Gene Therapy of Liver Disease with Lentiviral Vectors

Preclinical Studies in Models of Crigler-Najjar Disease and Hepatitis C

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Gentherapie van Leverziekte met Lentivirale Vectoren

Preklinische Studies in Modellen van de Ziekte van Crigler-Najjar en Hepatitis C

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Aan mijn ouders,

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Chapter I

Introduction

Introduction

The main theme of this thesis is the application of lentiviral vectors for the treatment of congenital and acquired liver disease. Gene therapy represents a relatively new and promising therapeutic tool with possible applications in a broad spectrum of medical disciplines. The underlying principle of gene therapy is based on the introduction of genetic material into living cells to achieve a therapeutic biological effect. Consequently, gene therapy focuses on treating the cause of disease rather than the symptoms. The first part of this introduction will review the current options and developments in the field of liver directed gene therapy (1.1). Next we will review the most widely used viral vector systems (1.2).

HIV-1 derived lentiviral vectors are among the most efficient gene therapy vectors that are currently available. The capacity of lentiviral vectors to stably integrate into the target cell genome of non-dividing cells is one of the main distinguishing features of this vector system. Clinical trials that involve the *ex vivo* transduction and retransplantation of autologous CD4+ T-cells or hematopoietic stem cells have been initiated [1-3]. In preclinical experiments, lentiviral vectors efficiently transduce differentiated liver parenchymal cells *in vivo* [4, 5]. Other major targets, such as the central nervous system and the airways are also under intense investigation [6, 7].

In the third part of this introduction, we will introduce this relatively new vector system that plays a central role in our studies (1.3). To learn how a dangerous pathogen such as HIV-1 was transformed into an efficient and safe gene transfer vehicle, the HIV-1 genome and virion composition and the viral lifecycle will be reviewed. Furthermore, we will discuss the lentiviral vector production procedure and transduction process. Finally, the scope of the thesis is outlined by introducing each chapter (1.4).

1.1 Gene therapy for liver disease

Hereditary metabolic liver disease

The liver is considered one of the main targets for gene therapeutic intervention. The organ plays a key role in many vital body processes, including carbohydrate and lipid metabolism, detoxification, plasma protein synthesis and micronutrient homeostasis. These processes rely on complex biochemical pathways mediated by various proteins with specific functions. As a consequence, the malfunction of one single gene can cause disease. Indeed, many forms of heritable monogenic liver disease have been described, which can be divided in two categories, hepatodegenerative disease and non-hepatodegenerative disease (Table 1). Hepatodegenerative disease induces general liver damage and dysfunction (chirrosis), usually due to the accumulation of toxic compounds in the liver and this process is associated with the induction of liver cancer. Non-hepatodegenative liver diseases affect hepatocyte function in such a way that it does not result in destruction of liver tissue, but in disease manifestations elsewhere in the body. For example, blood function can be impaired due to deficient synthesis of a specific blood-protein in the liver, as seen in hemophilia B. Other defects result in the accumulation of toxic compounds in the blood that are normally detoxificated by the liver, such as in Crigler-Najjar syndrome type I, which is the main focus of this study.

Due to their distinct disease characteristics, hepatodegenerative and non-hepatodegenerative hereditary metabolic diseases require different gene therapeutic approaches. In hepatodegenerative disease, gene complemented hepatocytes have a competitive advantage over non-corrected cells. As a consequence, high initial transduction efficiencies are not required and only a small fraction of successfully transduced hepatocytes can proliferate in time to give rise to healthy liver tissue. However, replacement of the diseased liver with these gene-corrected cells is never complete and with continuation of liver cirrhosis, the induction of liver cancer remains a risk. In several pre-clinical models of hepatodegenerative disease, liver

directed gene therapy was able to ameliorate disease symptoms, but did not prevent the development of liver cancer [8]. With current technology, it is not possible to achieve complete correction of all hepatocytes, especially in cirrhotic livers with a destroyed and fibrotic organ structure. Therefore, for this disease category, liver transplantation is still the preferred therapy.

