* 1997; **26**: 1054–1066.
31 Clarke DJ, Keen JN, Burchell B. Isolation and characterisation of a new hepatic bilirubin UDP-glucuronosyltransferase. Absence from Gunn rat liver. *FEBS Lett* 1992; **299**: 183–186.
32 Magdalou J, Antoine B, Ratanasavanh D, Siest G. Phenobarbital induction of cytochrome *P*-450 and UDP-glucuronosyltransferase in rabbit liver plasma membranes. *Enzyme* 1982; **28**: 41–47.
33 Kootstra NA, Matsumura R, Verma IM. Efficient production of human FVIII in hemophilic mice using lentiviral vectors. *Mol Ther* 2003; **7**: 623–631.
34 VandenDriessche T, Thorrez L, Naldini L, Follenzi A, Moons L, Berneman Z *et al*. Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells *in vivo*. *Blood* 2002; **100**: 813–822.
35 Follenzi A, Battaglia M, Lombardo A, Annoni A, Roncarolo MG, Naldini L. Targeting lentiviral vector expression to hepatocytes limits transgene-specific immune response and establishes long-term expression of human antihemophilic factor IX in mice. *Blood* 2004; **103**: 3700–3709.
36 Nguyen TH, Bellodi-Privato M, Aubert D, Pichard V, Myara A, Trono D *et al*. Therapeutic lentivius-mediated neonatal *in vivo* gene therapy in hyperbilirubinemic Gunn rats. *Mol Ther* 2005 (in press).
37 Peters WH, Allebes WA, Jansen PL, Poels LG, Capel PJ. Characterization and tissue specificity of a monoclonal antibody against human uridine 5′-diphosphate-glucuronosyltransferase. *Gastroenterology* 1987; **93**: 162–169.
38 Seppen J, Tada K, Ottenhoff R, Sengupta K, Chowdhury NR, Chowdhury JR *et al*. Transplantation of Gunn rats with autologous fibroblasts expressing bilirubin UDP-glucuronosyltransferase: correction of genetic deficiency and tumor formation. *Hum Gene Ther* 1997; **8**: 27–36.

Chapter 5

ABCB4 P-glycoprotein reduces infectivity of lentiviral particles by increasing their phosphatidylcholine content.

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Abstract
Human Immunodeficiency Virus-1 (HIV-1) particles are assembled and released from membrane microdomains (lipid rafts). The membrane composition of the viral particle, which is important for viral integrity and infectivity, is therefore determined by the raft lipid composition.
ATP binding cassette (ABC) transporters are transmembrane proteins, which require ATP to transport a broad array of compounds through membranes. The ABC transporter ABCB4 (MDR3 P-glycoprotein) is a phospholipid floppase that translocates phosphatidylcholine (PC) from the inner leaflet to the outer leaflet of the cellular membrane. ABCB4 is located in the apical membrane of hepatocytes; translocation of PC by ABCB4 promotes PC release into bile, which neutralizes the toxic effect of bile salts.
In a third generation lentiviral vector system based on HIV-1, we observed that the co-expression of ABCB4 during virus production inhibited viral infectivity, with no observed reduction in viral particles. Co-expression of an inactive ABCB4 mutant did not reduce viral infectivity. In addition, the ratio of phosphatidylcholine to cholesterol of lentiviral particles increased significantly from 0.4 in producer cells expressing no ABCB4 or a mutant ABCB4 to 1.3 if the wildtype ABCB4 protein was co-expressed.
We therefore conclude that the specific translocase activity of ABCB4 can affect viral infectivity negatively by changing the lipid membrane composition of the viral particles. Understanding of this mechanism may lead to novel treatment strategies of HIV patients.

Introduction
Lentiviral vectors based on human inmmunodeficiency virus 1 (HIV-1) hold great promise for the treatment of inherited diseases. These vectors can integrate in the host genome and give rise to long-term expression of the transgene in dividing and non-dividing cells [25,31]. Lentiviral particles are lipid-enveloped, they assemble and bud off in rafts, which are specific plasma membrane microdomains with distinct lipid and protein composition [18]. Entry into cells requires binding of the envelope proteins to receptors and co-receptors in lipid raft domains as well [18]. HIV-1 budding and entry requires cholesterol [46] and loss of virion-associated cholesterol reduces infectivity [9].
ATP-binding cassette (ABC) transporters belong to a large family of transmembrane proteins, which can actively transport compounds, such as lipids, bile salts, toxic compounds, and peptides for antigen presentation, through membranes by hydrolysis of ATP [5,14,23]. ABC transporters are characterized by ATP-binding cassettes, also called Nucleotide Binding Domains (NBD). These regions contain consensus motifs, such as the Walker-A domain. Single point mutations in the Walker-A motif at a key lysine residue have shown to reduce or abrogate the ATP hydrolysis activity and in some cases impair nucleotide binding [3,30,36,40].
The ABC transporter, multidrug resistance P-glycoprotein (P-gp) 3 (ABCB4) primarily mediates the translocation of phosphatidylcholine (PC) across the canalicular membrane of the hepatocyte, a process that is called flopping as opposed to the reverse translocation from the outer to the inner leaflet which is called flipping [6]. In the liver, the translocation of PC is necessary to neutralize the toxic detergent action of bile salts. Lack of ABCB4 expression leads to the severe liver disease Progressive Familial Intrahepatic Cholestasis type 3 (PFIC3).
Another ABC transporter, ABCC1 (Multidrugresistance related protein 1, MRP1), is an efflux pump that extrudes mostly anionic drugs, toxins, waste products and their conjugates, such as bilirubin glucuronides and glutathione S-conjugates [24,26,45].
Our attempts to develop a lentiviral vector expressing ABCB4 for the treatment of PFIC3 were unsuccessful. Because in this lentiviral vector the transgene product ABCB4 is also

expressed during lentiviral vector production, we hypothesized that the PC floppase activity of ABCB4 might have affected lentiviral vector production negatively. We co-expressed ABCB4 during lentiviral particle production and found a reduction in the number of infectious particles, but not the lentiviral particle production, as measured by p24 gag-antigen content. The expression of ABCB4 protein caused changes in phospholipid composition of the viral particles, indicating this may be the underlying process that was responsible for the reduced transduction efficiency. This was more strongly illustrated by the observation that ABCC1 and an inactive ABCB4 mutant did not affect viral vector production and infectivity.

Materials and methods

Construction of mammalian expression plasmids

The mammalian expression plasmid pcDNA3.1+ (Invitrogen) was used to express the ABC transporters ABCB4, mutant ABCB4 and ABCC1. For the preparation of pcDNA3.1+-ABCB4, human wildtype ABCB4 cDNA [42] was cloned as an AgeI and XbaI fragment into the mammalian expression plasmid pcDNA3.1+.
The human ABCC1 cDNA [45] was cloned as a BamHI and NotI fragment into pcDNA3.1+.

Development of ABCB4 mutant expression plasmid

To obtain the required ABCB4 mutant protein, a PCR was performed with the following oligonucleotide bearing a mismatch base CT CGA GCT AAC GTC AAG ATC TTG AAG GGC CTC AAC CTG AAG GTG CAG AGT GGG CAG ACG GTG GCC CTG GTT GGA AGT AGT GGC TGT GGG A<u>T</u>G AGC ACA ACG G and CAC GTC CAA TGG CGA TCC TC, to substitute nucleotide 1303 from adenine to thymine in the conserved Walker A domain, leading to an amino acid change at position 435 of a lysine into a methionine (mutation is underlined). This mutation was shown to completely inactivate the homologous transporter, ABCB1, as has been described previously [3,30]. The PCR product was cloned into pcR-TOPO2.1 (Invitrogen) and the correct substitution was confirmed by sequence analysis using conventional M13 forward and reverse primers. A 2271 bp fragment was removed from pcDNA3.1+-ABCB4 by ApaI digestion and cloned into the pcR-TOPO2.1 vector that contained the mutated PCR fragment. From this plasmid a 2640 bp fragment was taken out by XhoI and XbaI restriction enzymes and ligated in place in the pcDNA3.1+-ABCB4 plasmid resulting in the mutated ABCB4 (ABCB4mut).

Lentiviral vector production

Third generation lentiviral particles [15,47] pseudotyped with Vesicular Stomatitis Virus G-glycoprotein (VSV-G), were produced by using transient transfection of HEK 293T (human epithelial kidney cell line) by calcium phosphate precipitation, and were titrated on HeLa cells to determine the transducing units (TU) per ml as described before[37]. The transfer vector pRRLcpptpgkGFPpreSsin containing phosphoglycerate kinase promoter driving GFP expression, the hepatitis B virus posttranslational regulatory element, and central polypurine tract was used in all experiments, in addition to the packaging plasmids, pMDL-g/p, pVSV-g and pRSV-Rev [37]. Additionally, transfer vectors pRRLcpptcmvGFPpreSsin and pRRLcpptcmvdsREDpreSsin, containing the cytomegalovirus promoter driving expression of dsRED in the latter construct.
The cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (L-glu), 100 units/ml penicillin and 100 μg/ml streptomycin (P/S).
HEK 293T cells were seeded in ∅15cm dishes on day one, followed by transfection on day two. In addition to the lentiviral vector plasmids, 20μg of either pcDNA3.1+-ABCB4,

pcDNA3.1+-ABCB4mut, pcDNA3.1+-ABCC1 or pcDNA3.1+ (empty vector) were added to the transfection mixture.
On the third day, cells were washed with phosphate buffered saline (PBS), and replaced by serum-free medium (Optimem, Cambrex) with L-glu, P/S and 25mM HEPES, pH 7.5.
One day later, supernatant was harvested and centrifuged at 300*g* for 10 minutes to remove cell debris, followed by filtration through 0.45μm filters (Millipore).
For Western blot analysis and lipid assays only, an additional centrifugation (Rotor SW28, Optima L90K Ultracentrifuge, Beckman Coulter) step of 2 hours at 20,000 r.p.m. at 4°C was performed. The supernatant was removed after concentration, the inside of the tubes were thoroughly cleaned and the viral pellet was resuspended in 50μL PBS. Another 50μL of 2% sodium dodecyl sulphate (SDS) was added for inactivation of the viral pellet. Samples were stored and aliquoted at -80°C until further use.

Determination of lentiviral particles
The HIV-1 p24 Elisa kit (Perkin Elmer) was used to determine the amount of p24 in the viral supernatants. Samples were diluted in Optimem medium. The p24-content was used to calculate the number of HeLa transducing units per p24-gag-antigen for each sample.

Western blots
Transfected cells were harvested in 2% SDS, and an equal volume of lysis buffer (20mM KCl, 3mM $MgCl_2$-$6H_2O$, 20mM Tris/HCl, pH 7.4) with protease inhibitor mix (Roche) was added to the cell lysates. After sonication, protein content was determined by bicinchoninic acid protein assay kit (Sigma). For cell lysates, equal amounts of protein were loaded on a 10% polyacrylamide gel, which was confirmed by immunoreactivity for β-actin. For viral pellets, an equal amount of p24-gag antigen for each sample was loaded.
The gel was blotted on nitrocellulose membranes and subsequently blocked with PBS with 4% milkpowder and 0.05% Tween-20 for 1 hour.
The blots were washed with PBS and incubated with primary antibodies. The following antibodies were used: mouse anti-ABCB4 ($P3_{II}26$[13], 1:1000), rat anti-human ABCC1 ($R1$[17], 1:1000), rabbit anti-VSV-G (Sigma, 1:2500) and mouse anti-β-actin (AB-5, 1:1000, Neomarkers). The blots were washed three times for 5 minutes with PBS-0.05% Tween-20, followed by another incubation with secondary antibodies: goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (GAMPO, 1:1000, Biorad) or goat anti-rat IgG HRP conjugate (1:1000, Calbiochem). The blots were washed with PBS-Tween-20 and immune complexes were revealed by chemiluminescence substrate (Lumi-light, Roche).

Immunofluorescence staining of transfected cells
HEK 293T cells were seeded 1×10^5 in 6-well plates on rat-tail collagen coated glass slides. The cells were calcium phosphate transfected with 4μg of pcDNA3.1+-ABCB4, pcDNA3.1+-ABCB4mut, pcDNA3.1+-ABCC1 or empty plasmid. Three days later, cells were methanol/acetone (ratio 4:1) fixed and frozen at –20°C until further use. The fixed cells were thawed followed by washing with PBS-0.05% Tween20 for 10 minutes and subsequent incubation of $P3_{II}26$-antibody (1:200) or R1-antibody (1:100) for ABCB4 and ABCC1 respectively in PBS-0.05% Tween20/10% FBS for 1 hour.
Glass slides were washed and incubated with goat anti-mouse IgG antibody Alexa fluor 594 conjugate (Molecular Probes) or goat anti-rat IgG antibody Texas-Red conjugate (Rockland) respectively. The cells were washed with PBS and embedded in mounting

medium containing DAPI (Vector Laboratories). Pictures were taken with an inverse microscope (Leica DMRA2, Leica).

Generation of phosphatidylcholine vesicles and lentiviral transduction

To obtain PC vesicles, 100mg/ml L-α-phosphatidylcholine solution in chloroform (Sigma) was dried at 40°C in a glass tube under a nitrogen stream and then resuspended by vortexing in 5ml of Hank's Balanced Salt Solution (HBSS, Biowhittaker). The 500 μM solution was sonicated (amplitude 60, 50-60 Hz, Vibra Cell Sonicator, Sonics & Materials Inc) on ice for 10 minutes, filtered through a 0.45 μm filter and was then used immediately. The vesicle solution was added to the wells resulting in final concentrations of 1 and 10μM. Lentiviral supernatants were then added with a multiplicity of infection of 0.2. These mixtures were replaced by fresh medium 24 hours later. The transduction efficiency was determined after four days by flow cytometry.

Co-transduction of lentiviral vector produced with ABCB4 wildtype and mutant

Lentiviral vector (pRRLcpptcmvdsREDpreSsin) was produced during ABCB4 or ABCB4mut. In parallel, pRRLcpptcmvGFPpreSsin vector was produced. Subsequently, equal volumes were mixed and HeLa cells were incubated to determine transduction efficiency four days later.

Phosphatidylcholine and cholesterol measurement

The amount of phosphatidylcholine in lentiviral vector pellets was determined by measuring choline by enzymatic assay with phospholipase D and choline oxidase [34]. Cholesterol was determined using homovanillic acid and cholesterol oxidase as described before [1]. Both enzymatic reactions were measured with a Novostar analyzer (BMG Labtech).

Statistical analysis

Statistical analysis were performed using SPSS 10.0 software and significant difference were considered if $P < 0.05$ determined by One-Way ANOVA.

Results

ABCB4 protein expression decreases lentiviral vector titer

For the treatment of the inherited liver disease PFIC3, we constructed a lentiviral vector containing a cassette with PGK promoter driving ABCB4 expression. We were unable to produce infectious virus, as measured by immunofluorescence staining of several cell lines that had been incubated with this viral supernatant.

To test the hypothesis that function of ABCB4 may reduce lentiviral production and infectivity, VSV-G pseudotyped lentiviral particles were produced with co-expression of ABCB4, a mutant ABCB4 and another ABC transporter as control, ABCC1. ABC transporters have the highly conserved Walker-domains required for ATPase activity [44]. In the ABCB4 protein, we replaced the amino acid lysine435 to methionine. Previously, a this mutation has been shown to abolish ATPase activity in Multidrug Resistance P-glycoprotein 1 (ABCB1)[3,30] resulting in complete abrogation of the ability of ABCB1 to confer multidrug resistance.

In addition to the co-expression of the ATP transporter proteins during GFP lentivirus production, within separate transfection experiment, viral particles were also produced with co-transfection of empty plasmid. Titers obtained under these conditions were set as an arbitrary value of 100% and used to correct for variability between transfection experiments. The co-transfection of empty vector with the lentiviral plasmids led to an

average titer of $1.3 \pm 1.1 \times 10^6$ transducing units per ml (TU/ml) of GFP lentivirus. As shown in table 1, ABCB4mut or ABCC1 co-expression during lentiviral vector production resulted in similar titers. In contrast, co-expression of ABCB4 during lentiviral particle production decreased titers by more than 81% compared to co-expression of ABCB4mut, ABCC1 ($p<0.01$, Table 1).
However, the amount of p24 per ml was similar when ABCB4, ABCB4mut and ABCC1 were co-expressed during lentiviral vector production (Table 1).
The ratio of TU/pg p24 corresponds with the lentiviral particle quality. If lentiviral vectors were produced without the co-expression of transmembrane proteins the TU/pg p24 ratio was on average 9.9 TU/pg p24. This ratio was similar to an earlier published report about VSV-G pseudotyped third generation lentiviral vectors [15]. A significant reduction ($p<0.001$) of the TU/pg p24 was observed if ABCB4 was co-expressed during lentiviral vector production, which was more than 70% lower than ABCB4mut, ABCC1 or empty vector co-expression.

Presence of ABC transporter protein in transfected cells and in lentiviral vector preparations

Immunofluorescence staining was performed in 293T cells to display cellular localization of the expressed proteins. Immunofluorescence staining confirmed ABCB4, ABCB4mut and ABCC1 expression in transfected 293T cells (Figure 1). All proteins displayed a similar localisation, confirming expression of ABCB4mut in cellular membranes and subcellular location in intracellular vesicles. The similar localisation of ABCB4mut to ABCB4 was as expected, because similar mutations introduced in the ABC transporter ABCB1 did not affect targeting of the mutant proteins to the cellular membrane compartment [3].
To determine whether expression levels of ABCB4 and ABCB4mut were comparable, Western blots were performed. The upper panel of figure 2 shows Western blot analysis of 293T cells that produced lentiviral vector particles and were co-transfected with empty vector, ABCB4, ABCB4mut or ABCC1. ABCB4 protein was undetectable in the 293T cell line. The bands representing ABCB4 and ABCB4mut protein in polyacrylamide SDS/PAGE co-migrated (Figure 2A, sample 1,2). Liver lysate of ABCB4 transgenic mice (A63+ mouse strain [38]) was used as a positive control (data not shown). Bands representing ABCB4 and ABCB4mut protein were similar in intensity and mobility. In the transfectants, two bands were detected, possibly corresponding to different glycosylation patterns of ABCB4 protein.
As expected ABCC1 protein had a lower mobility than ABCB4 protein. No ABCC1 protein expression was detected in the untransfected cells.
Detection of protein in lentiviral preparations may result from incorporation in viral particles. Therefore, immunoreactivity of the expressed membrane proteins was measured in purified viral fractions. The lower panel of figure 2 shows the protein analysis of lentiviral vector preparations. When ABCB4 protein was expressed during lentiviral vector production, ABCB4 protein was also detected in lentiviral preparations. Although ABCB4 protein expression was similar to ABCB4mut protein expression in lentivirus producing cells, the amount of ABCB4mut protein in concentrated lentiviral vector preparations was undetectable. As mentioned above, only ABCB4 expression decreased the ratio of TU/p24 in viral supernatant. The presence of ABCB4 protein in the lentivirus particles *per se* cannot explain the reduction of infectious particles, because ABCC1 protein was also detected in lentivirus fractions, without affecting viral infectivity negatively. The fact that both ABCB4 and ABCC1 appear in concentrated virus preparations points in the direction that ABCB4 function, rather then its presence in viral particles, inhibits the production of infectious particles.

ABCB4 expression alters phosphatidylcholine and cholesterol composition of viral preparations

We also measured phosphatidylcholine and cholesterol content of lentiviral vector preparations. As mentioned above, ABCB4 co-expression and not ABCB4mut co-expression inhibited infectivity of lentiviral particles. One explanation for the decrease in the number of infectious lentiviral particles is that ABCB4 function affects the lipid composition of the lentiviral particle. Because ABCB4 is a specific phosphatidylcholine floppase [38], more of this lipid species might be incorporated in the lentiviral particles.

To investigate this, phosphatidylcholine and cholesterol concentration were determined in lentiviral vector preparations and corrected for the amount of p24-gag antigen (Table 2). Co-transfection of ABCB4mut plasmid or empty vector plasmid resulted in similar PC/p24-gag antigen and cholesterol/p24-gag antigen ratios. As expected, the ratio of PC/cholesterol was relatively high [2,7].

If ABCB4 was expressed during viral vector production, there was a significant increase in the content of PC per p24-gag antigen (51 $\pm$ 26 nmol/μg p24, $p<0.01$) compared to ABCB4mut co-expression or addition of empty vector. A concomitant increase was also observed in the presence of cholesterol per p24-gag antigen (40 $\pm$ 19 nmol/μg p24, $p<0.01$). The relative increase of PC content in lentiviral fractions obtained from ABCB4 positive 293T cells was larger than that of the cholesterol content compared to ABCB4 mutant co-expression (approximately 13-fold and 4-fold, respectively). The PC/cholesterol ratio in lentivirus produced by 293T cells increased from 0.4 co-expressing ABCB4mut to 1.3 in cells expressing wild type ABCB4. Hence, the specific floppase activity of ABCB4 may directly relate to an increase of phosphatidylcholine in the lentiviral particles.

No decrease of VSV-G incorporation in the lentiviral vector by ABCB4 expression.

A decrease in the incorporation of VSV-G in the lentiviral vector particles may reduce the infectivity. In figure 3, we show that equal amounts of p24 gag-antigen of lentiviral vector preps produced with ABCB4 or ABCB4mut did not alter the VSV-G content, suggesting that VSV-G is efficiently incorporated in the lentiviral vector particles. However, if equal numbers of transducing units were loaded, we observed a markedly increase in the VSV-G content if ABCB4 was expressed, which confirms that more particles were loaded, which were less infectious.

No significant inhibition by PC vesicles, presence of PC in the medium, or other components produced during co-expression of ABCB4 during lentiviral vector expression.

The increase of PC and cholesterol in viral preparations from cells expressing ABCB4 could also be caused by the generation of vesicles or membrane fragments by these cells. ABCB4 has been suggested to directly cause budding of phospholipid vesicles from the plasma membrane [11]. Such vesicles could indirectly cause the inhibition of lentiviral vector infectivity. To test this possibility, lentiviral vector transduction was performed in the presence of PC vesicles. The highest amount of PC that we measured in a viral preparation produced during ABCB4 co-expression was 514μM. Because the virus pellet was concentrated 350-fold, the concentration of PC vesicles in the unconcentrated supernatant would have been approximately 1.5μM. We produced PC liposomes and added these to lentiviral supernatants. Addition of PC did not significantly affect transduction of HeLa cells. Even a 7-fold higher concentration of PC than we observed in the viral preparation derived from ABCB4-expressing cells did not

significantly reduce transduction efficiency (Table 3a). Therefore, we conclude that presence of PC in viral supernatant cannot explain the reduced infectivity.
A second set of experiments was done to exclude that other factors in the medium produced by cells co-expressing ABCB4 had reduced viral infectivity. Supernatant was removed from 293T cells that where transfected to express ABCB4 and ABCB4mut protein and mixed with lentiviral supernatant. Subsequently, the mixture was used to transduce HeLa cells (Table 3b). There was no significant difference in transduction efficiency if supernatant of ABCB4 or ABCB4mut transfected cells was mixed with lentiviral vectors.
To exclude that the combination of the lentiviral vector production with ABCB4 had a significant effect on the infectivity we performed a co-transduction of lentiviral vector produced with ABCB4 wildtype and mutant. Lentiviral vector (pRRLcpptcmvdsREDpreSsin) was produced during ABCB4 or ABCB4mut. In parallel, pRRLcpptcmvGFPpreSsin vector was produced. Subsequently, equal volumes of CMVdsRED/ABCB4 or CMVdsRED/ABCB4mut were mixed with CMVGFP LV vector and HeLa cells were incubated and transduction efficiency was determined four days later.
As shown in table 3c, the percentage of GFP positive cells measured was similar if transduction was performed with CMVdsRED/ABCB4 or CMVdsRED/ABCB4mut supernatant. The three experiments described above make it very unlikely that PC vesicles or other factors produced by ABCB4 expressing cells inhibited infectivity of lentiviral particles. We therefore conclude that the lower transduction efficiency of lentiviral vectors co-produced with ABCB4 is due to increased PC content of the lentiviral particle.