In non-hepatodegenerative diseases, a selective advantage of corrected cells is absent, but the liver structure is fully intact and thus better accessible for gene therapy vectors from the circulation. A gene therapy solution for most of these diseases does not require transduction of the majority of hepatocytes and a transduction rate of 5-30% can have a significant therapeutic effect, either curing disease or ameliorating disease symptoms from a severe to a more moderate condition. Altogether, for this disease category, gene therapy may have considerable benefits over liver transplantation.

Table 1. Disease candidates for liver directed gene therapy.

Inherited monogenic liver disease (hepatodegenerative)	**Inherited monogenic liver disease (non-hepatodegenerative)**
Alpha 1-antitrypsin deficiency	Afibrinogenemia
Erythropoietic protoporphyria	Analbuminemia
Gaucher disease	Crigler-Najjar syndrome type I
Glycogen storage disease type Ia and type II	Familial hypercholesterolemia IIa
Mucopolysaccharidosis type I and type VII	Galactosemia
Niemann-Pick disease type C	Hemophilia B
Progressive familial intrahepatic cholestasis type II and type III	Ornithine transcarbamylase deficiency
Hereditary hemochromatosis	Phenylketonuria
Tyrosinemia type I	Primary hyperoxaluria
Wilson's disease	
Acquired liver disease (with or without inherited aspects)	**Non-liver disease**
Hepatitis B	Hemophilia A
Hepatitis C	Von Willebrand disease
Hyperlipidemia	Growth retardation
Liver fibrosis	Diabetes
Hepatic cancers	

Crigler-Najjar disease type I

Crigler-Najjar disease type I is a typical example of non-hepatodegenerative monogenic metabolic hereditary liver disease in which a successful liver directed gene therapy approach could be beneficial. Crigler-Najjar disease type I is caused by a mutation in the gene encoding uridine 5′-diphosphate-(UDP)-glucuronosyltransferase 1A1 (UGT1A1). This enzyme catalyses the hepatic glucuronidation of toxic bilirubin into more hydrophilic isoforms that can be excreted via the bile. The lack of efficient bilirubin clearance causes unconjugated hyperbilirubinemia, which is evident after birth by jaundice of the skin (icterus). Untreated, this condition induces kernicterus, a form of severe neuronal injury caused by deposition of unconjugated bilirubin in selected regions of the brain and is associated with temporary or permanent impairment of auditory, motor or mental function.

Treatment consists of exchange transfusions in neonates, to acutely reduce unconjugated bilirubin levels and prevent kericterus in the sensitive newborn brain. In infants, the serum bilirubin levels are maintained below critical values by intensive phototherapy that may be

required for up to 12 hours a day. Phototherapy with blue/green light of optimally 450 nm renders bilirubin that has build up in the skin into an iso-form that can be efficiently excreted. However, as the child gets older, phototherapy becomes less effective because of changes in the thickness and pigmentation of the skin and because of a reduction in the body's surface-to-volume ratio. Chronic skin irritation, and possibly UV induced carcinogenesis are further concerns associated with long-term phototherapy. Eventually liver transplantation is required to cure the disease and prevent serious and irreversible deterioration of the brain.

Crigler-Najjar syndrome type I is a candidate for gene therapy and despite its low prevalence (estimated at 0.6 - 1.0 per million live births), several factors make this disease one of the model diseases for liver directed gene therapy studies. These factors include: 1) the availability of a representative animal model in the form of the Gunn rat, 2) the low level of gene complementation required (estimated at 5% of the normal UGT1A1 expression level), 3) the straightforward determination of a gene-therapeutic effect in the blood and 4) the relatively short size of the UGT1A1 coding sequence (1.6 kb) that allows incorporation into many different gene therapy vector systems.