Discussion
We show that expression of the membrane protein ABCB4 during lentiviral vector production reduces titers. We also show that this inhibition of infectivity is caused by ABCB4 function, i.e. PC translocation, rather than by the presence of the protein: ABCB4 mutant protein in which the ATP-binding module has been mutated did not reduce viral titers, although it was expressed at the same level and with an identical localisation as the wild type protein. In contrast to the loss of infectivity, ABCB4 expression did not reduce the production of viral particles (as measured by p24-antigen values in viral vector supernatant). Whichever protein was expressed during virus production (ABCB4, ABCB4mut or ABCC1 plasmid or empty vector), the amount of viral particles produced was the similar.
A reduction of infectivity by the expression of ABC transporters has been shown previously in experiments with replicative HIV-1 [28,39]. In these experiments, ABCB1, an efflux pump that extrudes diverse hydrophobic drugs and peptides from cells, was overexpressed which led to a reduction in the production of HIV-1 [28]. In the studies of Lee *et al* [28], functional ABCB1 was not necessary for the decrease in HIV-1 production, because a mutant without ATPase activity also decreased reverse transcriptase activity in supernatant. Lee *et al* speculated that presence of protein in viral particles was responsible for the reduction in production and infectivity. However, we show that ABCC1 in lentiviral particle preparations did not reduce infectivity and furthermore that ABCB4mut had no negative effect on infectivity at all. Although the exact mechanism has not been elucidated some studies have indicated that ABCB1 can mediate flux of cholesterol from the cytosolic leaflet to the exoplasmic leaflet [19] and may expel analogs of phospholipids as well [43], including phosphatidylcholine, and has been shown to transport endogenous lipids [12,35].

HIV infection of ABCC1 overexpressing cells increased HIV-1 protein and virus production 50-fold compared to control cells [39]. In our studies, ABCC1 co-expression had no effect on viral titers. For both studies, the use of replicating virus may have contributed to the dissimilarity with our observations, and more insight in the underlying mechanisms is required to understand the precise process that takes place.

It has been described that many host cell-derived membrane proteins, such as major histocompatibility complex (MHC-I) and adhesion molecules can be HIV virion-associated [16,33,41]. However, it has not been shown that these membrane-associated proteins inhibit infectivity *in vitro*. In our study, we could detect ABCB4 and ABCC1 protein, but not ABCB4mut protein in the virus preparations. Thus, only functional ABCB4 protein may be incorporated into the virion and thereby reducing viral infectivity. Because ABCC1 was also detected in the viral preparations the incorporation of proteins in the viral particle itself does not seem to cause the reduced infectivity of lentiviral particles.
Additionally, the co-expression of ABCB4 may have an effect on the incorporation of VSV-g into the lentiviral particle, which may result in a lower infectivity. As shown by Western blot analysis (figure 3) equal amounts of p24 gag-antigen did not reduce the incorporation of VSV-g in the viral preparation, which excludes the possibility that less VSV-g per particle is responsible for the reduced infectivity.

It has been well described that the plasma membrane rafts play an important role in HIV-1 assembly and release [32]. These microdomains contain a high content of cholesterol and sphingomyelin [2]. Additionally, it has been shown that the cholesterol in the lentiviral particle is critical for HIV-1 integrity and infectivity [9,21,22]. In a recent paper the unusual composition of HIV-1 particles was shown with a marked decrease of PC in virions compared to cellular membranes of an infected T-cell line [7].
The ATP transporter ABCB4 is a phospholipid floppase and specifically translocates PC from the inner to the outer membrane in the apical membrane of the hepatocyte [38]. Because lipid composition of the viral particle is important for viral infectivity, ABCB4 may very well change lipid composition of the viral particles if present during viral vector production. The clear increase of the PC/cholesterol ratio in viral preparations that we observed strongly indicates that this process takes place.
Additionally, ABCC1 has been documented to be associated with lipid rafts [27], although this protein also has been reported not to be in glycolipid-enriched microdomains [39]. ABCB1 also appears to be associated with rafts and even retains function when reconstituted into sphingolipid- and cholesterol-rich environment [29]. ABCB4 is expressed at the apical membrane of hepatocytes, which contains relatively high levels of cholesterol and sphingolipid (rafts). ABCC1 and ABCB4 may have been incorporated in viral particles in our studies, because of their localisation in lipid rafts.

As has been shown previously, vesicles derived from cell membranes can be a major contaminant in gradient-enriched HIV-1 preparations [4,20]. Therefore, another explanation may be that ABCB4 increases PC vesicle formation and release *in vitro*, as has been shown to occur *in vivo* [11], which may indirectly interfere with viral transduction efficiency. Incubation of target cells with phosphatidylserine (PS) liposomes increased viral entry, but treatment of cells with PC did not increase viral entry [10]. In another study, HIV-1 replication was significantly inhibited with vesicles consisting of PS, but not PC [8]. To exclude that nonviral PC vesicles present in the preparation inhibited viral infectivity in our experiments, we generated PC vesicles, which were added before viral transduction. As shown in table 3a and in line with the literature cited above [8,10], there was no

significant decrease in viral transduction if we increased PC concentration 7-fold (10μM) compared to the average concentration of PC in culture medium harvested from cells co-expressing ABCB4 during lentivirus production (1.5μM).
In addition, to exclude that other factors in culture medium had influenced viral infectivity, harvested culture medium of ABCB4 or ABCB4mut expressing cells was mixed with lentiviral vector supernatant and incubated on HeLa cells. No reduction of lentiviral transduction efficiency was observed.
The additional production of lentiviral particles to ABCB4 expression may have reduced the infectivity. Lentiviral vector preparation (CMVdsRED) produced with co-expression of ABCB4 led to a reduced number of transducing units. A mix of CMVGFP with the CMVdsRED/ABCB4 preparation did not affect the transduction efficiency of the CMVGFP viral vector preparation.
These experiments above rule out that other factors in the culture medium had influenced viral infectivity negatively.

In conclusion, ABCB4 function decreases lentiviral vector infectivity, without any apparent effect on lentiviral vector production. Active ABC transport proteins and especially those that have the ability to influence the phospholipid cell membrane composition may alter the lipid composition of lentivirus resulting in impaired infectivity. More insight in the relation between lentiviral phospholipid composition and infectivity might lead to novel ways to combat HIV infection.

Recognition of personal assistance
We would like to thank Coen Paulusma (AMC Liver Center, Amsterdam) for performing the PC and cholesterol measurements of viral fractions.

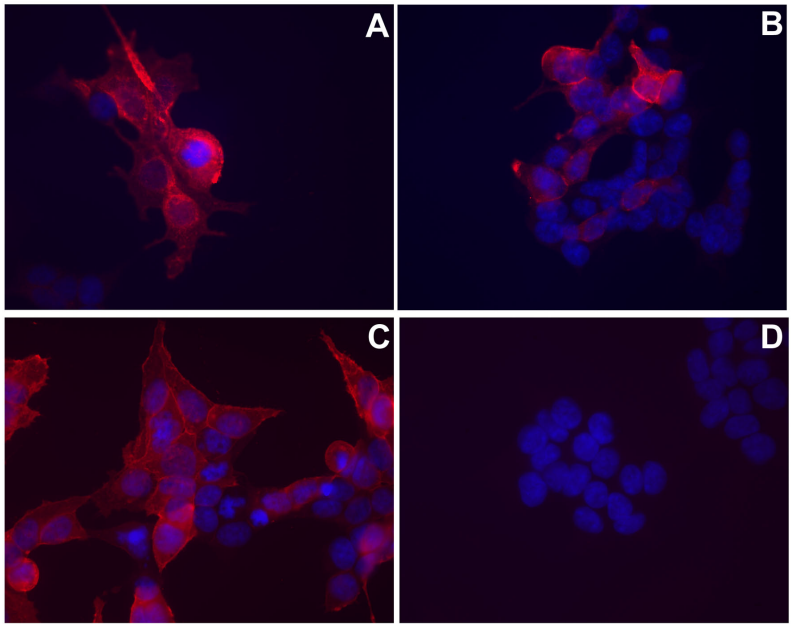

Figure 1. Cellular localisation of ABC transporter protein expression.
Immunofluorescence staining of transfected 293T cells using specific antibodies for ABCB4 and ABCC1 protein. Upper panel represent: (A) ABCB4 protein, and (B) ABCB4mut protein. Lower panel represent: (C) ABCC1 protein, and (D) 293T cell line (negative control). Nuclei were stained with DAPI. Strong immunoreactive staining was observed in cells expressing ABCB4, ABCB4mut and ABCC1. All three proteins displayed similar subcellular localization.

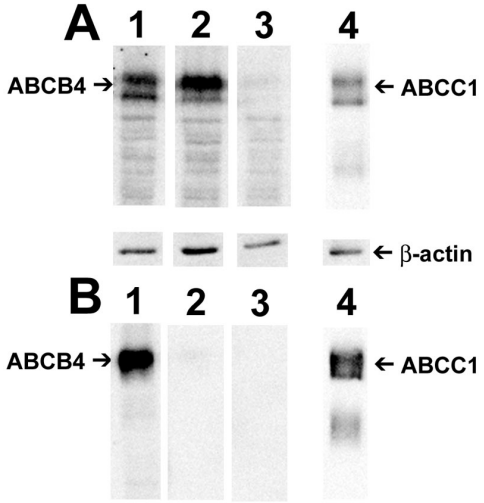

Figure 2. Western blot of ABC transporter proteins in cells and viral particles
(A) Lysates of lentiviral vector producing 293T cells with or without co-transfection of transmembrane proteins. Lanes represent: (1) ABCB4 and lentiGFP, (2) ABCB4mut and lentiGFP, (3) lentiGFP, and (4) ABCC1 and lentiGFP.
293T cells did not express ABCB4 or ABCC1 protein. ABCB4 immunoreactive protein of apparent molecular mass of 140 kDa was detected and ABCB4mut co-migrated with ABCB4 protein. ABCC1 protein migrated slower than ABCB4 protein in agreement with the higher molecular weight (170kD). The two apparent bands in both ABCB4 and ABCC1 lanes most likely correspond with glycosylated (upper) and non-glycosylated (lower) form. Similar amounts of protein were loaded as shown by β-actin immunoreactive signal.
(B) Lysates of lentiviral vector particle preparations are presented in similar order as panel A. Only in lentiviral particles produced during co-expression of ABCB4 or ABCC1 a band for the respective ABC transporter protein could be detected. No immunoreactivity was detected if ABCB4mut protein was expressed.

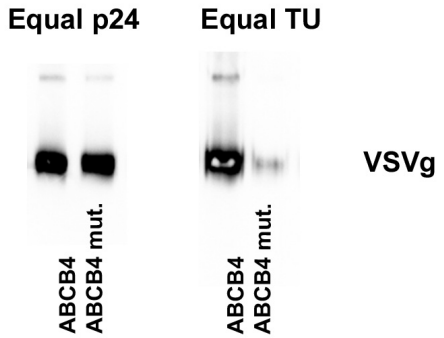

Figure 3. Western blot of VSVg content in viral vector preparations after ABCB4 co-expression.
Lentiviral vector preparations were produced with co-expression of ABCB4 or ABCB4mut protein. In the panel on the left hand side equal amount of p24 gag-antigen were loaded. On the right hand side equal numbers of transducing units were loaded. The amount of p24 gag-antigen, presenting the number of particles was not different between ABCB4 or ABCB4mut co-expression.

Figures and tables:

Table 1: Effect of co-expression of ABC transporters ABCB4 and ABCC1 on infectivity.

	TU/ml[a]	p24/ml[a]	TU/pg p24
Empty vector	100 %	100 %	9.9 ± 2.8** (n=6)
ABCB4 (n=8)	17.0 ± 9.0 %	90.5 ± 55.3 %	2.3 ± 0.7
ABCB4 mutant (n=8)	88.9 ± 39.8 %*	104.6 ± 30.3 %	8.9 ± 2.8**
ABCC1 (n=7)	106.1 ± 48.4 %**	119.3 ± 66.9 %	7.8 ± 1.1**

[a]The data for TU/ml and p24/ml are presented as a percentage, with the co-transfection of empty vector during the production of lentiviral vectors given an arbitrary value of 100%. Significant difference $p<0.01^{*}$ or $p<0.001$** compared to ABCB4 co-expression. Mean values are presented ± SD.

Table 2: Phosphatidylcholine and cholesterol content of viral fractions.

Transfer vector	Protein	nmol PC/μg p24	nmol cholesterol/μg p24	n
LentiGFP	ABCB4	51 ± 26	40 ± 19	6
LentiGFP	ABCB4mutant	4 ± 3*	9 ± 2*	6
LentiGFP	None	6 ± 4*	8 ± 1*	3

Table representing the PC and cholesterol content of lentiviral vector preparations produced with co-expression of ABCB4, ABCB4mut or no co-expression. The values were adjusted for the amount of p24-gag antigen per sample. *Significant difference $p<0.01$ compared to ABCB4 co-expression. Mean values are presented ± SD.

Table 3a: Effect of PC vesicles on transduction efficiency.

Phosphatidylcholine (μM)[a]	0	1	10
Transduction percentage	100	93.8 ± 4.7	85.1 ± 10.4

Table represents transduction efficiencies of lentiviral particles with or without addition of PC vesicles. The transduction efficiency without addition of PC vesicles was given an arbitrary value of 100%. [a]PC concentration in transduction medium. Mean values are presented ± SD (*n=3*).

Table 3b: No inhibition of transduction by medium of ABCB4 expressing 293T cells.

Supernatant	Empty medium	ABCB4	ABCB4 mutant
Transduction percentage	100	119 ± 3	98 ± 13

Supernatants harvested from ABCB4 or ABCB4mut expressing 293T cells were mixed with concentrated lentiviral vectors and transduction efficiencies were determined. The transduction efficiency of empty medium was given an arbitrary value of 100%. Mean values are presented ± SD (*n=3*).

Table 3c: No inhibition of transduction by LV-CMVdsRED/ABCB4 viral vector prep on CMVGFP lentiviral transduction.

Supernatant	CMVdsRED/ABCB4	CMVdsRED/ABCB4 mutant
Transduction percentage CMVGFP	100	104

Transduction at MOI 1with CMVdsRED/ABCB4 viral vector and CMVGFP viral vector were mixed and incubated with HeLa cells. The effect of CMVdsRED/ABCB4 lentivirus on CMVGFP transduction efficiency was determined by flow cytometry. The transduction efficiency of CMVdsRED/ABCB4 mix was given an arbitrary value of 100%. Samples were tested in duplo and mean values are presented ± SD (*n=2*).

References

1. **Allain, C. C., L. S. Poon, C. S. Chan, W. Richmond, and P. C. Fu**. 1974. Enzymatic determination of total serum cholesterol. Clin. Chem. **20**:470-475.
2. **Aloia, R. C., H. Tian, and F. C. Jensen**. 1993. Lipid composition and fluidity of the human immunodeficiency virus envelope and host cell plasma membranes. Proc. Natl. Acad. Sci. U. S. A **90**:5181-5185.
3. **Azzaria, M., E. Schurr, and P. Gros**. 1989. Discrete mutations introduced in the predicted nucleotide-binding sites of the mdr1 gene abolish its ability to confer multidrug resistance. Mol. Cell Biol. **9**:5289-5297.
4. **Bess, J. W., Jr., R. J. Gorelick, W. J. Bosche, L. E. Henderson, and L. O. Arthur**. 1997. Microvesicles are a source of contaminating cellular proteins found in purified HIV-1 preparations. Virology **230**:134-144.
5. **Borst, P. and R. O. Elferink**. 2002. Mammalian ABC transporters in health and disease. Annu. Rev. Biochem. **71**:537-592.
6. **Borst, P., N. Zelcer, and A. van Helvoort**. 2000. ABC transporters in lipid transport. Biochim. Biophys. Acta **1486**:128-144.
7. **Brugger, B., B. Glass, P. Haberkant, I. Leibrecht, F. T. Wieland, and H. G. Krausslich**. 2006. The HIV lipidome: A raft with an unusual composition. Proc. Natl. Acad. Sci. U. S. A.
8. **Callahan, M. K., P. M. Popernack, S. Tsutsui, L. Truong, R. A. Schlegel, and A. J. Henderson**. 2003. Phosphatidylserine on HIV envelope is a cofactor for infection of monocytic cells. J. Immunol. **170**:4840-4845.
9. **Campbell, S. M., S. M. Crowe, and J. Mak**. 2002. Virion-associated cholesterol is critical for the maintenance of HIV-1 structure and infectivity. AIDS **16**:2253-2261.
10. **Coil, D. A. and A. D. Miller**. 2005. Enhancement of enveloped virus entry by phosphatidylserine. J. Virol. **79**:11496-11500.
11. **Crawford, A. R., A. J. Smith, V. C. Hatch, R. P. Oude Elferink, P. Borst, and J. M. Crawford**. 1997. Hepatic secretion of phospholipid vesicles in the mouse critically depends on mdr2 or MDR3 P-glycoprotein expression. Visualization by electron microscopy. J. Clin. Invest **100**:2562-2567.
12. **Daleke, D. L.** 2003. Regulation of transbilayer plasma membrane phospholipid asymmetry. J. Lipid Res. **44**:233-242.
13. **de Vree, J. M., R. Ottenhoff, P. J. Bosma, A. J. Smith, J. Aten, and R. P. Oude Elferink**. 2000. Correction of liver disease by hepatocyte transplantation in a mouse model of progressive familial intrahepatic cholestasis. Gastroenterology **119**:1720-1730.
14. **Dean, M., A. Rzhetsky, and R. Allikmets**. 2001. The human ATP-binding cassette (ABC) transporter superfamily. Genome Res. **11**:1156-1166.
15. **Dull, T., R. Zufferey, M. Kelly, R. J. Mandel, M. Nguyen, D. Trono, and L. Naldini**. 1998. A third-generation lentivirus vector with a conditional packaging system. J. Virol. **72**:8463-8471.
16. **Esser, M. T., D. R. Graham, L. V. Coren, C. M. Trubey, J. W. Bess, Jr., L. O. Arthur, D. E. Ott, and J. D. Lifson**. 2001. Differential incorporation of CD45, CD80 (B7-1), CD86 (B7-2), and major histocompatibility complex class I and II molecules into human immunodeficiency virus type 1 virions and microvesicles: implications for viral pathogenesis and immune regulation. J. Virol. **75**:6173-6182.
17. **Flens, M. J., M. A. Izquierdo, G. L. Scheffer, J. M. Fritz, C. J. Meijer, R. J. Scheper, and G. J. Zaman**. 1994. Immunochemical detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. Cancer Res. **54**:4557-4563.
18. **Flint.S.J., L.W.Enquist, V.R.Racaniello, and A.M.Skalka**. 2004. Principles of Virology: Molecular Biology, Pathogenesis, and Control of Animal Viruses.
19. **Garrigues, A., A. E. Escargueil, and S. Orlowski**. 2002. The multidrug transporter, P-glycoprotein, actively mediates cholesterol redistribution in the cell membrane. Proc. Natl. Acad. Sci. U. S. A **99**:10347-10352.
20. **Gluschankof, P., I. Mondor, H. R. Gelderblom, and Q. J. Sattentau**. 1997. Cell membrane vesicles are a major contaminant of gradient-enriched human immunodeficiency virus type-1 preparations. Virology **230**:125-133.
21. **Graham, D. R., E. Chertova, J. M. Hilburn, L. O. Arthur, and J. E. Hildreth**. 2003. Cholesterol depletion of human immunodeficiency virus type 1 and simian immunodeficiency virus with beta-cyclodextrin inactivates and permeabilizes the virions: evidence for virion-associated lipid rafts. J. Virol. **77**:8237-8248.
22. **Guyader, M., E. Kiyokawa, L. Abrami, P. Turelli, and D. Trono**. 2002. Role for human immunodeficiency virus type 1 membrane cholesterol in viral internalization. J. Virol. **76**:10356-10364.
23. **Higgins, C. F.** 1992. ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. **8**:67-113.
24. **Jedlitschky, G., I. Leier, U. Buchholz, J. Hummel-Eisenbeiss, B. Burchell, and D. Keppler**. 1997. ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2. Biochem. J. **327 (Pt 1)**:305-310.
25. **Kafri, T., U. Blomer, D. A. Peterson, F. H. Gage, and I. M. Verma**. 1997. Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. Nat. Genet. **17**:314-317.
26. **Keppler, D., I. Leier, G. Jedlitschky, and J. Konig**. 1998. ATP-dependent transport of glutathione S-conjugates by the multidrug resistance protein MRP1 and its apical isoform MRP2. Chem. Biol. Interact. **111-112**:153-161.
27. **Klappe, K., J. W. Hinrichs, B. J. Kroesen, H. Sietsma, and J. W. Kok**. 2004. MRP1 and glucosylceramide are coordinately over expressed and enriched in rafts during multidrug resistance acquisition in colon cancer cells. Int. J. Cancer **110**:511-522.
28. **Lee, C. G., M. Ramachandra, K. T. Jeang, M. A. Martin, I. Pastan, and M. M. Gottesman**. 2000. Effect of ABC transporters on HIV-1 infection: inhibition of virus production by the MDR1 transporter. FASEB J. **14**:516-522.
29. **Modok, S., C. Heyward, and R. Callaghan**. 2004. P-glycoprotein retains function when reconstituted into a sphingolipid- and cholesterol-rich environment. J. Lipid Res. **45**:1910-1918.
30. **Muller, M., E. Bakos, E. Welker, A. Varadi, U. A. Germann, M. M. Gottesman, B. S. Morse, I. B. Roninson, and B. Sarkadi**. 1996. Altered drug-stimulated ATPase activity in mutants of the human multidrug resistance protein. J. Biol. Chem. **271**:1877-1883.

31. **Naldini, L., U. Blomer, P. Gallay, D. Ory, R. Mulligan, F. H. Gage, I. M. Verma, and D. Trono**. 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science **272**:263-267.
32. **Ono, A. and E. O. Freed**. 2001. Plasma membrane rafts play a critical role in HIV-1 assembly and release. Proc. Natl. Acad. Sci. U. S. A **98**:13925-13930.
33. **Ott, D. E.** 1997. Cellular proteins in HIV virions. Rev. Med. Virol. **7**:167-180.
34. **Oude Elferink, R. P., R. Ottenhoff, M. van Wijland, J. J. Smit, A. H. Schinkel, and A. K. Groen**. 1995. Regulation of biliary lipid secretion by mdr2 P-glycoprotein in the mouse. J. Clin. Invest **95**:31-38.
35. **Pohl, A., P. F. Devaux, and A. Herrmann**. 2005. Function of prokaryotic and eukaryotic ABC proteins in lipid transport. Biochim. Biophys. Acta **1733**:29-52.
36. **Ramjeesingh, M., C. Li, E. Garami, L. J. Huan, K. Galley, Y. Wang, and C. E. Bear**. 1999. Walker mutations reveal loose relationship between catalytic and channel-gating activities of purified CFTR (cystic fibrosis transmembrane conductance regulator). Biochemistry **38**:1463-1468.
37. **Seppen, J., M. Rijnberg, M. P. Cooreman, and R. P. Oude Elferink**. 2002. Lentiviral vectors for efficient transduction of isolated primary quiescent hepatocytes. J. Hepatol. **36**:459-465.
38. **Smith, A. J., J. M. de Vree, R. Ottenhoff, R. P. Oude Elferink, A. H. Schinkel, and P. Borst**. 1998. Hepatocyte-specific expression of the human MDR3 P-glycoprotein gene restores the biliary phosphatidylcholine excretion absent in Mdr2 (-/-) mice. Hepatology **28**:530-536.
39. **Speck, R. R., X. F. Yu, J. Hildreth, and C. Flexner**. 2002. Differential effects of p-glycoprotein and multidrug resistance protein-1 on productive human immunodeficiency virus infection. J. Infect. Dis. **186**:332-340.
40. **Szakacs, G., C. Ozvegy, E. Bakos, B. Sarkadi, and A. Varadi**. 2000. Transition-state formation in ATPase-negative mutants of human MDR1 protein. Biochem. Biophys. Res. Commun. **276**:1314-1319.
41. **Tremblay, M. J., J. F. Fortin, and R. Cantin**. 1998. The acquisition of host-encoded proteins by nascent HIV-1. Immunol. Today **19**:346-351.
42. **van der Bliek, A. M., P. M. Kooiman, C. Schneider, and P. Borst**. 1988. Sequence of mdr3 cDNA encoding a human P-glycoprotein. Gene **71**:401-411.
43. **van Helvoort, A., A. J. Smith, H. Sprong, I. Fritzsche, A. H. Schinkel, P. Borst, and G. van Meer**. 1996. MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. Cell **87**:507-517.
44. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay**. 1982. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1**:945-951.
45. **Zaman, G. J., M. J. Flens, M. R. van Leusden, M. de Haas, H. S. Mulder, J. Lankelma, H. M. Pinedo, R. J. Scheper, F. Baas, and H. J. Broxterman**. 1994. The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. Proc. Natl. Acad. Sci. U. S. A **91**:8822-8826.
46. **Zheng, Y. H., A. Plemenitas, C. J. Fielding, and B. M. Peterlin**. 2003. Nef increases the synthesis of and transports cholesterol to lipid rafts and HIV-1 progeny virions. Proc. Natl. Acad. Sci. U. S. A **100**:8460-8465.
47. **Zufferey, R., T. Dull, R. J. Mandel, A. Bukovsky, D. Quiroz, L. Naldini, and D. Trono**. 1998. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J. Virol. **72**:9873-9880.

Chapter 6

Novel autologous selection marker for mammalian cells in a lentiviral vector system.

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Abstract

Background Lentiviral vectors can transduce non-dividing cells for *ex vivo* gene therapy. To avoid rejection of transduced cells after transplantation, a non-immunogenic selection marker is necessary. RNA polymerase II is required for transcription of messenger RNA in all mammalian cells and can be selectively inhibited by the mushroom toxin α-amanitin at low concentrations leading to subsequent cell death. We investigated whether a mutated RNA polymerase II largest subunit (hRPII^r), which has been described to confer resistance to α-amanitin, could be used as a selection marker in a lentiviral vector system.

Methods The cDNA of this mutant was cloned into a lentiviral vector (lenti-hRPII^r) and was used to transduce HeLa cells, rat hepatoma cells and human fibroblasts to determine α-amanitin resistance.

Results The hRPII^r protein could be visualized by Western blot analysis 4 days after transduction. Transduced HeLa and h35 cells could be grown at 5μM α-amanitin, while more than 99% of non-transduced cells were killed after 4-day incubation. Titers of lenti-hRPII^r were 1.8×10^5 HeLa transducing units per ml. Both dividing and confluent human fibroblasts were resistant against α-amanitin after transduction with lenti-hRPII^r.

Conclusions We have shown that mutant RNA polymerase II can be used as a selection marker in a variety of mammalian cell-types. The toxin acts on the RNA polymerase II, which is a protein that is required in all mammalian cells. Over-expression of RNA polymerase is therefore not likely to be detrimental. Furthermore, because hRPII is an endogenous protein, an immune response is unlikely once *ex vivo* selected cells are transplanted.

Introduction

Lentiviral vectors can transduce non-dividing cells for *ex vivo* gene therapy [10,17]. The optimal transplantation procedure is transduction at a low multiplicity of infection (MOI) to minimize the risk of insertional mutagenesis. It has been shown that random insertion of murine retroviral vectors, multiple integrations and a bias for integration into transcriptionally active genes, may lead to leukemia in *ex vivo* gene therapy settings [3,9,14]. Although Human Immunodeficiency Virus and murine leukemia viruses have a different integration pattern [5], insertional mutagenesis may also be mediated by lentiviral vectors, because Human Immunodeficiency Virus-1 integrates preferentially near or in active genes [20].

Unfortunately, some cell types such as hepatocytes and cells of hematopoietic origin are not transduced with 100% efficiency at low MOI and selection of transduced cells is necessary. The majority of the conventional selection markers have the potential to elicit an immune response. These selection markers include enzymes that inactivate toxic levels of neomycin, hygromycin or puromycin. Reporter genes such as Green Fluorescent Protein (GFP) can be used to select transduced cells by flow cytometry, but this is not a mammalian protein and will induce an immune response as well. In conclusion, the ideal selection marker should be non-immunogenic, should normally be expressed in mammalian cells and act in both dividing and non-dividing cells. Therefore it is important to expand the availability of selectable markers for *ex vivo* gene therapy.

The mushroom *Amanita phalloides* produces two families of toxic peptides, of which one are the amatoxins [7]. A member of the amatoxins, α-amanitin, inhibits the RNA polymerase II in mammalian species [12,15,21]. It has been described that resistant clones of BALB/c 3T3 cells could be selected by low concentrations of α-amanitin. This mutant had a point mutation in RNA polymerase II gene resulting in an asparagine (residue 793)-to-aspartate change that was 500 times more resistant to α-amanitin than the wildtype form [2]. A similar mutation in human RNA polymerase II resulted in a protein that could not bind α-amanitin and therefore conferred resistance to this potent cytotoxin [18].

We investigated the use of this human mutant RNA polymerase II in a lentiviral vector system as an endogenous selection marker for gene therapy.

Materials and Methods

Plasmids

The plasmid pRRLcpptpgkGFPpreSsin containing the hepatitis B virus posttranslational regulatory element, central polypurine tract and the phosphoglycerate kinase promoter driving the expression of GFP (lenti-GFP) was used in our experiments as a negative control. This vector has been described before [23].

The plasmid pAT7h1αAm^r, kindly provided by Marc Vigneron [18], contained the cDNA corresponding to the coding sequences of a mutant RNA polymerase largest subunit (hRPII^r) in frame with six histidines (his-tag) that confers resistance to α-amanitin. The cDNA was excised by partial NheI (Roche) digestion and by XbaI (Pharmacia) digestion. This fragment was inserted into the XbaI site of the lentiviral transfer vector pRRLcpptpgkMCSpreSsin, which contains a multiple cloning site in place of the GFP gene, to obtain the pRRLcpptpgkhRPII^r-his-tagpreSsin vector (lenti-hRPII^r).

Lentiviral Vector production

Lentivirus vectors were produced as described [23] by transient transfection of 293T cells by calcium-phosphate precipitation, pseudotyped with Vesicular Stomatitis Virus-G envelope protein. Virus was titrated on HeLa cells and H35 rat hepatoma cells. The

number of single viable cells was counted to determine the transducing units (TU) per ml.

Cell culture
Human Epithelial Kidney 293T, HeLa cells (human cervical carcinoma), h35 rat hepatoma and F00-129 human fibroblast cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 100 units/ml of penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. HeLa and h35 cells were transduced by incubation of lentivirus for 4 hours with 10 μg/μL DEAE-Dextran (Pharmacia). F00-129 fibroblasts were prior to transduction grown to confluent monolayer and maintained for three days to ensure that all cells had stopped dividing or were seeded at low density. After transduction, cells were washed and selection of resistant cells was started 4 days later by incubation with α-amanitin (Sigma).

Colony stains
After 4 days incubation with α-amanitin, the medium was refreshed and one day later, the cells were fixed and stained in 50% methanol/10% glacial acetic acid/ H_2O containing 0.1% Coomassie Brilliant Blue® G250 and counted as described before [22].

Western blots
Selected cells were washed with phosphate buffered saline (PBS), dislodged in 5mM EDTA/PBS, and sonicated. Protein was determined by bicinchoninic acid solution (Sigma)/0.08% $CuSO_4$ solution (Merck). A total of 15μg protein was diluted in sample buffer containing 20mM dithiothreitol (DTT). Samples were boiled for 3 minutes, chilled on ice and loaded on an 8% polyacrylamide gel.
After one hour, the gels were blotted on nitrocellulose membrane and subsequently washed in 0.05% Tween 20/ 4% milkpowder in PBS for 1 hour. The membrane was washed three times with 0.05% Tween 20 in PBS and subsequently incubated with rabbit polyclonal anti-his-tag antibody (1:200, Santa Cruz) in 0.05% Tween/1% milkpowder in PBS.
The blot was washed three times with 0.05% Tween/1% milkpowder in PBS, followed by incubation with goat-anti-rabbit-alkaline phosphatase (AP, 1:3000) in 0.05% Tween/1% milkpowder in PBS. The blot was washed with 0.05% Tween in PBS for three times, followed by a wash in AP-buffer (100mM Tris, pH 9.5/100mM NaCl/50mM $MgCl_2$) and incubated with NBT/BCIP solution (Roche).

Quantification of proliferation and cell viability
Cell proliferation assay WST-1 (Roche) was used according to the manufacturer's protocol to determine proliferation and cell viability.

Results

H35 rat hepatoma cells were transduced with lentiviral hRPII^r expression vector. Western blot confirmed hRPII^r protein expression by detection of his-tag (Figure 1).

After 4 days, medium was refreshed and the cells were incubated with different concentrations of α-amanitin. Four days later the cells were fixed and stained (Figure 2). All lenti-GFP transduced negative control cells were killed at 10μM α-amanitin. At 5μM α-amanitin residual non-proliferating cells were observed. After withdrawal of α-amanitin, these cells resumed proliferation. To determine the lentiviral vector titers, we counted the number of viable cells after transduction with serially diluted viral supernatant, and selection with 10μM α-amanitin. (Table 1). This resulted in titers in h35 hepatoma cells and HeLa cells of 0.9×10^5 TU/ml and 1.8×10^5 TU/ml respectively. Some resistant cells formed colonies within 4 days of α-amanitin incubation.

To test viability, a WST-assay was performed in non-transduced h35 cells and α-amanitin selected cells (Figure 3A). Viable cells could not be detected with this assay at concentrations of 10μM α-amanitin and higher. At 5μM, less than 1% of WST signal was detected, corresponding with residual viable cells. In hRPII^r transduced h35 cells, more than 50% of cells survived at 5μM. Remarkably, even at a concentration of 160μM hRPII^r transduced h35 cells survived. Time course of α-amanitin induced cell death is shown in figure 3B, h35 cell viability decreased after an incubation of 2 days with 10μM α-amanitin, and all cells were killed after a 4 days.

Dividing and quiescent human fibroblasts were transduced with hRPII^r lentiviral vector (Table 2). Both dividing and non-dividing cells were resistant following transduction with hRPII^r lentiviral vector. The percentage of dividing resistant cells was slightly higher than non-dividing resistant cells. Control transduced cells were all killed after α-amanitin treatment (Table 2).

Discussion

It is important to generate selection markers for *ex vivo* gene therapy to be able to transduce cells with a low multiplicity of infection and still obtain a population of 100% transduced cells. The selection marker should act in a broad range of cell types and the cells should not have a natural resistance to the selection drug. Additionally, the selection marker should be endogenous to reduce immunogenicity and reduce the chance that the expression of the protein is detrimental to the cells.

We show that the recombinant hRPII^r can be used in a lentiviral vector system to select different mammalian cell-types. It is a highly selective procedure, relatively quick and efficient compared to conventional selection markers, such as the antibiotic resistance marker neomycin. Additionally, it acts on both dividing and non-dividing cells.

Lentiviral expression of foreign proteins can induce a rapid induction of an immune response [1,8,11,13,17]. The use of hRPII^r may prevent an immunologic response, because it is an endogenous protein. An endogenous protein with point mutations that affects a single amino acid is unlikely to trigger an immune response. This has been shown in gene therapy for missense mutations in β-glucuronidase deficient dogs [19]. In another hemophilia B model, hemophilic dogs have a missense mutations in the catalytic domain of factor IX (FIX) that changes Gly379 to a Glu residue [6]. These dogs rarely develop inhibitory antibodies to infused canine factor IX. On the other hand, all these animals developed inhibitory antihuman FIX antibodies, if these animals were infused with human FIX [4]. As a result of this, in a clinical trial to treat adult patients with severe hemophilia B by adeno-associated virus vectors, only patients with a missense mutation in FIX were included [16]. Since this selectable marker also features a missense mutation it is most likely that this protein is recognized as self, which makes it feasible for an *ex vivo* gene therapy approach. In addition, hRPII^r lentiviral vectors can yield sufficient titers.

Many primary cell types do not divide *in vitro*, or only if stimulated with growth factors. Therefore, we tested if human fibroblasts, grown to 100% confluence could be transduced and selected. The lentiviral vector rendered both dividing and non-dividing human fibroblasts resistant to α-amanitin. This endogenous protein may therefore provide an alternative for use in *ex vivo* gene therapy to select transduced cells of various origins.

Acknowledgements

We would like to thank Mark Vigneron for providing us with the plasmid pAT7h1αAm^r.

Table 1. Titration of lenti-hRPII^r virus.

	H35 TU/ml	HeLa TU/ml
Lenti-hRPII^r	0.9×10^5	1.8×10^5

H35 and HeLa cells were cultured in 10μM α-amanitin for 4 days to determine the transducing units per ml (TU/ml). HeLa TU/ml of lenti-hRPII^r viral vector preparations were approximately 10 times lower than control lenti-GFP viral vectors (2×10^6 TU/ml).

Table 2. Transduction and selection of human fibroblasts.

	Lentivirus	Lentivirus + α-amanitin	No lentivirus + α-amanitin
Dividing	100%	74%	0%
Non-dividing	100%	70%	0%

F00-129 human fibroblasts were transduced with hRPII^r lentiviral vectors, incubated with or without 10μM α-amanitin for 4 days. Subsequently, surviving fibroblasts were counted. The percentage of selected cells was

normalized to the number of cells cultured without α-amanitin. If cells were dividing, slightly more cells survived α-amanitin treatment. All non-transduced cells were killed.

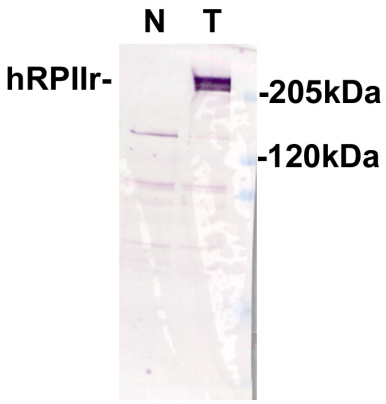


Figure 1. Western blot analysis for hRPII^r protein.
H35 rat hepatoma cells were transduced with lenti-hRPII^r viral vectors expressing the his-tagged hRPII^r (T). Negative control cells were transduced with lenti-GFP (N). Equal amounts of protein were loaded on the gel. In lenti-hRPII^r –transduced cells, bands were observed that migrated in the range of 240kDa (multiphosphorylated) and 214kDa (unphosphorylated) form [18].

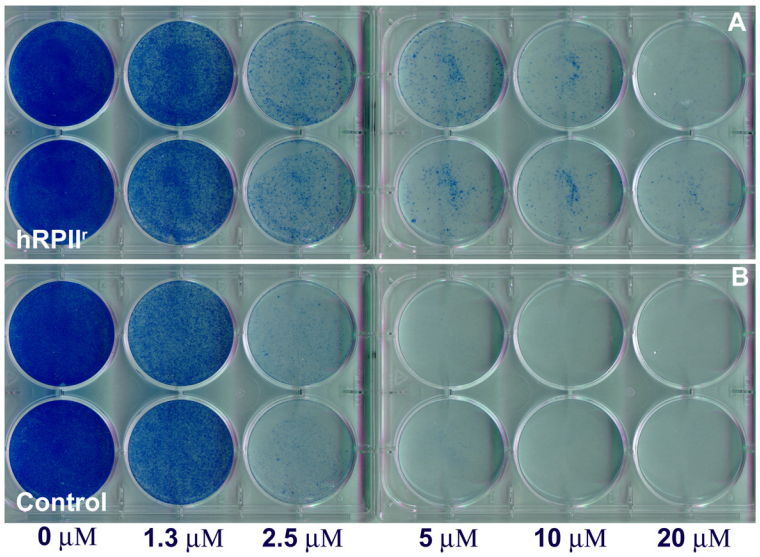


Figure 2. Lenti-hRPII^r confers resistance to α-amanitin.
In h35 hepatoma cells incubated with different concentrations of α-amanitin for 4 days all cells were killed at a concentration of 10μM. At 5μM only very few cells were left (lower panel). A proportion of cells transduced with lenti-hRPII^r viral vectors survived 20μM α-amanitin concentration for 4 days.

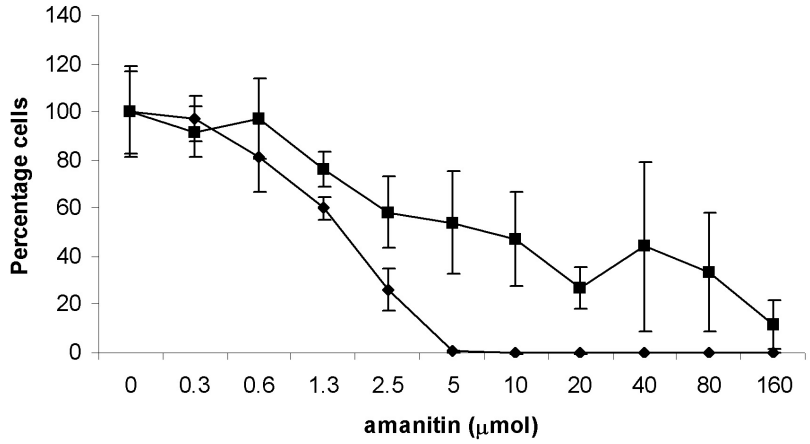
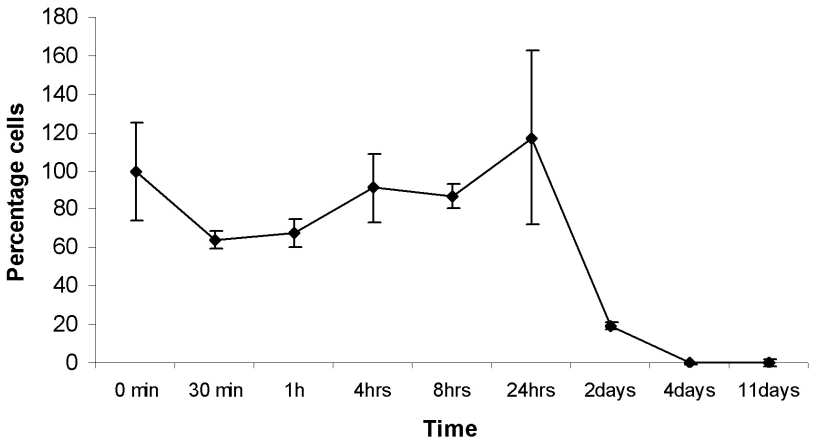


Figure 3. Cell proliferation and viability assay of hRPII^r-transduced h35 hepatoma cells.
(A) H35 rat hepatoma cells were incubated with increasing concentrations of α-amanitin with or without lenti-hRPII^r pre-treatment. At a concentration of 5μM less than 1 percent of cells in non-pretreated cells were viable. In hRPII^r-transduced cells, even at 160 μM cells were still viable.



(B) A significant reduction of cell survival was observed after a two-day incubation with 10μM α-amanitin. All h35 cells were killed after 4 days of 10μM α-amanitin incubation.