Indeed, in the past years, various vector systems have been applied to successfully reduce hyperbilirubinemia in the Gunn rat [9]. The majority of these approaches focus on gene complementation. However, one paper reports partial genomic repair of the UGT1A1 mutation in the Gunn rat with RNA/DNA chimeric oligonucletides [10]. This effect could not be efficiently reproduced by other labs, which questions the general value of this technique [11]. Another relatively successful approach with non-viral vectors includes hydrodynamic injection with a liver specific UGT1A1 expression plasmid [12]. Various viral vectors were shown to correct hyperbilrubinemia in the Gunn rat for at least one year after a single intravenous administration. These include: minimal adenoviral vectors [13], adeno-associated viral vectors [14], gammaretroviral vectors [15] and lentiviral vectors (see Chapter 2 and 3) [16, 17]. All these viral vector systems have their own specific advantages and constraints as discussed later in this chapter.

Viral hepatitis

Another promising target for liver directed gene therapy is the treatment of viral hepatitis by means of inhibitory RNA (RNAi) designed to interfere with the viral life cycle [18]. The World Health Organization (WHO) estimates that worldwide over 350 million people are chronically infected with hepatitis B virus (HBV) and 170 million with hepatitis C virus (HCV). These infections are the principal causes of severe liver disease, including cirrhosis-related end-stage liver disease and HCC [19]. Despite similar clinical manifestations, HBV infection can be better controlled by available treatment than HCV. Currently, the response rates of the most efficient HCV treatment regimen consisting of pegylated interferon alpha and ribavirin are 50-60% at best. Furthermore, treatment often has to be ceased due to severe drug related side effects or drug-resistance as a result of extremely high mutation rate of the virus.

For these reasons, HCV infection is the main indication for liver transplantation in the western world (approximately 40% of all transplantations). Post transplantation, HCV recurrence in the liver graft is a general problem and infection progression is enhanced by the immunosuppressive treatment necessary to avoid transplant rejection. Viral induced cirrhosis is reported to develop in 25-30% of patients within five years and in 50% within 10 years after transplantation. In the end, at least 10% of these patients will require re-transplantation due to HCV-related disease. However, due to the bad physical condition of these patients caused by the devastating effects of combined anti-HCV therapy and immunosupression, clinical perspectives are very poor.

The development of new pharmaceutical treatment options is hampered by the inability of wild-type HCV to replicate *in vitro* and to infect non-primate hepatocytes in *vivo*.
Models systems are limited to a HCV subgenomic replicon system in human hepatoma cells *in vitro* and a rather cumbersome *in vivo* model for HCV infection in mice that is based on chimeric mouse/human livers. Recently, a Japanese HCV isolate (JFH-1) has been reported to replicate in cell culture, which may accelerate HCV research [20].

Since the HCV lifecycle involves no nuclear phase and RNA replication takes place only in the cytoplasm, an RNAi based approach for HCV inhibition seems viable. Indeed, many groups have reported efficient inhibition of HCV replicon *in vitro* after siRNA and shRNA delivery by various synthetic and viral vector systems. This strategy should target multiple conserved nucleotide sequences to prevent mutational escape of the virus. Apart from the RNA genome, other candidate targets include several cellular co-factors of the viral life-cycle and the HCV co-receptors, while the main receptors remain to be identified. These approaches anticipate RNAi application in the diseased liver, prior to transplantation. Another approach is to render the liver graft resistant to HCV re-infection. Due to the expected selective advantage of cells resistant to HCV infection, the integrational properties of lentiviral vectors is expected to provide a robust source of anti HCV RNAi molecules and efforts in this direction are described in this thesis (Chapters 4 and 5).

Other targets for liver directed gene therapy

The high level of protein synthesis that takes place in the liver can render it into an efficient production unit for various therapeutic proteins. The produced proteins may act locally in the liver or systemically when secreted into the bloodstream. For example, for hereditary disease, this approach has been shown to ameliorate both liver-related and systemic disease symptoms in several animal models of lysosomal storage disease, including mucopoly-saccharidosis type I, II and VI and Gaucher's, Fabry's and Pompe's disease [21]. As the provided proteins do not pass the blood-brain and blood-nerve barrier, this form of treatment is most suited for non-neuropathic lysosomal storage diseases.