References

1. **Aubert, D., S. Menoret, E. Chiari, V. Pichard, S. Durand, L. Tesson, P. Moullier, I. Anegon, and N. Ferry**. 2002. Cytotoxic immune response blunts long-term transgene expression after efficient retroviral-mediated hepatic gene transfer in rat. Mol. Ther. **5**:388-396.
2. **Bartolomei, M. S. and J. L. Corden**. 1987. Localization of an alpha-amanitin resistance mutation in the gene encoding the largest subunit of mouse RNA polymerase II. Mol. Cell Biol. **7**:586-594.
3. **Baum, C., C. Von Kalle, F. J. Staal, Z. Li, B. Fehse, M. Schmidt, F. Weerkamp, S. Karlsson, G. Wagemaker, and D. A. Williams**. 2004. Chance or necessity? Insertional mutagenesis in gene therapy and its consequences. Mol. Ther. **9**:5-13.
4. **Brinkhous, K. M., J. L. Sigman, M. S. Read, P. F. Stewart, K. P. McCarthy, G. A. Timony, S. D. Leppanen, B. J. Rup, J. C. Keith, Jr., P. D. Garzone, and R. G. Schaub**. 1996. Recombinant human factor IX: replacement therapy, prophylaxis, and pharmacokinetics in canine hemophilia B. Blood **88**:2603-2610.
5. **De Palma, M., E. Montini, F. R. de Sio, F. Benedicenti, A. Gentile, E. Medico, and L. Naldini**. 2005. Promoter trapping reveals significant differences in integration site selection between MLV and HIV vectors in primary hematopoietic cells. Blood **105**:2307-2315.
6. **Evans, J. P., K. M. Brinkhous, G. D. Brayer, H. M. Reisner, and K. A. High**. 1989. Canine hemophilia B resulting from a point mutation with unusual consequences. Proc. Natl. Acad. Sci. U. S. A **86**:10095-10099.
7. **Faulstich, H. and T. Wieland**. 1996. New aspects of amanitin and phalloidin poisoning. Adv. Exp. Med. Biol. **391**:309-314.
8. **Follenzi, A., M. Battaglia, A. Lombardo, A. Annoni, M. G. Roncarolo, and L. Naldini**. 2004. Targeting lentiviral vector expression to hepatocytes limits transgene-specific immune response and establishes long-term expression of human antihemophilic factor IX in mice. Blood **103**:3700-3709.
9. **Hacein-Bey-Abina, S., C. Von Kalle, M. Schmidt, M. P. McCormack, N. Wulffraat, P. Leboulch, A. Lim, C. S. Osborne, R. Pawliuk, E. Morillon, R. Sorensen, A. Forster, P. Fraser, J. I. Cohen, B. G. de Saint, I. Alexander, U. Wintergerst, T. Frebourg, A. Aurias, D. Stoppa-Lyonnet, S. Romana, I. Radford-Weiss, F. Gross, F. Valensi, E. Delabesse, E. Macintyre, F. Sigaux, J. Soulier, L. E. Leiva, M. Wissler, C. Prinz, T. H. Rabbitts, F. Le Deist, A. Fischer, and M. Cavazzana-Calvo**. 2003. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science **302**:415-419.
10. **Herzog, R. W. and V. R. Arruda**. 2003. Update on gene therapy for hereditary hematological disorders. Expert. Rev. Cardiovasc. Ther. **1**:215-232.
11. **Izembart, A., E. Aguado, O. Gauthier, D. Aubert, P. Moullier, and N. Ferry**. 1999. In vivo retrovirus-mediated gene transfer to the liver of dogs results in transient expression and induction of a cytotoxic immune response. Hum. Gene Ther. **10**:2917-2925.
12. **Jacob, S. T., E. M. Sajdel, and H. N. Munro**. 1970. Specific action of alpha-amanitin on mammalian RNA polymerase protein. Nature **225**:60-62.
13. **Kung, S. K., A. Bonifacino, M. E. Metzger, G. E. Ringpis, R. E. Donahue, and I. S. Chen**. 2005. Lentiviral vector-transduced dendritic cells induce specific T cell response in a nonhuman primate model. Hum. Gene Ther. **16**:527-532.
14. **Li, Z., J. Dullmann, B. Schiedlmeier, M. Schmidt, C. Von Kalle, J. Meyer, M. Forster, C. Stocking, A. Wahlers, O. Frank, W. Ostertag, K. Kuhlcke, H. G. Eckert, B. Fehse, and C. Baum**. 2002. Murine leukemia induced by retroviral gene marking. Science **296**:497.
15. **Lindell, T. J., F. Weinberg, P. W. Morris, R. G. Roeder, and W. J. Rutter**. 1970. Specific inhibition of nuclear RNA polymerase II by alpha-amanitin. Science **170**:447-449.
16. **Manno, C. S., A. J. Chew, S. Hutchison, P. J. Larson, R. W. Herzog, V. R. Arruda, S. J. Tai, M. V. Ragni, A. Thompson, M. Ozelo, L. B. Couto, D. G. Leonard, F. A. Johnson, A. McClelland, C. Scallan, E. Skarsgard, A. W. Flake, M. A. Kay, K. A. High, and B. Glader**. 2003. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. Blood **101**:2963-2972.
17. **Nguyen, T. H. and N. Ferry**. 2004. Liver gene therapy: advances and hurdles. Gene Ther. **11 Suppl 1**:S76-S84.
18. **Nguyen, V. T., F. Giannoni, M. F. Dubois, S. J. Seo, M. Vigneron, C. Kedinger, and O. Bensaude**. 1996. In vivo degradation of RNA polymerase II largest subunit triggered by alpha-amanitin. Nucleic Acids Res. **24**:2924-2929.
19. **Ponder, K. P., J. R. Melniczek, L. Xu, M. A. Weil, T. M. O'Malley, P. A. O'Donnell, V. W. Knox, G. D. Aguirre, H. Mazrier, N. M. Ellinwood, M. Sleeper, A. M. Maguire, S. W. Volk, R. L. Mango, J. Zweigle, J. H. Wolfe, and M. E. Haskins**. 2002. Therapeutic neonatal hepatic gene therapy in mucopolysaccharidosis VII dogs. Proc. Natl. Acad. Sci. U. S. A **99**:13102-13107.
20. **Schroder, A. R., P. Shinn, H. Chen, C. Berry, J. R. Ecker, and F. Bushman**. 2002. HIV-1 integration in the human genome favors active genes and local hotspots. Cell **110**:521-529.
21. **Seifart, K. H. and C. E. Sekeris**. 1969. Alpha-amanitin, a specific inhibitor of transcription by mammalian RNA-polymerase. Z. Naturforsch. B **24**:1538-1544.
22. **Seppen, J., R. J. Kimmel, and W. R. Osborne**. 1997. Serum-free production, concentration and purification of recombinant retroviruses. Biotechniques **23**:788-790.
23. **Seppen, J., M. Rijnberg, M. P. Cooreman, and R. P. Oude Elferink**. 2002. Lentiviral vectors for efficient transduction of isolated primary quiescent hepatocytes. J. Hepatol. **36**:459-465.

Chapter 7

Novel immortalized human fetal liver cell line cBAL111 with partial hepatocyte specific function *in vitro*, fully differentiates *in vivo*.

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Abstract
Mature human hepatocytes are the preferred cells for *in vitro* applications that depend on hepatic function, such as pharmacological or toxicological assays and bioartificial liver systems. However, mature human hepatocytes are unsuitable for large-scale applications because of their limited availability. Therefore, a cell line that combines both *in vitro* hepatic function and proliferation capacity is desirable. In this study we investigated whether an immortalized human fetal liver cell derived line could be used as an alternative cell source for *in vitro* hepatocyte applications.
Cell clones were derived from human fetal liver cells and immortalized by over-expression of telomerase reverse transcriptase. One of the resulting cell lines, cBAL111, showed evidence of albumin and urea production, mRNA levels of hepatocyte-specific genes similar to the parental cells prior to immortalization, but did not show any evidence of growth in soft agar. Cell line cBAL111 expressed both cytokeratin 18 and 19 and showed high glutathione S transferase pi mRNA levels. In contrast to hepatic cell lines NKNT-3 and HepG2, all hepatic functions and markers were expressed in cBAL111, although there was considerable variation in their levels compared with primary mature hepatocytes. When transplanted in the spleen of immunodeficient mice, cBAL111 engrafted into the liver and differentiated into hepatocytes showing expression of carbamoyl phosphate synthetase without any signs of cell fusion.
We conclude that this novel immortalized fetal liver cell line has the potential to differentiate into mature hepatocytes to be used for *in vitro* hepatocyte applications.

Introduction

Most pharmacological or toxicological assays and bioartificial liver support systems require fully differentiated hepatocytes. The availability of mature human hepatocytes is variable and the numbers low, because they are usually isolated from donor livers not suitable for transplantation. In addition these cells hardly proliferate *in vitro* [2,9]. Since mature human hepatocytes cannot be used for large-scale applications, there is a pressing need for a cell line that combines highly differentiated hepatic functions while maintaining adequate proliferation capacity.

Several cell lines derived from human liver tumours, such as the hepatoma cell line HepG2 [17], as well as *in vitro* immortalized cell lines, like the NKNT-3 cell line, have been investigated [8,10]. In general, these cell lines proliferate adequately, but the levels of hepatocyte-specific functions (*e.g.* urea production from ammonia and cytochrome p450 detoxification activity) remain disappointedly low.

In tumour-derived cell lines, the mutations leading to immortalization are largely unknown. In an attempt to control the immortalization process and therefore prevent at least part of the dedifferentiation process, several immortalized cell lines have been developed. However, although certain genetic modifications in immortalized cell lines are known, spontaneous mutations contributing to the immortalization cannot be excluded.

For successful *in vitro* immortalization, overexpression of cell cycle stimulating genes is generally required. Due to the low proliferation capacity of mature hepatocytes, strong stimulation of cell cycle progression is necessary for immortalization. In the majority of *in vitro* immortalizations of primary human liver cells, the gene encoding Simian Virus 40 Large T antigen (SV40T), an inhibitor of the cell cycle inhibitors p53 and the Retinoblastoma protein, has been used [10,12,19]. In addition, overexpression of Cyclin D1, which stimulates cell cycle progression, and dominant negative mutants of p53 have also led to successful immortalization [29]. In some immortalized cell lines, proliferation was combined with stabilisation of the telomeres [16]. Critically short telomeres induce a terminal state of growth arrest called crisis [3]. Overexpression of the catalytic subunit of telomerase, hTERT, stabilizes telomere length, thereby avoiding cellular crisis. As a general principle immortalization by overexpression of hTERT only, minimises the reduction in functionality [27].

In contrast to mature human hepatocytes, fetal human hepatocytes have the ability to proliferate *in vitro* [11,27], thereby can be immortalized without cell cycle stimulation; in addition telomere stabilisation can immortalize these cells [27]. Wege *et al.* [27] showed that the immortalization of fetal human hepatocytes did not affect their differentiation potential, however, the functionality of the immortalized cells was not compared with mature human hepatocytes. Such comparison is essential to establish the suitability of these cells for hepatocyte applications *in vitro*. In our previous report we demonstrated that primary human fetal liver cells (HFLCs) in culture exhibit albumin production rates and hepatocyte specific mRNA levels comparable to those of primary mature human hepatocytes *in vitro* [5]. However, after eight population doublings most of these functions were decreased to less than 1% of the corresponding function of primary human hepatocytes *in vitro*. This functional loss can be, at least partly, attributed to the presence of non-parenchymal cells in the cell preparation, which eventually outnumber the functional hepatocytes. Therefore, selection of functional cells is necessary if HFLCs are extensively expanded *in vitro*.

In a previous study we already isolated HFLCs and selected specific clones based on their morphology and growth potential [5]. In this study, the telomerase based immortalization technique and the selection of functional human fetal liver cells were combined to obtain new hepatic cell lines. In addition, the hTERT gene was introduced in these cells by lentiviral transduction to restore telomerase activity. The resulting

immortalized cell line was tested for *in vitro* hepatic functions and compared with other well-known hepatic cell lines, more specifically the conditionally immortalized NKNT-3 [10] and the tumour derived HepG2 cells [17]. Furthermore to test whether the resulted novel immortalized cell line had the ability to differentiate into functional hepatocytes; the cell line was transplanted into the spleen of immunodeficient mice.

Materials and Methods

Cell isolation and culture

Human fetal livers were obtained from elective abortions. Gestational age was determined by ultrasonic measurement of the diameter of the skull and ranged from 14 to 18 weeks. The use of this tissue was approved by the Medical Ethical Committee of the Academic Medical Center, Amsterdam, the Netherlands, subject to informed patient consent. We isolated HFLCs on three independent occasions; in each case four fetal livers were pooled. Cells were isolated as described previously [5]. HFLCs were seeded in DMEM culture medium (Dulbecco's modified Eagle's medium, BioWhittaker) containing 10% heat-inactivated fetal bovine serum (HI-FBS, BioWhittaker), 2 mM L-glutamine (BioWhittaker), 1 µM dexamethason (Sigma), 10 µg/mL insulin, 5.5 µg/mL transferrin, 6.7 ng/mL selenium-X (ITS mix, Life Technology), 100 U/mL penicillin, 100 µg/mL streptomycin (penicillin/streptomycin mix, BioWhittaker) at a density of approximately $3*10^5$ cells/cm^2 in Primaria 6-well plates (BD Falcon). Clonal derivatives were obtained by limiting dilution. The selection procedure used and the functionality of the clonal derivatives are described elsewhere [5]. Near-confluent cultures were detached by 5 min incubation with 0.25 % trypsin/0.03% EDTA (BioWhittaker) and split at 1:4 ratios. The number of population doublings (PD) was calculated as $PD = \log (N_f/N_i) / \log 2$, in which N_f is the final number of cells harvested and N_i is the number of cells initially seeded. No corrections were made for cells that did not re-attach after passaging, since their proportion was negligible. The period in which PD number progressed linear with culture time was used to calculate the PD time (T_{PD}).

Mature primary human hepatocytes were isolated from seven patients undergoing partial hepatectomy, because of metastatic carcinoma. The tumour free liver tissue used in each case for the hepatocyte isolation ranged between 2 to 10 grams. The procedure was approved by the Medical Ethical Committee of the Academic Medical Center subject to informed patient consent. The hepatocyte isolation method was adapted from the protocol described by Seglen [22] as previously described [5 4].

NKNT-3 cells were kindly donated by Prof. I. Fox, University of Nebraska, USA. The NKNT-3 cells were cultured on Primaria 6-well culture plates (BD Falcon) and in 75 cm^2 culture flasks using CS-C complete serum free medium (Cell Systems Corporation) with 0.2mg/ml hygromycin B (Invitrogen) and 1 U/ml penicillin/streptomycin (BioWhittaker). Cultures were passaged with a split ratio of 1:5 according to instructions for CS-C medium. Cre-mediated recombination to revert immortalization of NKNT-3 cells [10] was carried out by transduction of the adenoviral vector AxCANCre (Riken DNA Bank (Tsukuba Life Science Center, Japan) as described previously [8]. We analysed both reverted, hence transduced with AxCANCre and selected with G418, and unreverted *i.e.* untreated cells. HepG2 cells were obtained from ATCC (HB-8065) and cultured in Primaria tissue culture flasks in DMEM culture medium as described above for HFLCs.

All cultures were maintained at 37ºC in a humidified atmosphere (95% air, 5% CO_2) and the medium was changed every 2-3 days.

Introduction of hTERT and Green Fluorescent Protein genes

The cDNA of the human telomerase reverse transcriptase (hTERT) gene, kindly provided by R.L. Beijersbergen, Netherlands Cancer Institute, the Netherlands, was

introduced in the cells by lentiviral transduction. The lentiviral vector backbone was described as LTRCMVR2 by Markusic et al. [14] and was produced as previously described [23]. In brief, HEK 293T cells were transiently transfected by calcium phosphate precipitation with a third generation lentiviral vector system. Virus containing supernatant was collected at 24 and 48 hours following transfection, filtered through 0.45 µm Millipore filters, concentrated by centrifugation and added to the culture medium of the HFLCs. The lentiviral vector contained a cytomegalovirus promoter controlling the expression of a reverse tetracycline (Tet) responsive transcriptional activator and a Tet responsive element controlling the expression of the hTERT gene. In this system hTERT transcription was increased by adding 1 µg/mL doxycyclin to the medium. The hTERT cDNA was introduced in three independent HFLC cultures and in five clonal derivatives, *i.e.* cBAL08, cBAL09, cBAL20, cBAL21 and cBAL29. Cells were passaged twice before integration of the lentiviral vector was confirmed by PCR using genomic DNA of transduced cells as template.
In the transplantation experiment cBAL111 cells were marked with Green Fluorescent Protein (GFP) by transduction using lentiviral construct pRRLcpptPGKGFPpreSsin [23] carrying the GFP gene under control of a phosphoglycerate kinase promoter. By fluorescence-activated cell sorting it was demonstrated that $\geq$ 95% of the transduced cells were GFP positive. There were at least five passages between transduction with GFP and transplantation of the cells.

Hepatocyte function tests
Hepatocyte function tests were performed at confluence in 6-well plates. After washing the cells twice with phosphate buffered saline (PBS, NBPI International) culture medium was replaced by 2.5 mL of test medium (William's E medium with 4% HI-FBS, 2 mM L-glutamine, 1 µM dexamethason, 20 mU/mL insulin (Novo Nordisk), 2 mM ornithine (Sigma-Aldrich), 100 U/mL penicillin, 100 µg/mL streptomycin, 0.5 mM NH_4Cl). Medium samples were taken after 0 and 72 hours of incubation. The cells were then washed twice with PBS and stored at -20°C for protein determination. All experiments were performed in triplicate.

Biochemical assays
Urea concentrations were determined using the blood urea nitrogen test (Sigma Chemical Co). Albumin concentrations were determined via enzyme linked immunosorbent assays using cross-absorbed goat-anti-human albumin antibodies (Bethyl). Total protein/well was quantified by spectrometry using Coomassie blue (Bio-Rad). Production rates were established by calculating the changes in concentration during time and corrected for protein content.

RT-PCR
RNA was isolated from the cell lines by using TRIzol (Boehringer Mannheim). As a reference, human liver samples were included in the analyses. First strand cDNA was generated from 500 ng of total RNA using 20 pmol of gene-specific RT primers specific for the mRNA of Albumin, α-1-Antitrypsin (AAT), Transferrin, Alpha-fetoprotein (AFP), π class Glutathione S transferase (GST π) and hTERT in combination with 5 pmol of RT primer for 18S ribosomal RNA and 134 units Superscript III (Invitrogen). Real-time reverse transcription PCR (RT-PCR) using SYBR green I (Roche) was performed as described previously [7]. The sequences of the RT and PCR primers and PCR conditions are given in Table 1.
Starting levels of mRNA, except for hTERT, were calculated by analyzing linear regression on the Log (fluorescence) per cycle number data using LinRegPCR software

[21]. Starting levels of hTERT mRNA were calculated by standard curve analysis, using serial dilutions of hTERT containing plasmid ranging from 10^2 to 10^9 copies/reaction and LightCycler software (Roche). The mRNA starting levels of Albumin, AAT, Transferrin, AFP, GST π, and hTERT were normalised for the starting levels of 18S ribosomal RNA. Normalised mRNA levels, except for hTERT mRNA levels, are expressed as a percentage of the mean mRNA starting levels of the two liver samples normalised for 18S ribosomal RNA starting levels.

Immunocytochemistry

For the detection of glutamine synthetase (GS), cBAL111 cells were cultured on 8-wells culture-slides (BD Falcon) for two days. Then the cells were washed twice with PBS and fixed by a 10-minutes incubation with ice-cold methanol-acetone-water mixture (2:2:1). Cells were incubated with 70% ethanol for 5 minutes, washed with PBS and subsequently incubated overnight with monoclonal GS antibody (Transduction Laboratories, Lexington, KY, G45020) diluted 1:1000. Antibody binding was visualized with the indirect unlabelled antibody peroxidase anti-peroxidase (PAP) method [24].

For the detection of cytokeratin (CK) 18 and 19, cBAL111 cells were seeded on Immunoslides (ICN, Aurora, Ohio, USA) and cultured for 3 days. Cells were washed once in PBS with 0.1% Tween-20 and fixated in 4% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were washed as before and incubated with a blocking buffer (3% BSA, 0.2% Fishgelatin (Sigma), 2%FCS) for one hour at room temperature. After a further washing step, the cells were incubated with the primary antibody for one hour at room temperature. As primary antibodies we used mouse-anti-human CK18 (sc-6259, Santa Cruz) and mouse-anti-human CK19 (Santa Cruz) for detection of CK 18 and 19, respectively. Cells were washed again as before and incubated with 28 µg/mL Cy2 conjugated goat-anti-mouse IgG and 1 µg/mL tetramethylrhodamine isothiocyanate (TRITC) conjugated phalloidin for 1 hour in a humidified chamber at room temperature. Forty minutes before the end of this incubation period, 20 ng/mL Diamidinophenylindoldiacetate (DAPI) was added. Cells were washed again as before and embedded in Polymount (Polyscience, Washington, USA) and covered with a coverslip. Slides were analysed using an Axiovert 200 fluorescence microscope.

Soft Agar assay

Cells were added to 0.35% low-melting-temperature agarose (Seaplaque) containing DMEM culture medium as described above and transferred at a density of 5000 cells/well to 6-well plates previously lined with 0.5% agar DMEM culture medium. After 15 days, the colonies were stained with 0.005% Crystal violet and counted.

Transplantation

The cBAL111 cells overexpressing GFP were transplanted into 6 week old $Rag2^{-/-}\gamma c^{-/-}$ mice [28]. The mice were anesthetized with an intraperitoneal injection of FFM mixture (2.5 mg Fluanisone/0.105 mg Fentanyl citrate/0.625 mg Midazolam HCl/kg in H_2O, 7 mL/kg). One million GFP-marked cBAL111 cells suspended in 100 µL HBSS were injected into the inferior tip of the spleen as described [20]. Nine or 34 days after transplantation, liver and spleen were harvested after *in vivo* fixation. For *in vivo* fixation, mice were anesthetized as described above and 25 mL PBS was flushed trough the circulation, followed by 25 mL 2% paraformaldehyde (PFA) in PBS. Subsequently, liver and spleen were harvested and cut into pieces of approximately 0.2 cm^3. The tissue pieces were incubated in 4% PFA in PBS for four hours, followed by an overnight incubation in 30% sucrose solution. Tissues were snap frozen in liquid nitrogen and stored at –80°C.

Immunohistochemistry
Cryosections were 6 µm thick and were mounted on poly-L-lysine coated slides. Sections were incubated in Teng-T (10 mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin and 0.05% Tween-20, pH 8.0) for 30 minutes before incubation with primary antibodies. Human mitochondria were visualized with a mouse-anti-human mitochondria antibody (Chemicon International) in a 150-fold dilution; vimentin was visualized with a mouse-anti vimentin antibody, clone 9 (Boehringer Mannheim) in a 1000-fold dilution; carbamoylphosphate synthetase (CPS) was visualized with rabbit-anti-CPS antibody in a 1500-fold dilution and glutamine synthetase (GS) was visualized with monoclonal GS antibody (Transduction Laboratories) in a 500-fold dilution. As a secondary antibody Alexa594 conjugated goat-anti mouse IgG (Molecular Probes) was used in a 1000-fold dilution for the detection of vimentin and human mitochondria and in a 250-fold dilution for detection of GS. CPS antibodies were detected with Alexa594 conjugated goat-anti rabbit IgG (Molecular Probes) in a 250-fold dilution. Slides were mounted in Vectashield containing 1 µg/mL 4,6-diaminidino-2-phenylindole (DAPI) to counterstain DNA.

FISH analysis
FISH analysis was performed on 6 µm sections of paraffin embedded liver tissue as described before [15]. Briefly, sections were treated to remove paraffin and the sections were denatured in 70% formamide for 2.5 minutes. 150 ng Biotin 11-dUTP labeled human genomic DNA, 200ng digoxigenin 11-dUTP labeled murine genomic DNA and 5 µg salmon sperm DNA were denatured together and hybridized overnight at 37 ºC with the sections. After washing the slides, human DNA was visualized with FITC anti-avidin followed by biotinylated anti-avidin antibodies (Vector laboratories), whilst mouse genomic DNA was visualized with sheep rhodamine anti-digoxigenin followed by Texas red anti-sheep antibodies (Vector laboratories). Slides were mounted in Vectashield (Vector laboratories) containing 1 µg/mL DAPI to counterstain DNA.

Results
Human TERT introduction in HFLCs and clonal derivatives
The maximum number of PDs between the HFLC preparations and clonal derivatives were different. Expression of hTERT was detected in some of the clonal derivatives analysed, however, all HFLCs and clonal derivatives eventually entered a state of terminal growth arrest (Table 2).
After lentiviral introduction of the hTERT gene, the presence of the hTERT cDNA was confirmed in HFLCs and clonal derivatives by PCR on genomic DNA (results not shown). However, the HFLC cultures did not overcome the terminal growth arrest after the introduction of the hTERT gene; only one of the HFLC cultures' lifespan was extended by 30%. In addition, only one of the clonal derivatives was able to overcome the terminal growth arrest after the introduction of hTERT. This clone, cBAL08, previously showed the longest life span of 61 PDs and a relatively high endogenous hTERT expression (Table 2). Because the transduced cell line was capable of more than 120 PDs, which is twice the life span of the parental cell line cBAL08, and still did not show any sign of growth arrest, we considered this cell line to be immortalized and named it cBAL111. The hTERT mRNA levels of cBAL111 were $1x10^5$-fold higher as compared to its parental cell line cBAL08 (Table 2).

Characterisation of cBAL111 in vitro
The *in vitro* functionality of cBAL08 and cBAL111 was compared after reaching confluence. No significant difference was detected between cBAL08 and cBAL111 for urea production. Furthermore the mRNA levels of Albumin, AAT, Transferrin, GSTπ and

AFP differed less than 2.5-fold (Table 3). So, the immortalization of cBAL08 maintained most of the investigated hepatic functions.
When cBAL111 cells were cultured for 2-3 days, all cells stained positive for the hepatocyte markers GS and CK 18, and the cholangiocyte marker CK 19 (Fig. 1). The CK18 staining was predominantly around the nucleus reaching into the cytoplasm, which is similar to the CK18 staining of primary human hepatocytes that are dedifferentiating *in vitro* [1]. These data suggest that under these conditions the cBAL111 cells were not fully differentiated into hepatocytes, but rather expressed progenitor cell characteristics.
To investigate the putative tumorigenicity of cBAL111, the cells were seeded in soft agar. The cBAL111 cells were not able to form colonies in soft agar, where HepG2 as positive control, formed 61 ± 21 colonies from 5000 cells. So, cBAL111 cells could not grow in an anchorage independent way suggesting that the cBAL111 cells are not tumorigenic.