Via a natural pathway, a fraction of lysosomal pro-enzymes escapes sorting and goes through a cycle of secretion and recovery by receptor mediated endocytosis. Currently, this pathway facilitates treatment of several lysosomal storage diseases by enzyme replacement therapy (ERT) [22]. ERT involves intravenous administration of the missing or defective lysosomal pro-enzyme that is subsequently taken up by deficient cells. However, ERT is not effective in all patients and associated with several drawbacks including repeated intravenous enzyme administration, fluctuating therapeutic enzyme levels, expensive enzyme preparations and immune reactions that may ameliorate treatment. In principle, a safe form of liver directed gene therapy that gives rise to stable enzyme levels in the blood could be an alternative for ERT.

Another experimental approach for treatment of lysosomal storage disorders consists of allogeneic hematopoietic stem cell transplantation (HSCT). This has been performed in patients for over 20 lysosomal storage diseases and is a standard treatment option for about a dozen disease forms. Despite significant progress in medical procedures and the availability of banked umbilical cord blood, HSCT is still associated with significant risks of graft failure or graft-versus-host disease that can lead to death. Transplantation of autologous hematopoietic stem cells genetically modified to express the deficient protein may circumvent the majority of the problems associated with allogeneic HSCT.

Other examples of liver directed gene therapy for non-liver or systemic disease could aid in treating diseases such as hyperlipidemia (obesity), growth retardation, familial hypercholesterolemia, diabetes and liver fibrosis [23-25].

Liver cancer

The liver is a major site of tumor metastasis, especially for tumors originating in the abdomen. Also tumors that originate in the liver, most importantly hepatocellular carcinoma, are frequent as a complication of liver cirrhosis caused by hepato-degenerative disease. In general, liver tumors are difficult to resect due to the extensive vascularisation of the organ and are therefore associated with high morbidity. In theory, liver directed gene therapy targeting these malignant cells with tumor modulator genes, i.e. suicide genes, genes encoding cytokines such as IL-2 or TNF-alpha or tumor suppressor genes like p53 could alleviate tumor cells or limit their growth. However, tumor vasculature has often lost its fenestration which makes it difficult for gene therapy vectors to gain access to individual tumor cells. Efforts targeting lentiviral vectors to melanoma metastases in the lung *in vivo* have been described, and

further development of this approach may enable treatment of liver tumors [26]. Another application of lentiviral vectors for cancer treatment includes immunotherapy (see Carroll et al, 2007 for a review) [27]. In this approach, lentiviral vectors are used to program bone marrow-derived dendritic progenitors to self-differentiate *in vitro* and induce immunogenicity against a tumor antigen *in vivo* [28]. A clinical trial using lentiviral vector based genetic vaccination of autologous dendritic cells is in preparation.

Alternative strategies, liver transplantation and liver cell transplantation
Orthotopic liver transplantation (OLT) has become a standard procedure and is the only treatment option for various forms hereditary and acquired liver disease. Since the early years of liver transplantation, this field recorded enormous progress regarding patient survival and the prevention of graft rejection. However, despite the development of novel techniques such as split liver transplantation, living donor liver transplantation and the use of suboptimal liver grafts from non-heart beating donors, the shortage of donor organs remains a huge problem. The mortality rate for patients on the waiting list for liver transplantation is approximately fifteen percent and while waiting lists are growing, the number of donor organs that becomes available has reached a plateau.

Despite all progress, the liver transplantation procedure is still associated with considerable rates of morbidity and graft rejection, especially in HCV infected patients. After “standard” OLT, five-year patient and graft survivals in adults are 83.5 % vs. 74.6 %, and 80.6 % vs. 69.9 % in the HCV negative and positive patients respectively [29]. However, in children, especially for non-hepatodegenerative metabolic disease, five-year survival rates of over 90 % are achieved [30]. Furthermore, lifelong immunosuppression with cyclosporine is associated with several complications including the induction of chronic renal failure, fibrosis of the liver graft, de novo malignancy and, when applicable, hepatitis and hepatocellular carcinoma recurrence [31].