Comparison of cBAL111 with other hepatic cell lines and mature hepatocytes
The novel *in vitro* immortalized fetal hepatocyte cell line cBAL111 was subsequently compared with the hepatoblastoma derived cell line HepG2, the conditionally immortalized hepatocyte cell line NKNT-3 and primary (mature) human hepatocytes two days after seeding. HepG2, cBAL111 and NKNT-3 cells, both un-reverted and reverted for immortalization, synthesized urea, at a level 11-32 fold lower than mature hepatocytes. The HepG2 cells and to a lesser extent the cBAL111 cells produced albumin in contrast to NKNT-3 cells. In cBAL111 and NKNT-3 cells, the mRNA levels of albumin, AAT and transferrin, (markers of hepatocyte differentiation) were less than 1% of the corresponding levels in mature hepatocytes *in vivo*. HepG2 cells showed mRNA levels for albumin, AAT and transferrin that were comparable to mature hepatocytes *in vivo*. As markers associated with immature hepatocytes, AFP mRNA levels of cBAL111 and NKNT-3 cells were comparable to the level of mature hepatocytes *in vivo,* while the GST π mRNA levels were 10-55 times higher. In HepG2 cells, mRNA levels for AFP were 1000-fold higher than in mature hepatocytes *in vivo* and GST π levels were undetectable.
In summary cBAL111 was positive for all tested hepatocyte parameters, whereas HepG2 and the NKNT-3 cells in all but one.

cBAL111 cells differentiate into functional hepatocytes in murine liver
To determine whether cBAL111 cells have the potential for hepatic differentiation, the cells were marked with GFP by lentiviral transduction and transplanted in the spleen of 4 immunodeficient mice. Nine and 34 days after transplantation, GFP expressing cells were detected in the murine spleen (results not shown) and liver (Fig. 2). The majority of these cells exhibited an elongated morphology, however a small number of cells (~1%) had the morphological characteristics of hepatocytes, given their cuboid appearance. No differences were observed between the livers harvested at nine and 34 days after transplantation. The GFP positive cells were confirmed to be from human origin by immunohistochemistry using a human specific antibody binding to mitochondria (Fig. 2). Immunohistochemistry using an antibody against vimentin, a marker for dedifferentiated and mesenchymal cells [18], indicated a high expression in cBAL111 *in vitro* (data not shown) and in the elongated cBAL111 cells found in the mouse liver, whereas the cBAL111 cells with cuboid appearance did not or hardly expressed vimentin (Fig. 2). Furthermore these GFP positive hepatocyte-looking cells with cuboid morphology were indistinguishable from the surrounding mice hepatocytes with regards to CPS expression, which is expressed periportally [6]. In contrast, elongated cBAL111 cells did not express CPS. *In vitro* GS expression (Fig. 1), was absent in the transplanted cells in

the periportal areas. No GFP positive hepatocytes were detected in pericentral areas, the site of GS expression in normal liver. A possible explanation for this is that the cells may have entered the liver via the portal vein and engrafted before reaching the pericentral area.
We then tested whether the GFP positive hepatocytes were the result of fusion between human cBAL111 cells and murine hepatocytes [26], by using FISH analysis. The results showed that nuclei reacting to the human probe (Fig. 3 A and D) were negative for the murine probe (Fig. 3 C and F). From the shape of the nuclei and the localization of cells, we concluded that these were cBAL111 derived hepatocytes.

Discussion

In this study we have shown that the immortalized human fetal liver cell line cBAL111 displays hepatocyte-specific functions, but is not fully differentiated *in vitro*. However, the cBAL111 cell line has the potential to fully differentiate into hepatocytes when transplanted into immunodeficient mice. We therefore conclude that our novel immortalized fetal liver cell line cBAL 111 has the potential to differentiate to mature hepatocytes, but that current culture conditions do not sufficiently support full hepatic differentiation for *in vitro* hepatocyte applications.
In contrast to Wege *et al*. we could not immortalise unselected HFLC preparations [27], although we were able to extend the life span of the cells by about 30%. In our study we selected cells with cuboid morphology from the initial cell preparation and of these selected cells only the cell line with the highest proliferation capacity, cBAL08, became immortal by overexpression of hTERT. The reduced ability of immortalization in the present study may be the result of differences in transduction levels observed in the two studies. Wege *et al.* used a constitutively active Moloney murine leukemia promoter to drive hTERT expression [27]; we used a tetracycline inducible expression system, stimulated with 1 µM doxycyclin, to drive hTERT expression. Because Moloney murine leukaemia vectors are prone to transduce rapidly dividing cells, this might have resulted in the selection of cells prone to immortalization.
In line with the conclusion of Wege *et al.* that telomerase-induced immortalization of HFLCs does not affect their differentiation potential [27], immortalization of cBAL08 did not change the urea production rate and the hepatocyte specific mRNA levels of the cells. Furthermore, cBAL111 was not able to form colonies in soft agar and no tumours were found in immunodeficient mice 34 days after transplantation, suggesting that cBAL111 is not tumorigenic. We conclude that telomerase reconstitution can immortalize fetal human hepatocytes without loss of function and without resulting into tumorigenicity.
To our knowledge this is the first hTERT-immortalized human fetal liver cell line. The clonal origin of the cell line is most important for preservation of the phenotype during long-term culturing. The stability in phenotype was previously shown for cBAL08 [5]. This is in contrast to cultures of hepatocyte isolates from mature or fetal origin, either immortalized or not.
In addition to the description of cBAL111, we compared its functionality with that of mature human hepatocytes kept under the same culture conditions. It is rather surprising that such comparison is rarely seen in similar studies describing hepatic cell lines. Mature hepatocytes are currently the only cells that meet the criteria for *in vitro* applications, and therefore are the gold standard when evaluating alternative hepatic cell lines. The performance of our novel hepatocyte line cBAL111 was also compared with two well-known and widely used hepatic cell lines, NKNT-3 [10] and HepG2 [17]. Admittedly all three cell lines, performed considerably less well compared with primary mature human hepatocytes in all the liver parameters tested in this study. The levels of hepatocyte specific functions exhibited by the cBAL111 cell were superior compared

with the other two lines (NKNT-3, HepG2), although still insufficient for most *in vitro* applications.
In concordance with the low level of hepatic differentiation, relatively high mRNA levels of AFP and particularly GST π were observed in cBAL111. Moreover the cBAL111 cells expressed CK19, a marker for cholangiocytes, and in addition CK18, a marker for hepatocytes, in a pattern characteristic of de-differentiated human hepatocytes. Therefore it can be concluded that cBAL111 cells are not fully differentiated *in vitro* into mature hepatocytes, but rather should be regarded as a progenitor liver cell line that has the full potential to differentiate into hepatocytes.
The liver provides an optimal environment for hepatic differentiation of cBAL111. When cBAL111 cells were labelled with GFP and transplanted into the spleen of immunodeficient mice the cells migrated to the liver. This was already shown with human fetal hepatocytes, both immortalized and freshly isolated [13,27]. However, in this study we showed that following engraftment a number of these cells went to become hepatocytes morphologically indistinguishable from murine hepatocytes. These cells had very low or no expression of vimentin, a marker for mesenchymal cells and for the undifferentiated cBAL111 cells. In addition, the cuboid cells adapted the zonal expression pattern of CPS and GS characteristic for the surrounding cells [6]. With FISH analysis, we excluded the possibility that these GFP positive hepatocytes were the result of fusion between GFP labelled cBAL111 cells and murine hepatocytes. Fusion between host liver cells and transplanted stem cells, specifically haematopoietic stem cells, has been widely reported to account for high rates of transdifferentiation [25,26]. Our experiments confirm that the cBAL111 line is able to differentiate into hepatocytes when the right differentiation stimuli are present.
However, the fact that a significant number of the transplanted cells did not adapt the hepatocyte morphology and expressed high levels of vimentin suggests that either not all cBAL111 cells were equally sensitive to differentiation stimuli or that not all cells were exposed to the same levels of differentiation stimuli due to micro-environmental variations. This requires further investigation.

In conclusion the development of a cell line that combines both *in vitro* hepatic function and proliferation capacity is important for large-scale applications that depend on *in vitro* hepatic functionality. In this study we present evidence of a novel cell line cBAL111, which is a telomerase immortalized fetal human hepatocyte cell line capable to differentiate into mature hepatocytes *in vivo*.
The potential of this novel cell line merits further investigation. The challenge is to define the best possible experimental conditions *in vitro* to mimic as closely as possible the differentiation stimuli present *in vivo* aiming to achieve a high degree of differentiation into mature hepatocytes *in vitro*.

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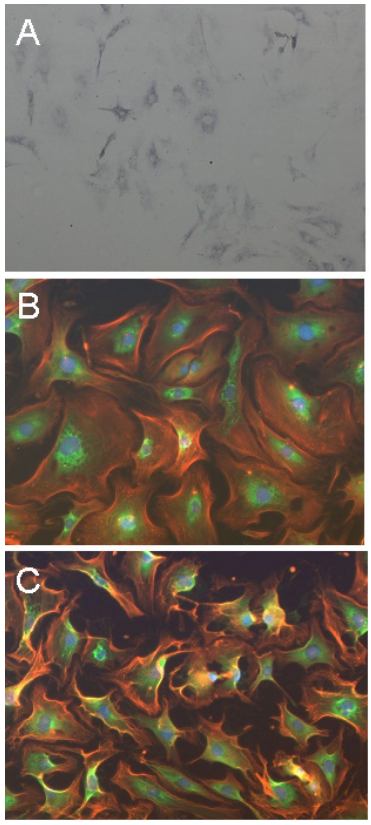

Figure 1. Immunostainings of cBAL111 cells *in vitro*.
The cells were stained using antibodies against GS (A) (blue, 40x magnification), CK18 (B) or CK19 (C) (both green, 200x magnification). In the CK18 and CK19 staining, cells were further visualized by phalloidin, binding to actin (red), and DAPI, binding to the nuclei (blue).

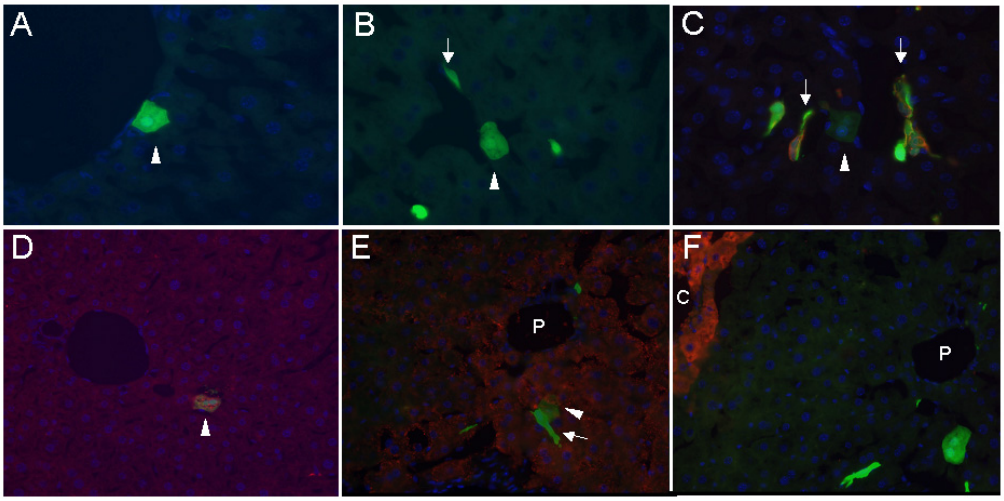

Figure 2. Immunofluorescensce of mouse livers.
(40x magnification), harvested 9 days after transplantation of GFP marked cBAL111 cells (green). All nuclei were visualized by DAPI staining (blue). The majority of cells exhibited an elongated morphology (arrows); a small number of cells adapted hepatocyte morphology (arrowheads, A and B). With hepatic differentiation, the cells lost the expression of vimentin, a marker of undifferentiated mesenchymal cells (red) (C). Human origin of the cells was confirmed using an antibody against human mitochondria (red) (D). Hepatocytes originating from cBAL111 are undistinguishable from the surrounding murine hepatocytes in CPS expression (red) (E). No cells originating from cBAL111 were found in pericentral regions; cBAL111 *in vivo* did not express GS (F). In figure E and F the portal vein is indicated with P, the central vein with C.

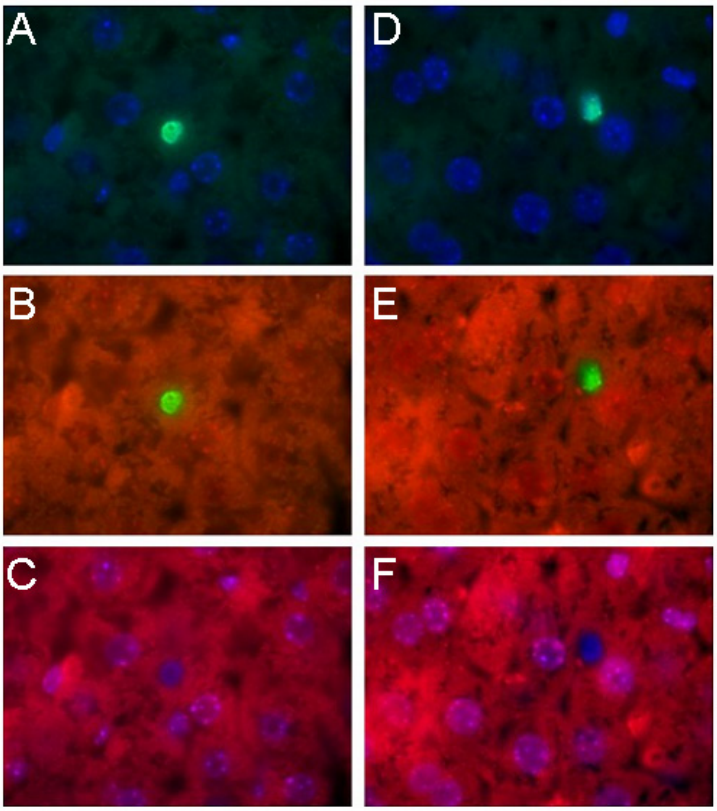

Figure 3. FISH analysis of mouse livers after transplantation of GFP marked cBAL111 cells.
(40x magnification) The probe hybridizing with human DNA was visualized using a FITC labeled antibody (green, A, B, D and E), the probe hybridizing with murine DNA was visualized using a Texas Red labeled antibody (red, B, C, E and F). All nuclei were counterstained using DAPI (blue A, C, D and F). Picture sets A-C and D-F both show a human cell that is negative for the murine DNA.

Table 1: Primers and conditions used in RT-PCR analysis

Gene	Sense primer 5'→ 3'	Antisense primer 5'→ 3'	Application	Size amplicon (bp)	PCR conditions	
					Dilution template	Annealing temp. (°C)
18S rRNA		CGAACCTCCGACTTTCGTTT	RT	Not applicable		
AAT		GGGGGATAGACATGGGTATGG	RT			
AFP		CGTTTTGTCTTCTCTTCCCC	RT			
Albumin		ACTTCCAGAGCTGAAAAGCATGGTC	RT			
GSTπ		AGCAGGTCCAGCAGGTTG	RT			
hTERT		CAGAGCAGCGTGGAGAGGATG	RT			
Transferrin		CCAGACCACACTTGCCCGCTATG	RT			
18S rRNA	TTCGGAACTGAGGCCATGAT	CGAACCTCCGACTTTCGTTT	PCR	151	1000x	68→63
AAT	ACAGAAGGTCTGCCAGCTTC	GATGGTCAGCACAGCCTTAT	PCR	181	-	68→63
AFP	TKCCAACAGGAGGCYATGC	CCCAAAGCAKCACGAGTTTT	PCR	306	-	62→55
Albumin	TGAGCAGCTTGGAGAGTACA	GTTCAGGACCACGGATAGAT	PCR	189	-	68→63
GSTπ	GCCAGAGCTGGAAGGAGG	TTCTGGGACAGCAGGGTC	PCR	333	10x	70→63
hTERT	CGTACTGCGTGCGTCGGTAT	GGTGGCACATGAAGCGTAGG	PCR	233	-	68→63
Transferrin	GAAGGACCTGCTGTTTAAGG	CTCCATCCAAGCTCATGGC	PCR	310	-	68→63

Sequences of the primers used in Reverse Transcriptase (RT) reaction or during real-time PCR reaction (PCR) and the conditions used in the real-time PCR reactions.

Table 2. The life span and the hTERT mRNA levels of three different HFLC isolates, clonal derivatives and cBAL111

Cell source	Maximal PDs	hTERT mRNA copies / 18S rRNA copies
HFLCs, 16 weeks	57.6 ± 10.2	Undetectable (n=4)
cBAL08	61	$2.7 * 10^4$ (n=1)
cBAL09	30	Not determined
cBAL20	38	Undetectable (n=1)
cBAL21	31	Undetectable (n=1)
cBAL29	42	$2.4 * 10^5 \pm 1.9 * 10^5$ (n=3)
cBAL111	Immortal	$2.6 * 10^9 \pm 3.2 * 10^8$ (n=4)

The life span and the hTERT mRNA levels of three different HFLC isolates, clonal derivatives and cBAL111. Life span is indicated as the maximum number of population doublings (PDs).

Table.3: Hepatic functions of different hepatic cell lines and primary mature human hepatocytes.

Function / Cell	Hepatic function		mRNA levels Mature hepatic marker			mRNA levels Immature hepatic marker	
	Albumin production (ng/h/mg protein)	Urea production (nmol/h/mg protein)	Albumin	Transferrin	AAT	GSTπ	AFP
Mat Hep	37.7 ± 7.7	91.5 ± 33.7	130	245	121	535	ND
cBAL08	0.3 ± 0.4	4.0 ± 1.6	0.02 ± 0.01	0.20 ± 0.14	0.32 ± 0.14	1000 ± 565	78 ± 61
cBAL111	0.7 ± 0.8	8.0 ± 6.6	0.02 ± 0.01	0.18 ± 0.16	0.13 ± 0.13	1944 ± 1010	89 ± 72
HepG2	2.8 ± 0.3	4.7 ± 0.2	63 ± 6	896 ± 110	199 ± 56	Undetectable	93353 ± 13228
NKNT-3 reverted	Undetectable	4.9 ± 9.4	0.24 ± 0.22	0.16 ± 0.12	0.92 ± 1.52	952 ± 1164	117 ± 166
NKNT-3 unreverted	Undetectable	2.9 ± 5.2	0.42 ± 0.66	0.20 ± 0.13	0.26 ± 0.34	4427 ± 5366	65 ± 79

Hepatic functions of different hepatic cell lines and primary mature human hepatocytes (Mat Hep). Messenger RNA levels are expressed as a percentage of the mean mRNA levels of the two liver samples. ND = not determined.

References

1. **Blaheta, R. A., B. Kronenberger, D. Woitaschek, M. K. Auth, M. Scholz, S. Weber, H. Schuldes, A. Encke, and B. H. Markus**. 1998. Dedifferentiation of human hepatocytes by extracellular matrix proteins in vitro: quantitative and qualitative investigation of cytokeratin 7, 8, 18, 19 and vimentin filaments. J. Hepatol. **28**:677-690.
2. **Chen, H. L., H. L. Wu, C. C. Fon, P. J. Chen, M. Y. Lai, and D. S. Chen**. 1998. Long-term culture of hepatocytes from human adults. J. Biomed. Sci. **5**:435-440.
3. **Colgin, L. M. and R. R. Reddel**. 1999. Telomere maintenance mechanisms and cellular immortalization. Curr. Opin. Genet. Dev. **9**:97-103.
4. **Deurholt, T., L. Ten Bloemendaal, A. A. Chhatta, A. C. W. A. van Wijk, K. Weijer, J. Seppen, R. P. J. Oude Elferink, R. A. F. M. Chamuleau, and R. Hoekstra**. Cell Transplant., in press.
5. **Deurholt, T., L. ten Bloemendaal, A. A. Chhatta, A. C. W. A. van Wijk, K. Weijer, J. Seppen, R. P. J. Oude Elferink, R. A. F. M. Chamuleau, and R. Hoekstra**. 2006. In vitro functionality of human fetal liver cells and clonal derivatives under proliferative conditions. Analytical Biochemistry.
6. **Haussinger, D., W. H. Lamers, and A. F. Moorman**. 1992. Hepatocyte heterogeneity in the metabolism of amino acids and ammonia. Enzyme **46**:72-93.
7. **Hoekstra, R., T. Deurholt, P. P. Poyck, L. ten Bloemendaal, and A. A. Chhatta**. 2005. Increased reproducibility of quantitative reverse transcriptase-PCR. Anal. Biochem. **340**:376-379.
8. **Hoekstra, R., T. Deurholt, L. ten Bloemendaal, M. Desille, A. C. van Wijk, B. Clement, R. P. Oude Elferink, T. M. van Gulik, and R. A. Chamuleaut**. 2006. Assessment of in vitro applicability of reversibly immortalized NKNT-3 cells and clonal derivatives. Cell Transplant. **15**:423-433.
9. **Katsura, N., I. Ikai, T. Mitaka, T. Shiotani, S. Yamanokuchi, S. Sugimoto, A. Kanazawa, H. Terajima, Y. Mochizuki, and Y. Yamaoka**. 2002. Long-term culture of primary human hepatocytes with preservation of proliferative capacity and differentiated functions. J. Surg. Res. **106**:115-123.
10. **Kobayashi, N., T. Fujiwara, K. A. Westerman, Y. Inoue, M. Sakaguchi, H. Noguchi, M. Miyazaki, J. Cai, N. Tanaka, I. J. Fox, and P. Leboulch**. 2000. Prevention of acute liver failure in rats with reversibly immortalized human hepatocytes. Science **287**:1258-1262.
11. **Lazaro, C. A., E. J. Croager, C. Mitchell, J. S. Campbell, C. Yu, J. Foraker, J. A. Rhim, G. C. Yeoh, and N. Fausto**. 2003. Establishment, characterization, and long-term maintenance of cultures of human fetal hepatocytes. Hepatology **38**:1095-1106.
12. **Li, J., L. J. Li, H. C. Cao, G. P. Sheng, H. Y. Yu, W. Xu, and J. F. Sheng**. 2005. Establishment of highly differentiated immortalized human hepatocyte line with simian virus 40 large tumor antigen for liver based cell therapy. ASAIO J. **51**:262-268.
13. **Mahieu-Caputo, D., J. E. Allain, J. Branger, A. Coulomb, J. P. Delgado, M. Andreoletti, S. Mainot, R. Frydman, P. Leboulch, J. P. Di Santo, F. Capron, and A. Weber**. 2004. Repopulation of athymic mouse liver by cryopreserved early human fetal hepatoblasts. Hum. Gene Ther. **15**:1219-1228.
14. **Markusic, D., R. Oude-Elferink, A. T. Das, B. Berkhout, and J. Seppen**. 2005. Comparison of single regulated lentiviral vectors with rtTA expression driven by an autoregulatory loop or a constitutive promoter. Nucleic Acids Res. **33**:e63.
15. **Newsome, P. N., I. Johannessen, S. Boyle, E. Dalakas, K. A. McAulay, K. Samuel, F. Rae, L. Forrester, M. L. Turner, P. C. Hayes, D. J. Harrison, W. A. Bickmore, and J. N. Plevris**. 2003. Human cord blood-derived cells can differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion. Gastroenterology **124**:1891-1900.
16. **Nguyen, T. H., G. Mai, P. Villiger, J. Oberholzer, P. Salmon, P. Morel, L. Buhler, and D. Trono**. 2005. Treatment of acetaminophen-induced acute liver failure in the mouse with conditionally immortalized human hepatocytes. J. Hepatol. **43**:1031-1037.
17. **Nyberg, S. L., R. P. Remmel, H. J. Mann, M. V. Peshwa, W. S. Hu, and F. B. Cerra**. 1994. Primary hepatocytes outperform Hep G2 cells as the source of biotransformation functions in a bioartificial liver. Ann. Surg. **220**:59-67.
18. **Pagan, R., I. Martin, A. Alonso, M. Llobera, and S. Vilaro**. 1996. Vimentin filaments follow the preexisting cytokeratin network during epithelial-mesenchymal transition of cultured neonatal rat hepatocytes. Exp. Cell Res. **222**:333-344.
19. **Pfeifer, A. M., K. E. Cole, D. T. Smoot, A. Weston, J. D. Groopman, P. G. Shields, J. M. Vignaud, M. Juillerat, M. M. Lipsky, B. F. Trump, and .** 1993. Simian virus 40 large tumor antigen-immortalized normal human liver epithelial cells express hepatocyte characteristics and metabolize chemical carcinogens. Proc. Natl. Acad. Sci. U. S. A **90**:5123-5127.
20. **Ponder, K. P., S. Gupta, F. Leland, G. Darlington, M. Finegold, J. DeMayo, F. D. Ledley, J. R. Chowdhury, and S. L. Woo**. 1991. Mouse hepatocytes migrate to liver parenchyma and function indefinitely after intrasplenic transplantation. Proc. Natl. Acad. Sci. U. S. A **88**:1217-1221.
21. **Ramakers, C., J. M. Ruijter, R. H. Deprez, and A. F. Moorman**. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. **339**:62-66.
22. **Seglen, P. O.** 1979. Hepatocyte suspensions and cultures as tools in experimental carcinogenesis. J. Toxicol. Environ. Health **5**:551-560.
23. **Seppen, J., M. Rijnberg, M. P. Cooreman, and R. P. Oude Elferink**. 2002. Lentiviral vectors for efficient transduction of isolated primary quiescent hepatocytes. J. Hepatol. **36**:459-465.
24. **Sternberger, L. A., P. H. Hardy, Jr., J. J. Cuculis, and H. G. Meyer**. 1970. The unlabeled antibody enzyme method of immunohistochemistry: preparation and properties of soluble antigen-antibody complex (horseradish

peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J. Histochem. Cytochem. **18**:315-333.

25. **Vassilopoulos, G., P. R. Wang, and D. W. Russell**. 2003. Transplanted bone marrow regenerates liver by cell fusion. Nature **422**:901-904.
26. **Wang, X., H. Willenbring, Y. Akkari, Y. Torimaru, M. Foster, M. al Dhalimy, E. Lagasse, M. Finegold, S. Olson, and M. Grompe**. 2003. Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature **422**:897-901.
27. **Wege, H., H. T. Le, M. S. Chui, L. Liu, J. Wu, R. Giri, H. Malhi, B. S. Sappal, V. Kumaran, S. Gupta, and M. A. Zern**. 2003. Telomerase reconstitution immortalizes human fetal hepatocytes without disrupting their differentiation potential. Gastroenterology **124**:432-444.
28. **Weijer, K., C. H. Uittenbogaart, A. Voordouw, F. Couwenberg, J. Seppen, B. Blom, F. A. Vyth-Dreese, and H. Spits**. 2002. Intrathymic and extrathymic development of human plasmacytoid dendritic cell precursors in vivo. Blood **99**:2752-2759.
29. **Werner, A., S. Duvar, J. Muthing, H. Buntemeyer, U. Kahmann, H. Lunsdorf, and J. Lehmann**. 1999. Cultivation and characterization of a new immortalized human hepatocyte cell line, HepZ, for use in an artificial liver support system. Ann. N. Y. Acad. Sci. **875**:364-368.

Chapter 8

General discussion

Gene therapy may provide an alternative treatment for patients with inherited liver diseases. We investigated whether lentiviral gene therapy could be used to efficiently transduce the liver *in vivo* and explored *ex vivo* lentiviral gene therapy strategies. The results of the individual chapters are discussed below.

Lentiviral vector barriers for efficient hepatocyte transduction (Chapter 2)

There are several routes of administration of lentiviral vectors to the liver, with different limitations for efficiently transducing hepatocytes. Direct injection into the parenchyma is not really an option, because the area of transduction will be local and damage of the site of injection can be considerable [35]. Intraperitoneal injection of lentiviral vectors can lead to hepatocyte transduction [38], as has been shown by both *in utero* and neonatal administration [69,70], but other organs residing in the peritoneal cavity are likely to be transduced as well, such as pancreas and spleen.

Another common route of lentiviral vector administration in mice is through tail vein injection [23,60], but the viral vectors first have to pass through the lungs and the heart before they can enter the liver. A more direct route to target the liver is to inject viral vectors into the portal vein [23,67] or the hepatic artery [46]. In our experiments we chose the portal vein, because in mice it is easily accessible and larger than the hepatic artery. The liver will be the primary organ of passage with the highest chance of transducing liver cells first before extrahepatic transduction takes place. In chapter 2 we show that Kupffer cells form a major barrier with respect to hepatocyte transduction. In contrast, the liver sinusoidal endothelium did not affect hepatocyte transduction. A recently published report showed that enlargement of the size of LSEC fenestrae increased adenoviral gene transfer to hepatocytes in rabbits [42], which was as expected, because in these rabbits the fenestrae were shown to be smaller than the size of the adenoviral vector particles.

On the other hand, because the size of LSEC fenestrae [8,9,73,82] are estimated to be similar of size (100-200 nm) as compared to the size of HIV particles (120-200 nm)[10], the lentiviral particles should not be limited in their passage to hepatocytes. Indeed, we show in chapter 2 that LSEC did not form a barrier for lentiviral vectors to pass to the Space of Disse and transduce hepatocytes. The presence of Kupffer cells, however, was shown to be a major factor that reduced hepatocyte transduction in the liver. The Kupffer cells themselves could be easily transduced and sequestration of viral vector probably caused the reduction of hepatocyte transduction. This is in agreement with studies using other viral vectors, i.e. adenoviral vectors [41,68,74].

We also explored the ability of lentiviral vectors to transduce hepatocytes by injection into the bile duct system, as was previously performed with adenoviral vectors [77] or retroviral vector in regenerating liver [16]. We show that both bile duct cells and hepatocytes could be transduced. The number of transduced hepatocytes was low, possibly due to the inactivation of the lentiviral particles by bile acids and because the bile canaliculi are the dead end of the biliary tree [65], so perfusion is not possible.

The experiments described in chapter 2 indicate that agents that transiently deplete or inactivate the liver of Kupffer cells may be helpful for liver directed lentiviral gene therapy.

Alteration of lentiviral vector tropism to increase hepatocyte transduction (Chapter 3)

Another alternative to direct viral particles to the liver is to change the properties of the therapeutic vehicle. Wildtype HIV-1 can infect T-lymphocytes and macrophages, because its envelope protein gp160 binds to the cellular receptor CD4, which is present

on both these cell-types [22]. Combining properties of different viruses, such as the alteration of the lentiviral vectors by pseudotyping with exogenous envelope proteins may lead to delivery to the site of interest. One of these envelope proteins, VSV-G is often used to replace the gp160 envelope protein to broaden the tropism of the lentiviral particle, and also provides stability to the particle so that concentration of the viral vector by ultracentrifugation is possible. For *in vivo* gene therapy, the broad tropism of VSV-G is unwanted, because expression of the therapeutic gene should be confined to hepatocytes. If viral particles can be targeted to the hepatocyte, the loss of transgene expression in hepatocytes by transduction and activation of antigen-presenting cells (APC's) against cells expressing the transgene product can also be reduced. Because it has been shown that APC's can be easily transduced by VSV-G pseudotyped lentiviral vectors [80]; hence preventing gene expression in these APCs will allow long-term gene expression *in vivo* [11]. Thus, an optimal viral vector should only transduce liver cells and must be stable enough to allow concentration to the high titers necessary for *in vivo* applications.

Besides replacing the HIV-1 envelope protein with exogenous envelope proteins, another option to create hepatocyte specific tropism is to combine properties of different envelope proteins to make a stable and liver-specific envelope protein. One strategy is to fuse a stable envelope protein with an external domain that recognizes a specific receptor on the target cell. To target hepatocytes, the external domain should be from an envelope protein that favors hepatocyte transduction. A member of the family *Paramyxoviridae*, the Sendai virus contains an envelope protein that binds to the asialoglycoprotein receptor, which is present on hepatocytes [48]. This envelope protein has been shown to pseudotype both retroviral [72] and lentiviral vectors [39] resulting in hepatocyte specific gene transfer. The use of the Sendai envelope protein has been hampered by its inability to be concentrated to high vector titer. To circumvent this problem, we fused the external Sendai envelope protein part to the envelope glycoprotein GP64, the baculovirus envelope protein of *Autographa californica* nucleopolyhedrovirus. We chose this strategy, because GP64 is a stable envelope protein, producing a viral vector that can be concentrated to high titers. It has been previously used to co-display the complement-regulatory, decay accelerating factor (DAF, CD55) in HIV-based lentiviral vectors [25].

In our experiments, the use of GP64/Sendai fusion protein to pseudotype lentiviral vectors resulted in low titers, but with specificity towards HepG2 hepatoma cells. Co-expression of the wildtype GP64 glycoprotein during viral vector production increased the titers while retaining specificity. When these GP64/Sendai vectors were injected in mice, there was no increase in hepatocyte transduction, but solely a decrease in Kupffer cell transduction when compared to wildtype GP64.

Our results show that incorporation of the wildtype GP64 glycoprotein into the lentiviral particles in combination with the GP64/Sendai fusion protein does not target to the hepatocyte, but detargets Kupffer cell transduction. Because the GP64/Sendai fusion protein had the highest specificity for HepG2 cells, further modifications may provide a potentially usable vector for the clinic with *in vivo* hepatocyte specificity.

In the future, other viral envelope proteins or chimeric proteins may be generated for liver-directed gene therapy. Possible candidates for chimeric envelope proteins for liver directed lentiviral vectors are proteins displayed on the surface of *Plasmodium* protozoa during malaria liver infection, or combinations of viral envelope proteins derived from hepatitis B [12,15] or C [81], because these proteins already have a liver tropism.

In order to specifically develop envelope proteins that may be suitable for hepatocyte gene therapy, systems that select for the evolution of envelope proteins can be used.

Powell *et al* described a technique in which they used DNA shuffling of envelope genes of ecotropic murine leukemia virus, of which particles are sensitive to stress forces during purification and concentration. Subsequently, this DNA shuffled genes are ligated back in replication-competent retroviral backbones to obtain a retroviral library. Amplification of these clones and repetitive ultracentrifugation resulted in stable clones [62]. The envelope protein, which renders stable viral particles, can be cloned for use in viral vectors.

Problems relating the immune response to viral vector and non-self therapeutic protein (Chapter 4).

The use of viral vectors for gene therapy may have several problems that could influence its efficacy. First of all, because the viral vectors are derived from pathogens, there may be immunity against the viral vector components itself. This may hold for adenoviral vectors and lead to adverse effects for use in the clinic as has been shown in patient trials [63]. In fact, adenovirus vectors are the most immunogenic of all the viral vector groups and this property is the largest hurdle for gene therapists using adenovirus vectors to overcome. Adenovirus vectors induce multiple components of the immune response: cytotoxic T-lymphocyte (CTL) responses can be elicited against 'foreign' transgene products that are produced by transduced cells, and the capsid itself (in the absence of viral gene expression) induces virus-neutralizing antibody responses. Incoming adenovirus capsid components can also enter the major histocompatibility complex (MHC) class I processing pathway and target transduced cells for recognition by pre-existing CTLs [36,76].

In the adenovirus vector field, progress has been made in reducing T-cell responses against viral gene products. These immune associated problems resulted in the development of 'gutted' or 'helper-dependent' (HD) vectors that are stripped of all viral genes [51]. Although these vectors have reduced vector-mediated cytokine responses after systemic administration [14,19], these highly disabled vectors still retained the potential to induce a capsid-mediated inflammatory response in rat brain [76].

Immune-mediated response to components of AAV-vectors has also been described. In a patient trial for the treatment of hemophilia by AAV-factor IX, an immune response was directed to the components of the AAV-2 serotype leading to destruction of the transduced hepatocytes with disappearance of the therapeutic effect [46]. But in general, AAV vectors and also lentiviral vectors are less inflammatory and immunogenic than adenovirus vectors. Immunogenicity to viral vectors may depend and be influenced by serotype differences for AAV and on the pseudotype for lentiviral vectors. Pre-exposure to the wildtype virus is almost certainly detrimental for successful treatment. The use of envelope proteins for *in vivo* lentiviral-mediated gene therapy, such as VSV-G may therefore be risky, because people, such as farmers, could have been pre-exposed to this virus and indeed been infected, although VSV's primary hosts are horses, cattle and pigs [40,64].

Another major problem is the immune response to the transgene product that has been observed in many animal model studies. However, both cell-mediated response and humoral response to the delivered gene depend on a number of variables; including the nature of the transgene, the promoter used, the route and site of administration, vector dose and host factors.

The immune response is most often observed with high viral dose in combination with a ubiquitous promoter controlling transgene expression. If such viral vectors can transduce antigen presenting cells, such as dendritic cells, generation of an immune response to the transgene can occur as has been shown with lentiviral vectors [80].

Viral vector systems based on adenovirus or AAV are less likely to transduce antigen-presenting cells, but earlier exposure in life to adenovirus or AAV may generate an immune response to viral particles [44,46,54]. If HIV-based lentiviral vectors are used for clinical application in patients with hepatic disorders, these patients most likely have not been exposed to wildtype HIV. Hence, generation of an immune response to the lentiviral vector will not be an issue.

The immune response to viral structural components or the transgene [83] can be limited by the use of immunosuppressants, but this is associated with a higher morbidity for the patient, who is more likely to develop infections. The need for immunosuppressants is not easy to solve, but depends on the antigenicity of the therapeutic protein. The immunogenicity of a protein can vary however, as has been observed by the development of neutralizing antibodies in 10-40% of hemophilia A patients to factor VIII and only about 5% of hemophilia B patients to factor IX [79]. For patients with a missense mutation that results in wrong protein folding affecting function, the addition of the functional protein may not lead to an immunological reaction, because the protein is still expressed in these patients, albeit in its mutated form. These patients seem therefore well suited for first enrolment into clinical trials.
The use of a tissue-specific promoter has been shown to reduce immunogenicity and may lead to immune tolerance. One clear example has been the injection of a liver specific promoter containing AAV-vector expressing factor IX in mice [49]. These mice did not develop an immune response to the new protein. In an earlier mentioned study in a clinical trial [46], no immune response was developed against the transgene with the use of a liver specific chimeric promoter. Other studies show similar results. Long-term expression of BUGT was observed by intravenous injection of albumin promoter containing lentiviral vectors into juvenile Gunn rats as a model for Crigler-Najjar disease [78]. Intravenous injection of lentiviral vectors in newborn Gunn rats [55] or in neonatal hemophilia B mice [84], with the appropriate gene (UGT1A1 and factor IX, respectively) driven by hepatocyte-specific promoters resulted in long-term correction of the disease.
The investigation of treatment of neonatal animals is of great importance, because of ethical reasons, i.e. early intervention to correct the genetic disorder to increase quality of life, but also because of practical reasons. In some diseases, the disease development cannot be reversed anymore after achieving a certain disease state. In liver diseases, development of cirrhosis may preclude gene therapy treatment and then the only remaining curative option may be OLT.
We tried to develop gene therapy for the treatment of Crigler Najjar. Crigler Najjar patients have high bilirubin levels, because of a defect in bilirubin UDP-glucuronosyltransferase (BUGT) expression, leading to kernicterus. Phototherapy and liver transplantation are the main treatment options at present, but other treatment modalities are required [7,34].
As described above, the use of liver specific promoters can induce immune tolerance to the transgene. Start of treatment at early time in development may also circumvent an immune response, because the immune system has not been completely matured yet. We therefore investigated whether treatment of *in utero* or neonatal injection into the peritoneum may lead to partial or complete correction of bilirubin levels in Gunn rats. Treatment was successful, because serum bilirubin levels were stably decreased to about 50% of diseased Gunn rats levels [69,70]. But as elaborated in chapter 3, even though initiation of treatment was started at the embryonic or neonatal stage, antibodies were generated against the therapeutic protein.

This can be due to the route of administration, which largely determines the cell-types that are transduced and, of course, another cause could have been our choice to use a ubiquitous promoter in our experimental setup.
The recently published papers of van der Wegen *et al* [78] and Nguyen *et al* [55] show that early intervention in Gunn rats can lead to complete and permanent correction of hyperbilirubinemia with lentiviral vectors. In these studies they did not test whether antibodies were generated against BUGT1. In a comparable study using recombinant retroviral vectors the investigators did check for anti-BUGT1 antibodies, but these were not detected during follow-up of treated animals [5]. Similar results were found in neonatal gene transfer of retroviral vectors expressing human factor IX, which resulted in tolerance to the new protein [84].
The antigen presenting cells (APCs) are the major contributors to induction of an immune response following gene transfer. Although the strategy of tissue-specific promoters to target gene expression to hepatocytes reduces the incidence and extent of the transgene-specific immune response, neutralizing antibodies against the transgene product and immune-mediated vector clearance can still be observed.
Lentiviral vectors integrate *at random*, but with a preference to actively transcribed genomic regions, because these regions are accessible for integration. It has been shown that promoter-enhancer trapping at insertions near active regions of transcription can lead to nonspecific activity. The inclusion of a liver-specific promoter in a lentiviral vector to limit transgene expression to hepatocytes may therefore still lead to transgene expression in APCs depending on the dose used. Brown *et al* demonstrated that specific regulation of microRNAs (miRNAs) to repress translation of cellular transcripts in APCs enabled stable gene transfer in immunocompetent mice [11].
These recently published findings point out the importance of viral vector design and the clinical trial set-up for successful treatment of Crigler Najjar patients.

Future treatment modalities for PFIC3 patients (Chapter 5).
Progressive Familial Intrahepatic Cholestasis type 3 (PFIC3) is caused by mutations in the ABCB4 gene [17]. PFIC3 can be treated with ursodeoxycholic acid (UDCA), but this treatment does not lead to an improved liver function in all patients [6,31] and end-stage PFIC3 can only be treated with liver transplantation. For treatment of PFIC3 several future perspectives and treatment modalities are under development. One such option is the transplantation of hepatocytes or cells that may differentiate to functional hepatocytes. In diseases, such as PFIC3, corrected cells can repopulate the diseased liver, because the transplanted cells have a protective advantage against toxic bile salts. In a mouse model for PFIC3, the Abcb4 knockout mouse model, transplantation of 2×10^6 healthy MDR3 expressing hepatocytes could achieve a repopulation level of about 30% of total hepatocytes [18], which mitigated the effect of toxic bile salts.
Other repopulation models, such as in the Fah knockout mouse model, transplantation of as few as a 1000 *ex vivo* retrovirally transduced hepatocytes showed more than 90% of hepatocytes containing Fah protein after repopulation, and function was restored to normal [58]. Unfortunately, the FAH knockout mice develop tumors over time, which are derived from the remaining endogenous hepatocytes and not the transplanted ones. Tumorigenesis also occurs in the PFIC3 mouse model, although the number of tumors that develop after hepatocyte transplantation is lower [18]. The stop of repopulation in the PFIC 3 mouse model may be due to a certain threshold at which sufficient neutralization of toxic bile salt occurs in the bile duct system and surrounding hepatocytes.
In the development of viral vectors for PFIC3, adenoviral vectors were not considered suitable, because they do not integrate into the host genome and may also elicit an immune response to the viral components. The use of AAV vectors to treat PFIC3 is

hampered by their small size, which cannot include all DNA-elements required for ABCB4 protein production, i.e. the cis-acting elements, such as promoter and trans-acting elements.

As shown in chapter 5, the production of infectious lentiviral vectors is reduced by the co-expression of ABCB4 in the 293T producer cell line. We concluded that this was due to the flippase activity of ABCB4, because we observed an increase of phosphatidylcholine in the lentiviral vector preparations. It is well known that viral membrane composition is critical for maintenance of HIV-1 structure and infectivity, and this has been clearly demonstrated by removal of one such component, i.e. cholesterol of the HIV-1 envelope [13,26].

The first observation of reduced infectivity by expression of ABCB4 was in a lentiviral vector containing the *ABCB4* cDNA driven by a phosphoglycerate kinase promoter. The 293T producer cells expressed ABCB4 protein after transfection, both confirmed by Western blot and immunofluorescence staining (data not shown), but ABCB4 protein could not be detected by these detection methods in transduced target cells, such as HeLa or polarized cells (MDCKII) (data not shown), suggesting that either no viral vector was produced or that the viral vector was not infectious. The *ABCB4* cDNA was also cloned into other lentiviral vector constructs, i.e. with CMV (cytomegalovirus) promoter with IRES (internal ribosome entry site) driving GFP expression and in a lentiviral vector with the liver specific albumin promoter (Figure 1). In the first mentioned lentiviral construct both ABCB4 and GFP were expressed in 293T cells after transfection. However, no infective virus was produced, because after transduction of MDCKII cells, no ABCB4 or GFP expression could be detected. Therefore we designed a construct containing the albumin promoter to reduce expression of ABCB4 protein in the producer cells, and also to achieve hepatocyte specific expression after *in vivo* administration. Protein expression during lentiviral vector production also comes from the genomic viral transcript, because the albumin promoter and transgene are in the same orientation as the RSV (Rous sarcoma virus) promoter, driving production of full-length genomic lentiviral vector messenger with subsequent ABCB4 protein expression. Additionally, protein expression will also come from integrated viral genomic DNA caused by co-transduction during production. Indeed, with the albumin promoter also no ABCB4 lentiviral vector titer was measured.

We conclude that the presence of active ABCB4 during virus production generates viral particles with very poor infectivity. Apparently, proper virus production requires a very strict phospholipid composition of the plasma membrane.

Other phospholipid translocase deficiencies (e.g. PFIC type 1 in which the flippase ATP8B1 is lacking) may give similar problems with respect to the production of lentiviral vectors for gene therapy, because they might interfere with phospholipid composition of the cellular membrane, from which the lentiviral particles bud off. Future modifications in lentiviral vector design may lead to an adequate production of therapeutic vehicle for treatment of PFIC3 patients.

In figure 2, an alternative plasmid design is depicted that may lead to higher vector titer. As shown in the upper part, transfection of the plasmid containing the transfer vector in 293T cells produce the viral RNA strand under control of the RSV promoter containing the cDNA of ABCB4. Because HIV is a positive strand RNA, transcription can directly take place. Additionally, the ABCB4 cDNA being under the control of the internal promoter (e.g. PGK) is also transcribed from the plasmid itself. These processes lead both to high expression of ABCB4 protein influencing the viral vector production negatively.

As shown in the lower part, an improved viral vector can be made by first changing the orientation of the internal cassette in a reverse orientation to stop all translation of ABCB4 protein from the viral transcript. This is commonly used in β-globin lentiviral vectors, in which the regulatory elements, such as introns and exons are necessary for optimal regulation of the therapeutic protein expression [66]. Because the gene contains exons, they are not spliced out of the genomic lentiviral transcript. The HIV Rev/RRE element has to reduce this process as well. These β-globin lentiviral vectors can give high titers to treat thallassemias in animal models [1]. In another lentiviral vector system, containing the mifepristone-inducible system, the trans-activator expression cassette performed optimally when cloned in a reverse-orientation [71]. The reverse orientation should therefore allow efficient production of the lentiviral vector system for *in vivo* applications.
Secondly, it is required to replace the ubiquitous promoter with a liver specific promoter, such as the albumin promoter, to reduce the expression of the transgene (e.g. ABCB4) in 293T cells. The expression of this promoter is expected to be lower than a constitutive promoter, such as PGK, because tissue-specific promoters are generally weaker and the albumin promoter is not supposed to be active in 293T cells. This will also reduce the expression from lentiviral vectors by transduction during production, which can affect viral vector production especially on later harvest days.
Thirdly, to reduce the expression of ABCB4 even more, an RNAi cassette [27,28,59] can be included into the transfer plasmid, directed against the residual ABCB4 transcript. This strategy can only lead to a productive titer if the therapeutic cassette is in the reverse orientation, otherwise the RNA sequences will bind to the viral transcript, which may results in degradation of the viral transcript and low titers.
In the final lentiviral vector other adjustments may be necessary to optimize regulatory expression of ABCB4, because its expression is tightly regulated in the healthy liver and its expression has to move within a narrow window [57].

Another option to increase viral titers of ABCB4 lentivirus is to use a different producer cell line. We found that the above described ABCB4 lentiviral vector with cassettes containing PGK, albumin promoter or CMV promoter could lead to detectable titers if transfection was performed in C33A (human cervical carcinoma, kindly provided by Georgios Pollakis, Laboratory of Experimental Virology, AMC) instead of 293T cells. Unfortunately, we observed that titers of PGK-GFP lentivirus were in general 10-fold lower if produced in C33A cells compared to production in 293T cells. It is therefore likely that this C33A cell line cannot be used to obtain the high titers as with 293T cells.

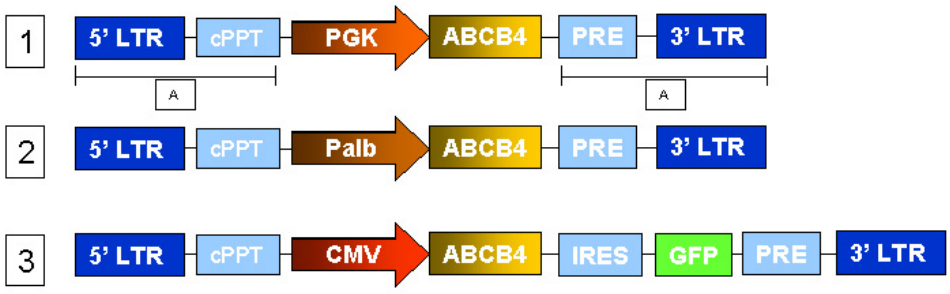

Figure 1. The ABCB4 lentiviral vectors. The backbone (A) of the vectors is the same in all three viral vector constructs. Vector 1 contains the constitutive PGK promoter, vector 2 contains the liver-specific albumin promoter and vector 3 contains the constitutive viral CMV promoter driving both ABCB4 expression and GFP linked by IRES.

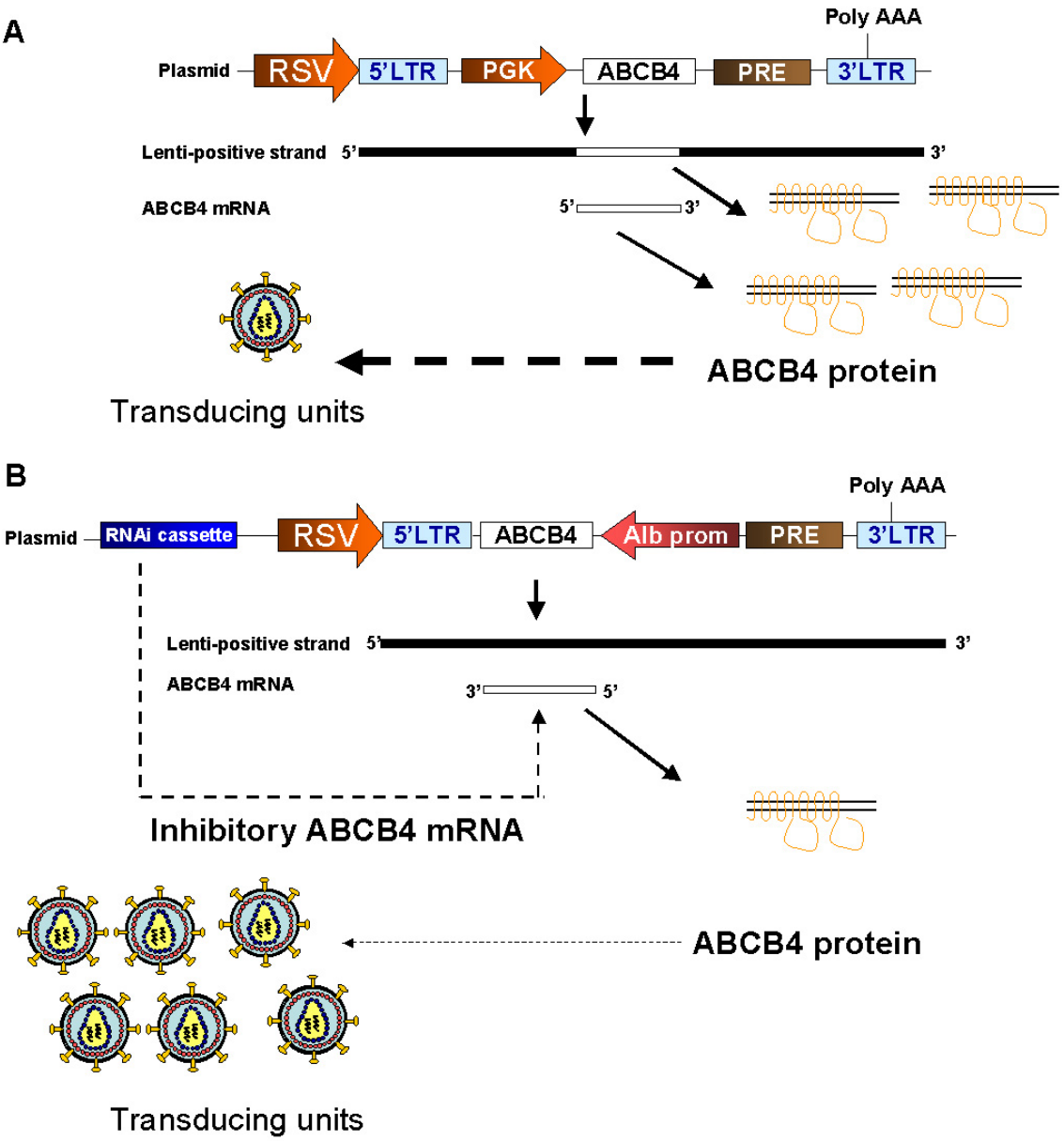

Figure 2. Modifications in ABCB4 lentiviral vector design for efficient titer production. (A) The basic lentiviral vector plasmid containing the viral transfer vector LTR sequences necessary for integration. The therapeutic cassette contains the ubiquitous and constitutive promoter PGK driving ABCB4 expression. The PRE element increases the expression of the viral vector transcript and the ABCB4 transcript. The 3' LTR contains a poly A signal terminating transcription. The RSV promoter activates expression of the complete viral transcript. From both the viral transcript and the ABCB4 transcript translation of ABCB4 protein takes place leading to a reduced number of transducing units.
(B) Modifications in the vector design include a tissue specific promoter, e.g. albumin promoter, which should be inactive in 293T cells. The therapeutic cassette has to be cloned in the reverse orientation. In this setting only background/leakiness of the tissue specific promoter may lead to residual ABCB4 protein expression from the plasmid, but not anymore from the viral transcript. RNAi cassettes can be used to reduce the remaining ABCB4 transcripts. As a consequence of the therapeutic cassette being in the reverse orientation, the RNAi loops will not target the viral transcript. This should lead to higher ABCB4 lentiviral vector titer.

A repopulation model based on an endogenous selection marker (Chapter 6)
In some metabolic liver diseases, transplantation of corrected hepatocytes may give these cells an intrinsic survival advantage over the host hepatocytes, which may lead to an increase in number of the transplanted cells in the liver. However, in the majority of the inherited liver disorders this will not happen, because the defect affects the patient, but not the liver itself. An artificial repopulation system may provide a therapeutic solution for the low grafting efficiency of hepatocytes after administration to the liver. The repopulation system should have its effect at the liver only, with an advantage for the transplanted cells; no other organs should be affected. We were interested in the expression of an endogenous protein that is non-immunogenic with a mutation leading to resistance in hepatocytes.
One such option was the use of the mutated human α-amanitin resistant RNA polymerase II largest subunit (hRPB1^r) [56], which was based on a earlier described mutation in RNA polymerase II that leads to resistance of mouse cells to α-amanitin [2]. RNA polymerase II is expressed in all cells and is mandatory for the production of messenger RNA. All cells require RNA polymerase II, so it is most likely not detrimental if this protein is overexpressed temporarily or long-term. We tested the toxicity of α-amanitin in mice to make sure that the selection system based on hRPB1^r could be applied in an *in vivo* setting. Liver and kidney toxicity by α-amanitin has been described in mice before [20,21,47]. We observed α-amanitin toxicity in FVB mice, which led to the death of these mice within 5 days after administration (data not shown). This was probably also due to the earlier reported development of liver and kidney damage in mice. The intake of the mushroom *Amanita phalloides*, which is often mistaken as an edible mushroom, can also lead to liver and kidney damage in man, but because this mushroom contains a cocktail of toxins (amatoxins and phalloidin) [32], the action of α-amanitin in humans, and its liver specificity, are not 100% clear. It is likely though, that the kidneys in man are also affected by α-amanitin. To still be able to use a system of repopulation by hRPB1^r overexpressing cells and to target α-amanitin to the liver only, it may be conjugated to bile acids to direct the uptake of the toxin into hepatocytes with a concomitant decrease in extrahepatic toxicity.
Because the repopulation system could not easily be applied *in vivo*, an *ex vivo* gene therapy approach to use the selection marker was investigated. We showed in chapter 6, that human fibroblasts and hepatoma cells expressing hRPB1^r could be selected at low concentrations of α-amanitin. For several primary cell types that are difficult to transduce with lentiviral vectors, this selection marker may be useful to select for corrected cells, before these cells are transplanted in the patient.
The use of this system may especially be advantageous to transduce hematopoietic cells at low MOI, because *ex vivo* transduction of retroviral vectors can lead to tumorigenesis by activation of oncogenes [4]. This reduces the risk to develop uncontrolled cell-cycling by insertional mutagenesis.

Transplantation of liver cell lines to correct inherited liver disorders (Chapter 7)
Besides the conventional OLT to treat patients with inherited liver diseases, hepatocyte transplantation has been used to ameliorate the symptoms [24,29,52]. Unfortunately, there remains a shortage of liver donors to use hepatocytes in these patients. Alternatives are therefore necessary. One such alternative is the generation of an unlimited source of cells suitable for transplantation into the liver, e.g. a hepatocyte cell-line. One drawback of using fully differentiated cells, such as hepatocytes, is that these cells quickly dedifferentiate and divide at the most only a couple of times *in vitro*. Because fetal liver cells have the potential to differentiate into hepatocytes and also divide, these cells

might be suitable for the generation of a cell line for transplantation purposes. In chapter 7, we showed that a cell line could be generated from human fetal liver cells (HFLC) by introduction of telomerase reverse transcriptase. Clones of the immortalized HFLCs were selected on their *in vitro* hepatocyte function. One clone with a relatively good *in vitro* function was called cBAL111 and was selected for testing its *in vivo* differentiation potential. Transplantation of this clone into the spleen of immunodeficient mice showed that this cell line still exhibited the potential to fully differentiate into hepatocytes *in vivo*. The low number of cells that differentiated into hepatocytes in our experiments may be due to the fact that the majority of the transplanted cells resided in the portal vein areas and did not migrate into the liver parenchyma. Removal of liver sinusoidal endothelium by cyclophosphamide has been shown to increase hepatocyte engraftment in rats [45] and may also promote differentiation of transplanted cBAL111 cells. One of the main advantages of the use of cells instead of viral vectors to correct a genetic disorder is the advantage that the gene can be regulated in a normal fashion, compared to addition of a viral vector with a constitutive promoter.

Other concerns with gene therapy vectors.
A lot of research for treatment of inherited diseases has been focused on cures for a certain defect through addition of a gene. In fact, all viral vectors act by supplementing additional therapeutic genes to the diseased cell. For lentiviral vectors these include the long terminal repeats that are required for integration, the Rev Responsive Element (RRE), central polypurine tract (cppt), posttranslational regulatory element (PRE). All these sequences are required for optimal viral vector production and high transgene expression, but our goal is to get normal regulation of the corrected gene. Furthermore, by using viral vectors, there will always be a risk to develop tumors by insertional mutagenesis, as long as we cannot regulate the integration pattern of the viral vectors [3,4]. Themis *et al* [75] have shown that even retroviral vector gene therapy may result in oncogenesis in the liver. In this study, nonprimate lentiviral vectors derived from equine infectious anemia virus (EIAV) were injected in fetal and neonatal mice in which hepatocytes still rapidly divide. In the adult liver, however, the risk of integration in a tumor-suppressor gene or the activation of a proto-oncogene is reduced, because the majority of the hepatocytes are in quiescent state. Notably, in the study of Themis *et al* HIV-1 derived vectors did not lead to tumorigenesis. A recent paper shows data of low genotoxicity of lentiviral vectors in a tumor-prone mouse model [50]. Integration in more than one gene involved in cell-cycling is generally considered to be crucial for oncogenesis; therefore, usage of low MOI of viral vector is preferred. Therefore, the consequences of viral vector integration is largely determined by the gene transfer vector, the number of copies per cell, the cell-type and number of treated cells and the rate of proliferation in a given, potentially disease-specific environment.

Another concern for the use of lentiviral vectors in the clinic has been specific safety and ethical issues. Concerns include the possible generation of replication competent lentiviruses during vector production or mobilization of the vector by endogenous retroviruses in the genomes of patients. No replication competent lentiviruses have been detected using third generation lentiviral vectors during production in human cells or after administration in animals. Unfortunately, it has been shown by Logan *et al* [43] that these vectors may still be able to produce full-length vector transcripts after integration in the target cell. To reduce the risk of wildtype reconstitution even more, other vectors have been constructed for Rev-independent production.

Ethical concerns include the possibility of germline transmission of viral vectors and may raise questions whether *in vivo* gene therapy should be applied. MMLV-based vector for *in utero*-transduced rams led to 6 out of 19 PCR positive for provirus in purified sperm, although in very low numbers [61]. Lentiviral vectors were also able to transduce Sertoli cells, but not germ cells in other experiments [30,53]. AAV vectors have not been shown to transduce semen in a human clinical trial [37]. In another study, AAV vectors were detected in gonads and persisted for more than a year, but neither in isolated sperm nor in their offspring were vector sequences detectable [33].

In the near future, therapeutic vehicles that add a certain therapeutic gene most likely will be used to treat inherited diseases, but the preferred treatment is correction of the defective gene itself. Correct expression of the endogenous defect gene will not solve any problems associated with the immune system to the foreign gene product, but is important to correct regulation of the therapeutic gene. For optimal regulation of gene expression splicing of exons and introns and other regulatory elements are often required, which may be too large to be incorporated into lentiviral vector particles.

References

1. **Bank, A., R. Dorazio, and P. Leboulch**. 2005. A Phase I/II Clinical Trial of {beta}-Globin Gene Therapy for {beta}-Thalassemia. Ann. N. Y. Acad. Sci. **1054**:308-316.
2. **Bartolomei, M. S. and J. L. Corden**. 1987. Localization of an alpha-amanitin resistance mutation in the gene encoding the largest subunit of mouse RNA polymerase II. Mol. Cell Biol. **7**:586-594.
3. **Baum, C., O. Kustikova, U. Modlich, Z. Li, and B. Fehse**. 2006. Mutagenesis and oncogenesis by chromosomal insertion of gene transfer vectors. Hum. Gene Ther. **17**:253-263.
4. **Baum, C., C. von Kalle, F. J. Staal, Z. Li, B. Fehse, M. Schmidt, F. Weerkamp, S. Karlsson, G. Wagemaker, and D. A. Williams**. 2004. Chance or necessity? Insertional mutagenesis in gene therapy and its consequences. Mol. Ther. **9**:5-13.
5. **Bellodi-Privato, M., D. Aubert, V. Pichard, A. Myara, F. Trivin, and N. Ferry**. 2005. Successful gene therapy of the Gunn rat by in vivo neonatal hepatic gene transfer using murine oncoretroviral vectors. Hepatology **42**:431-438.
6. **Beuers, U., J. L. Boyer, and G. Paumgartner**. 1998. Ursodeoxycholic acid in cholestasis: potential mechanisms of action and therapeutic applications. Hepatology **28**:1449-1453.
7. **Bosma, P. J.** 2003. Inherited disorders of bilirubin metabolism. J. Hepatol. **38**:107-117.
8. **Braet, F., M. Muller, K. Vekemans, E. Wisse, and D. G. Le Couteur**. 2003. Antimycin A-induced defenestration in rat hepatic sinusoidal endothelial cells. Hepatology **38**:394-402.
9. **Braet, F. and E. Wisse**. 2002. Structural and functional aspects of liver sinusoidal endothelial cell fenestrae: a review. Comp Hepatol. **1**:1.
10. **Briggs, J. A., T. Wilk, R. Welker, H. G. Krausslich, and S. D. Fuller**. 2003. Structural organization of authentic, mature HIV-1 virions and cores. EMBO J. **22**:1707-1715.
11. **Brown, B. D., M. A. Venneri, A. Zingale, L. S. Sergi, and L. Naldini**. 2006. Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. Nat. Med. **12**:585-591.
12. **Bruss, V.** 2004. Envelopment of the hepatitis B virus nucleocapsid. Virus Res. **106**:199-209.
13. **Campbell, S. M., S. M. Crowe, and J. Mak**. 2002. Virion-associated cholesterol is critical for the maintenance of HIV-1 structure and infectivity. AIDS **16**:2253-2261.
14. **Chuah, M. K., G. Schiedner, L. Thorrez, B. Brown, M. Johnston, V. Gillijns, S. Hertel, N. Van Rooijen, D. Lillicrap, D. Collen, T. VandenDriessche, and S. Kochanek**. 2003. Therapeutic factor VIII levels and negligible toxicity in mouse and dog models of hemophilia A following gene therapy with high-capacity adenoviral vectors. Blood **101**:1734-1743.
15. **Cooper, A., N. Paran, and Y. Shaul**. 2003. The earliest steps in hepatitis B virus infection. Biochim. Biophys. Acta **1614**:89-96.
16. **De Godoy, J. L., R. Malafosse, M. Fabre, M. Mehtali, D. Houssin, and O. Soubrane**. 1999. In vivo hepatocyte retrovirus-mediated gene transfer through the rat biliary tract. Hum. Gene Ther. **10**:249-257.
17. **de Vree, J. M., E. Jacquemin, E. Sturm, D. Cresteil, P. J. Bosma, J. Aten, J. F. Deleuze, M. Desrochers, M. Burdelski, O. Bernard, R. P. Oude Elferink, and M. Hadchouel**. 1998. Mutations in the MDR3 gene cause progressive familial intrahepatic cholestasis. Proc. Natl. Acad. Sci. U. S. A **95**:282-287.
18. **de Vree, J. M., R. Ottenhoff, P. J. Bosma, A. J. Smith, J. Aten, and R. P. Oude Elferink**. 2000. Correction of liver disease by hepatocyte transplantation in a mouse model of progressive familial intrahepatic cholestasis. Gastroenterology **119**:1720-1730.
19. **Ehrhardt, A. and M. A. Kay**. 2002. A new adenoviral helper-dependent vector results in long-term therapeutic levels of human coagulation factor IX at low doses in vivo. Blood **99**:3923-3930.
20. **Faulstich, H., K. Kirchner, and M. Derenzini**. 1988. Strongly enhanced toxicity of the mushroom toxin alpha-amanitin by an amatoxin-specific Fab or monoclonal antibody. Toxicon **26**:491-499.
21. **Fiume, L., V. Marinozzi, and F. Nardi**. 1969. The effects of amanitin poisoning on mouse kidney. Br. J. Exp. Pathol. **50**:270-276.
22. Flint.S.J., L.W.Enquist, V.R.Racaniello, and A.M.Skalka. Principles of Virology: Molecular Biology, Pathogenesis, and Control of Animal Viruses. 2004.
23. **Follenzi, A., G. Sabatino, A. Lombardo, C. Boccaccio, and L. Naldini**. 2002. Efficient gene delivery and targeted expression to hepatocytes in vivo by improved lentiviral vectors. Hum. Gene Ther. **13**:243-260.
24. **Fox, I. J., J. R. Chowdhury, S. S. Kaufman, T. C. Goertzen, N. R. Chowdhury, P. I. Warkentin, K. Dorko, B. V. Sauter, and S. C. Strom**. 1998. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. N. Engl. J. Med. **338**:1422-1426.
25. **Guibinga, G. H. and T. Friedmann**. 2005. Baculovirus GP64-pseudotyped HIV-based lentivirus vectors are stabilized against complement inactivation by codisplay of decay accelerating factor (DAF) or of a GP64-DAF fusion protein. Mol. Ther. **11**:645-651.
26. **Guyader, M., E. Kiyokawa, L. Abrami, P. Turelli, and D. Trono**. 2002. Role for human immunodeficiency virus type 1 membrane cholesterol in viral internalization. J. Virol. **76**:10356-10364.
27. **Hannon, G. J. and D. S. Conklin**. 2004. RNA interference by short hairpin RNAs expressed in vertebrate cells. Methods Mol. Biol. **257**:255-266.
28. **Hannon, G. J. and J. J. Rossi**. 2004. Unlocking the potential of the human genome with RNA interference. Nature **431**:371-378.
29. **Horslen, S. P., T. C. McCowan, T. C. Goertzen, P. I. Warkentin, H. B. Cai, S. C. Strom, and I. J. Fox**. 2003. Isolated hepatocyte transplantation in an infant with a severe urea cycle disorder. Pediatrics **111**:1262-1267.
30. **Ikawa, M., V. Tergaonkar, A. Ogura, N. Ogonuki, K. Inoue, and I. M. Verma**. 2002. Restoration of spermatogenesis by lentiviral gene transfer: offspring from infertile mice. Proc. Natl. Acad. Sci. U. S. A **99**:7524-7529.

31. **Jacquemin, E.** 2000. Progressive familial intrahepatic cholestasis. Genetic basis and treatment. Clin. Liver Dis. **4**:753-763.
32. **Jaeger, A., F. Jehl, F. Flesch, P. Sauder, and J. Kopferschmitt**. 1993. Kinetics of amatoxins in human poisoning: therapeutic implications. J. Toxicol. Clin. Toxicol. **31**:63-80.
33. **Jakob, M., C. Muhle, J. Park, S. Weiss, S. Waddington, and H. Schneider**. 2005. No evidence for germ-line transmission following prenatal and early postnatal AAV-mediated gene delivery. J. Gene Med. **7**:630-637.
34. **Jansen, P. L.** 1999. Diagnosis and management of Crigler-Najjar syndrome. Eur. J. Pediatr. **158 Suppl 2**:S89-S94.
35. **Kafri, T., U. Blomer, D. A. Peterson, F. H. Gage, and I. M. Verma**. 1997. Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. Nat. Genet. **17**:314-317.
36. **Kafri, T., D. Morgan, T. Krahl, N. Sarvetnick, L. Sherman, and I. Verma**. 1998. Cellular immune response to adenoviral vector infected cells does not require de novo viral gene expression: implications for gene therapy. Proc. Natl. Acad. Sci. U. S. A **95**:11377-11382.
37. **Kay, M. A., C. S. Manno, M. V. Ragni, P. J. Larson, L. B. Couto, A. McClelland, B. Glader, A. J. Chew, S. J. Tai, R. W. Herzog, V. Arruda, F. Johnson, C. Scallan, E. Skarsgard, A. W. Flake, and K. A. High**. 2000. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. Nat. Genet. **24**:257-261.
38. **Kootstra, N. A., R. Matsumura, and I. M. Verma**. 2003. Efficient production of human FVIII in hemophilic mice using lentiviral vectors. Mol. Ther. **7**:623-631.
39. **Kowolik, C. M. and J. K. Yee**. 2002. Preferential transduction of human hepatocytes with lentiviral vectors pseudotyped by Sendai virus F protein. Mol. Ther. **5**:762-769.
40. **Letchworth, G. J., L. L. Rodriguez, and J. Del cbarrera**. 1999. Vesicular stomatitis. Vet. J. **157**:239-260.
41. **Lieber, A., C. Y. He, L. Meuse, D. Schowalter, I. Kirillova, B. Winther, and M. A. Kay**. 1997. The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. J. Virol. **71**:8798-8807.
42. **Lievens, J., J. Snoeys, K. Vekemans, S. Van Linthout, R. de Zanger, D. Collen, E. Wisse, and B. De Geest**. 2004. The size of sinusoidal fenestrae is a critical determinant of hepatocyte transduction after adenoviral gene transfer. Gene Ther **11**:1523-1531.
43. **Logan, A. C., D. L. Haas, T. Kafri, and D. B. Kohn**. 2004. Integrated self-inactivating lentiviral vectors produce full-length genomic transcripts competent for encapsidation and integration. J. Virol. **78**:8421-8436.
44. **Lozier, J. N., M. E. Metzger, R. E. Donahue, and R. A. Morgan**. 1999. Adenovirus-mediated expression of human coagulation factor IX in the rhesus macaque is associated with dose-limiting toxicity. Blood **94**:3968-3975.
45. **Malhi, H., P. Annamaneni, S. Slehria, B. Joseph, K. K. Bhargava, C. J. Palestro, P. M. Novikoff, and S. Gupta**. 2002. Cyclophosphamide disrupts hepatic sinusoidal endothelium and improves transplanted cell engraftment in rat liver. Hepatology **36**:112-121.
46. **Manno, C. S., V. R. Arruda, G. F. Pierce, B. Glader, M. Ragni, J. Rasko, M. C. Ozelo, K. Hoots, P. Blatt, B. Konkle, M. Dake, R. Kaye, M. Razavi, A. Zajko, J. Zehnder, H. Nakai, A. Chew, D. Leonard, J. F. Wright, R. R. Lessard, J. M. Sommer, M. Tigges, D. Sabatino, A. Luk, H. Jiang, F. Mingozzi, L. Couto, H. C. Ertl, K. A. High, and M. A. Kay**. 2006. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat. Med. **12**:342-347.
47. **Marinozzi, V. and L. Fiume**. 1971. Effects of -amanitin on mouse and rat liver cell nuclei. Exp. Cell Res. **67**:311-322.
48. **Markwell, M. A., A. Portner, and A. L. Schwartz**. 1985. An alternative route of infection for viruses: entry by means of the asialoglycoprotein receptor of a Sendai virus mutant lacking its attachment protein. Proc. Natl. Acad. Sci. U. S. A **82**:978-982.
49. **Mingozzi, F., Y. L. Liu, E. Dobrzynski, A. Kaufhold, J. H. Liu, Y. Wang, V. R. Arruda, K. A. High, and R. W. Herzog**. 2003. Induction of immune tolerance to coagulation factor IX antigen by in vivo hepatic gene transfer. J. Clin. Invest **111**:1347-1356.
50. **Montini, E., D. Cesana, M. Schmidt, F. Sanvito, M. Ponzoni, C. Bartholomae, L. S. Sergi, F. Benedicenti, A. Ambrosi, C. Di Serio, C. Doglioni, C. von Kalle, and L. Naldini**. 2006. Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. Nat. Biotechnol. **24**:687-696.
51. **Morsy, M. A. and C. T. Caskey**. 1999. Expanded-capacity adenoviral vectors--the helper-dependent vectors. Mol. Med. Today **5**:18-24.
52. **Muraca, M., G. Gerunda, D. Neri, M. T. Vilei, A. Granato, P. Feltracco, M. Meroni, G. Giron, and A. B. Burlina**. 2002. Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. Lancet **359**:317-318.
53. **Nagano, M., D. J. Watson, B. Y. Ryu, J. H. Wolfe, and R. L. Brinster**. 2002. Lentiviral vector transduction of male germ line stem cells in mice. FEBS Lett. **524**:111-115.
54. **Nathwani, A. C., A. M. Davidoff, H. Hanawa, Y. Hu, F. A. Hoffer, A. Nikanorov, C. Slaughter, C. Y. Ng, J. Zhou, J. N. Lozier, T. D. Mandrell, E. F. Vanin, and A. W. Nienhuis**. 2002. Sustained high-level expression of human factor IX (hFIX) after liver-targeted delivery of recombinant adeno-associated virus encoding the hFIX gene in rhesus macaques. Blood **100**:1662-1669.
55. **Nguyen, T. H., M. Bellodi-Privato, D. Aubert, V. Pichard, A. Myara, D. Trono, and N. Ferry**. 2005. Therapeutic Lentivirus-Mediated Neonatal in Vivo Gene Therapy in Hyperbilirubinemic Gunn Rats. Mol. Ther.
56. **Nguyen, V. T., F. Giannoni, M. F. Dubois, S. J. Seo, M. Vigneron, C. Kedinger, and O. Bensaude**. 1996. In vivo degradation of RNA polymerase II largest subunit triggered by alpha-amanitin. Nucleic Acids Res. **24**:2924-2929.

57. **Oude Elferink, R. P. and C. C. Paulusma**. 2006. Function and pathophysiological importance of ABCB4 (MDR3 P-glycoprotein). Pflugers Arch.
58. **Overturf, K., M. al Dhalimy, R. Tanguay, M. Brantly, C. N. Ou, M. Finegold, and M. Grompe**. 1996. Hepatocytes corrected by gene therapy are selected in vivo in a murine model of hereditary tyrosinaemia type I. Nat. Genet. **12**:266-273.
59. **Paddison, P. J., A. A. Caudy, R. Sachidanandam, and G. J. Hannon**. 2004. Short hairpin activated gene silencing in mammalian cells. Methods Mol. Biol. **265**:85-100.
60. **Pan, D., R. Gunther, W. Duan, S. Wendell, W. Kaemmerer, T. Kafri, I. M. Verma, and C. B. Whitley**. 2002. Biodistribution and toxicity studies of VSVG-pseudotyped lentiviral vector after intravenous administration in mice with the observation of in vivo transduction of bone marrow. Mol. Ther **6**:19-29.
61. **Porada, C. D., P. J. Park, J. Tellez, F. Ozturk, H. A. Glimp, G. Almeida-Porada, and E. D. Zanjani**. 2005. Male germ-line cells are at risk following direct-injection retroviral-mediated gene transfer in utero. Mol. Ther. **12**:754-762.
62. **Powell, S. K., M. A. Kaloss, A. Pinkstaff, R. McKee, I. Burimski, M. Pensiero, E. Otto, W. P. Stemmer, and N. W. Soong**. 2000. Breeding of retroviruses by DNA shuffling for improved stability and processing yields. Nat. Biotechnol. **18**:1279-1282.
63. **Raper, S. E., N. Chirmule, F. S. Lee, N. A. Wivel, A. Bagg, G. P. Gao, J. M. Wilson, and M. L. Batshaw**. 2003. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol. Genet. Metab **80**:148-158.
64. **Rodriguez, L. L.** 2002. Emergence and re-emergence of vesicular stomatitis in the United States. Virus Res. **85**:211-219.
65. **Roskams, T. A., N. D. Theise, C. Balabaud, G. Bhagat, P. S. Bhathal, P. Bioulac-Sage, E. M. Brunt, J. M. Crawford, H. A. Crosby, V. Desmet, M. J. Finegold, S. A. Geller, A. S. Gouw, P. Hytiroglou, A. S. Knisely, M. Kojiro, J. H. Lefkowitch, Y. Nakanuma, J. K. Olynyk, Y. N. Park, B. Portmann, R. Saxena, P. J. Scheuer, A. J. Strain, S. N. Thung, I. R. Wanless, and A. B. West**. 2004. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. Hepatology **39**:1739-1745.
66. **Sadelain, M., L. Lisowski, S. Samakoglu, S. Rivella, C. May, and I. Riviere**. 2005. Progress Toward the Genetic Treatment of the {beta}-Thalassemias. Ann. N. Y. Acad. Sci. **1054**:78-91.
67. **Schauber, C. A., M. J. Tuerk, C. D. Pacheco, P. A. Escarpe, and G. Veres**. 2004. Lentiviral vectors pseudotyped with baculovirus gp64 efficiently transduce mouse cells in vivo and show tropism restriction against hematopoietic cell types in vitro. Gene Ther **11**:266-275.
68. **Schiedner, G., S. Hertel, M. Johnston, V. Dries, N. Van Rooijen, and S. Kochanek**. 2003. Selective depletion or blockade of Kupffer cells leads to enhanced and prolonged hepatic transgene expression using high-capacity adenoviral vectors. Mol. Ther. **7**:35-43.
69. **Seppen, J., R. R. van der, N. Looije, N. P. van Til, W. H. Lamers, and R. P. Oude Elferink**. 2003. Long-term correction of bilirubin UDPglucuronyltransferase deficiency in rats by in utero lentiviral gene transfer. Mol. Ther. **8**:593-599.
70. **Seppen, J., N. P. van Til, R. R. van der, J. K. Hiralall, C. Kunne, and R. P. Elferink**. 2005. Immune response to lentiviral bilirubin UDP-glucuronosyltransferase gene transfer in fetal and neonatal rats. Gene Ther.
71. **Sirin, O. and F. Park**. 2003. Regulating gene expression using self-inactivating lentiviral vectors containing the mifepristone-inducible system. Gene **323**:67-77.
72. **Spiegel, M., M. Bitzer, A. Schenk, H. Rossmann, W. J. Neubert, U. Seidler, M. Gregor, and U. Lauer**. 1998. Pseudotype formation of Moloney murine leukemia virus with Sendai virus glycoprotein F. J. Virol. **72**:5296-5302.
73. **Steffan, A. M., J. L. Gendrault, and A. Kirn**. 1987. Increase in the number of fenestrae in mouse endothelial liver cells by altering the cytoskeleton with cytochalasin B. Hepatology **7**:1230-1238.
74. **Tao, N., G. P. Gao, M. Parr, J. Johnston, T. Baradet, J. M. Wilson, J. Barsoum, and S. E. Fawell**. 2001. Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. Mol. Ther. **3**:28-35.
75. **Themis, M., S. N. Waddington, M. Schmidt, C. von Kalle, Y. Wang, F. Al Allaf, L. G. Gregory, M. Nivsarkar, M. Themis, M. V. Holder, S. M. Buckley, N. Dighe, A. T. Ruthe, A. Mistry, B. Bigger, A. Rahim, T. H. Nguyen, D. Trono, A. J. Thrasher, and C. Coutelle**. 2005. Oncogenesis following delivery of a nonprimate lentiviral gene therapy vector to fetal and neonatal mice. Mol. Ther. **12**:763-771.
76. **Thomas, C. E., G. Schiedner, S. Kochanek, M. G. Castro, and P. R. Lowenstein**. 2001. Preexisting antiadenoviral immunity is not a barrier to efficient and stable transduction of the brain, mediated by novel high-capacity adenovirus vectors. Hum. Gene Ther. **12**:839-846.
77. **Tominaga, K., S. Kuriyama, H. Yoshiji, A. Deguchi, Y. Kita, F. Funakoshi, T. Masaki, K. Kurokohchi, N. Uchida, T. Tsujimoto, and H. Fukui**. 2004. Repeated adenoviral administration into the biliary tract can induce repeated expression of the original gene construct in rat livers without immunosuppressive strategies. Gut **53**:1167-1173.
78. **van der Wegen, P., R. Louwen, A. M. Imam, R. M. Buijs-Offerman, M. Sinaasappel, F. Grosveld, and B. J. Scholte**. 2006. Successful treatment of UGT1A1 deficiency in a rat model of Crigler-Najjar disease by intravenous administration of a liver-specific lentiviral vector. Mol. Ther **13**:374-381.
79. **VandenDriessche, T., D. Collen, and M. K. Chuah**. 2001. Viral vector-mediated gene therapy for hemophilia. Curr. Gene Ther. **1**:301-315.
80. **VandenDriessche, T., L. Thorrez, L. Naldini, A. Follenzi, L. Moons, Z. Berneman, D. Collen, and M. K. Chuah**. 2002. Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells in vivo. Blood **100**:813-822.
81. **Voisset, C. and J. Dubuisson**. 2004. Functional hepatitis C virus envelope glycoproteins. Biol. Cell **96**:413-420.

82. **Wisse, E., F. Braet, D. Luo, R. de Zanger, D. Jans, E. Crabbe, and A. Vermoesen**. 1996. Structure and function of sinusoidal lining cells in the liver. Toxicol. Pathol. **24**:100-111.
83. **Ye, P., A. R. Thompson, R. Sarkar, Z. Shen, D. P. Lillicrap, R. J. Kaufman, H. D. Ochs, D. J. Rawlings, and C. H. Miao**. 2004. Naked DNA transfer of Factor VIII induced transgene-specific, species-independent immune response in hemophilia A mice. Mol. Ther. **10**:117-126.
84. **Zhang, J., L. Xu, M. E. Haskins, and P. K. Parker**. 2004. Neonatal gene transfer with a retroviral vector results in tolerance to human factor IX in mice and dogs. Blood **103**:143-151.

Summary
Samenvatting

Summary
Gene therapy may be used in the near future to treat patients with inherited liver diseases. Many researchers are exploring the possibility to use vectors based on viruses, because viruses have evolved to efficiently infect mammalian cells. These viruses should therefore contain the required properties to insert foreign DNA into cells. We used lentiviral vectors, because these vectors can stably integrate into non-dividing cells *in vivo* and *ex vivo*, which is a major advantage compared to many other viral vector systems, such as commonly used adenoviral vectors.

Chapter 1 gives an overview of the current developments of viral vector systems for treatment of inherited liver diseases. It also gives an overview of the animal models for liver repopulation and other cell-based strategies to correct for a defective gene.
In **chapter 2** we show that liver sinusoidal endothelial cells do not form a major barrier for *in vivo* lentiviral gene therapy of hepatocytes, but the Kupffer cells do. In addition, hepatocytes can be transduced by injection of a lentiviral vector dose into the common bile duct.
In **chapter 3** detargeting of lentiviral vectors was acquired by pseudotyping the viral particles with a fusion envelope protein between GP64 and Sendai envelope proteins (GP64/Sendai fusion). Because Sendai envelope protein binds to asialoglycoprotein, which is present on hepatocytes, the expectation was that hepatocyte specific transduction would take place. Pseudotyping lentiviral particles with GP64/Sendai fusion envelope led to stable particles and a lower Kupffer cell transduction after administration *in vivo*.
In utero and neonatal lentiviral gene therapy was performed in the Gunn rat, a model for Crigler Najjar syndrome. In **chapter 4**, we show that at this early age, we markedly reduced the plasma bilirubin levels long-term. However, injection of lentiviral vectors via the peritoneum led to generation of antibodies against the transgene.
In another inherited liver disease, Progressive Familial Intrahepatic Cholestasis type 3, we show in **chapter 5** that lentiviral vector design is crucial to generate infectious lentiviral particles for possible PFIC3 gene therapy. Our lentiviral vectors expressed ABCB4 during viral vector production, which affected infectivity by interfering with the lipid membrane composition of the viral vector particle. There was a decrease of the cholesterol content, which has been shown to be crucial for viral infectivity and integrity.
In **chapter 6**, we show that the use of a lentiviral vector containing a cassette that expresses α-amanitin resistant RNA polymerase II largest subunit could be used as a selection marker in cell-lines and in dividing and quiescent human fibroblasts. This strategy may be used to select primary cells *ex vivo* that poorly transduce, such as hepatocytes.
In **chapter 7**, a cloned cell-line was made from fetal liver cells that could differentiate in the liver into hepatocytes after administration in the spleen. The development of such cell lines may also be used in the future as an unlimited source for transplantation into patients with inherited liver diseases.
The general discussion in **chapter 8** summarizes our findings and provides possibilities to further develop lentiviral gene therapy of inherited liver diseases.

Samenvatting
Gentherapie zou in de nabije toekomst gebruikt kunnen gaan worden in de kliniek voor de behandeling van patiënten met erfelijke leverziekten. Veel onderzoekers willen hiervoor gebruik maken van virale vectoren, die gebaseerd zijn op virussen. Omdat virussen tijdens evolutie gespecialiseerd zijn in het overbrengen van hun genetisch materiaal van cel tot cel, kan er gebruik gemaakt worden van deze eigenschappen voor gentherapie. Wij hebben gebruik gemaakt van lentivirale vectoren, omdat deze vectoren stabiel kunnen integreren in niet-delende cellen *in vivo* en *ex vivo*. Vele andere virale vectoren, zoals adenovirale vectoren, bezitten deze eigenschappen niet.

In **hoofdstuk 1** wordt een overzicht gegeven van de ontwikkelingen van virale vectoren voor de behandeling van erfelijke leverziekten. Ook wordt er een overzicht gegeven van de diermodellen van lever repopulatie en andere cel-gebaseerde strategieën om een defect gen te kunnen corrigeren.
In **hoofdstuk 2** laten we zien dat lever sinusoidaal endotheel niet de efficiëntie van hepatocyt transductie *in vivo* beïnvloedt, maar dat Kupffer cellen weldegelijk invloed uitoefenen op de transductie efficiëntie door het mogelijk wegvangen van virale vectoren, en doordat deze cellen beter getransduceerd worden dan hepatocyten. Daarbij laten we zien dat hepatocyten ook getransduceerd kunnen worden door injectie van lentivirale vectoren in de galgang.
In **hoofdstuk 3** worden lentivirale vectoren gepseudotypeerd met een fusie envelop eiwit, dat gedeeltelijk bestaat uit cellulair deel van GP64 envelop eiwit en een extern deel van het Sendai envelop eiwit. Omdat het Sendai virus bindt aan de asialoglycoprotein receptor, dat op hepatocyten aanwezig is, zou de virale vector gestuurd kunnen worden naar deze cellen. Echter, wij zagen geen toename van de hepatocyt transductie, maar wel een afname in de Kupffer cel transductie.
In utero en neonatale gentherapie kan toegepast worden in Gunn ratten, een model voor het syndroom van Crigler Najjar. In **hoofdstuk 4** beschrijven we dat injectie van lentivirale vectoren die bilirubine UDP glucuronyl transferase (UGT1A1) tot expressie brengen leidt tot een reductie in plasma bilirubine waarden, maar dat deze injectie route via het peritoneum als gevolg heeft dat er antilichamen tegen UGT1A1 gegenereerd worden.
In een andere erfelijke leverziekte, Progressieve Familiaire Intrahepatische Cholestase type 3, laten we zien in **hoofdstuk 5** dat de expressie van ABCB4 eiwit een negatief effect heeft op het verkrijgen van infectieuze virale vector deeltjes. ABCB4 komt tot expressie tijdens de virale vector produktie en beïnvloedt de lipiden samenstelling van de lentivirale vector door afname in de cholesterol fractie, waarvan bekend is dat het cruciaal is voor de integriteit en infectiviteit van HIV deeltjes. Aanpassingen zijn nodig voor het genereren van een bruikbare lentivirale vector dosis voor PFIC3 behandeling.
In **hoofdstuk 6** beschrijven we een lentivirale vector met α-amanitine resistente RNA polymerase II als een selectiemarker die effectief is in zowel delende als niet-delende humane fibroblasten. Deze strategie zou eventueel gebruikt kunnen worden voor selectie van primaire cellen die *ex vivo* slecht te transduceren zijn, zoals hepatocyten.
In **hoofdstuk 7** wordt een gekloneerde cellijn beschreven, afkomstig van geïsoleerde humane foetale cellen. Deze cellen konden differentiëren naar hepatocyten in de lever na toediening via de milt. De ontwikkeling van deze cellijnen zou in de toekomst tot ongelimiteerde bronnen kunnen leiden voor cel transplantaties in patiënten met erfelijke leverziekten.
In **hoofdstuk 8** worden alle hoofdstukken nog eens tegen het licht gehouden, wat betreft de gevonden resultaten en ingegaan op mogelijkheden voor het verder ontwikkelen van lentivirale gentherapie voor de behandeling van erfelijke leverziekten.

Abbreviations

AAV	Adeno-associated vector
AAT	α-1-antitrypsin
ABC	ATP binding cassette
AFP	a-fetoprotein
ATCC	American Type Culture Collection
BAL	Bio Artificial Liver
BUGT	bilirubin UDP-glucuronosyltransferase
CK	cytokeratin
CMV	cytomegalovirus
CPS	carbamoylphosphate synthetase
CTL	cytotoxic T-lymphocyte
CD	cluster of differentiation
DAPI	diamidinophenylindoldiacetate
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	ethylene diamine tetra-acetic acid
EF1α	elongation factor 1α
EPO	erythropoietin
FAH	fumaryl acetoacetate dehydrolase
FISH	fluorescent In-Situ Hybridisation
FITC	fluorisceine isothiocyanate
GFP	green fluorescent protein
GS	glutamin synthetase
GST π	gluthathion S transferase π subunit
HD	helper dependent
HFLC	human fetal liver cell
HI-FBS	heat inactivated-fetal bovine serum
HIV	human immunodeficiency virus
hTERT	human telomerase reverse transcriptase
IRES	internal ribosome entry site
LSEC	liver sinusoidal endothelial cells
MDCKII	Madin-Darby canine kidney
MHC	major histocompatibility complex
MOI	multiplicity of infection
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	population doubling
PFA	paraformaldehyde
PFIC3	progressive familial intrahepatic cholestasis
PGK	phosphoglycerate kinase
RNAi	RNA interference
RRV	Ross river virus
RSV	Rous sarcoma virus
RT	reverse transcriptase
SIN	self-inactivating vectors
uPA	urokinase plasminogen activator
SV40T	Simian virus 40 large T antigen
T_{PD}	population Doubling Time
TRITC	Tetramethylrhodamine isothiocyanate
VSV-G	Vesicular stomatitis virus G protein

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