Since liver transplantation is not always indicated or available, an alternative treatment has recently entered the clinic in the form of liver cell transplantation (LCT) [32]. In LCT, patients are typically transplanted with donor hepatocytes equivalent to 5-10 % of the patient’s liver mass. Successful LCT could prevent the risks involved with liver transplantation, reduce medical costs, and will certainly reduce the shortage for donor organs. LCT can be applied in children and adults and indications include hereditary metabolic liver disease, fulminant hepatic failure and decompensated end-stage cirrhosis. Until present, transient, yet significant, effects were observed after LCT without serious side effects. The procedure appears most effective in children with hereditary metabolic liver disease. The most successful case reports a 50% decrease in the plasma bilirubin levels of a Criler-Najjar disease type I patient that lasted up to nine months, but could not prevent the requirement for liver transplantation [33]. Therefore, LCT can aid in the alleviation of disease burden and prevent systemic organ damage while the patient waits for liver transplantation [34]. In the coming years, improvements in terms of cell quality and rejection prevention are expected to further improve the procedure. Future integration with stem cell technology and lentiviral gene transfer can be anticipated and these developments are likely to provide novel opportunities for treatment of many forms of liver disease.

1.2 Gene therapy approaches and vector systems

As mentioned earlier, gene therapy involves the introduction of genetic material into living cells to achieve a therapeutic biological effect. The majority of current gene therapy strategies involve gene addition rather than the correction or replacement of defective genes. Gene therapy can be performed on several different levels. First, one can discriminate between germ-line and somatic gene therapy. Germ line gene therapy aims at genetic modification of reproductive cells in such a way that the transgene is carried over to future generations. Under current law these procedures are considered unethical and illegal so in practice only somatic gene therapy can be performed in clinics. Somatic gene therapy aims at the modification of non-reproductive cells and thus the gene therapy-derived trait dies out with the death of the patient. Another divergence can be made between *ex vivo* and *in vivo* gene therapy. The *ex vivo* approach involves the isolation of autologous (stem)cells that are

genetically modified and possibly expanded *in vitro*, after which they are reintroduced into the patient. The *in vivo* gene therapy approach is performed directly in the patient's body.

Gene therapeutic material is usually delivered as RNA or DNA sequences. The actual gene therapeutic intervention is often mediated via a functional RNA species that can be either coding or non-coding. The definition of this RNA depends on the genetic basis of the disease that is to be treated and which intervention on gene expression is aimed for. Where coding sequences in the form of mRNA facilitate expression of an existing or completely novel protein, non-coding RNAs such as ribozymes, RNA decoys, short interfering RNAs, short hairpin RNAs, modified primary microRNAs or antisense molecules are used to inhibit gene expression.

Functional RNA can be delivered directly across the target cell membrane into the cytosol where it is able to mediate a rapid effect on gene expression. However, due to the short half-life of RNA this effect is very transient. For this reason, the majority of gene therapeutic approaches use more stable DNA elements containing a transgene expression cassette that transcribes the therapeutic RNA in the nucleus of the cell. This expression cassette can be delivered in the form of plasmid DNA, DNA minicircles, artificial chromosomes, transposons or virus-derived sequences. Here, the properties of the transgene expression cassette largely determines the level, specificity and duration of transgene expression, which, together with the efficiency of gene delivery determine the eventual gene therapeutic effect.

Under normal conditions, the uptake of "naked" RNA or DNA by eukaryotic cells is not efficient, but can be enhanced by changing the physical parameters of the cell's environment. Examples of physical gene delivery methods (reviewed by Gao et al. [35]) include the gene gunn that uses high velocity to "shoot" small particles with bound genetic material into cells. Other methods are electroporation and ultrasound facilitated gene transfer, where high voltages or high frequency sound waves temporarily disrupt the cell membrane to allow uptake of foreign genetic material. An efficient method regularly used in small rodents to target the liver i*n vivo* is hydrodynamic injection which is based on the injection of a large volume (6-10% body weight) in a single bolus into the blood stream. Overall, these methods lack the efficiency or induce too much tissue damage to be translated from a pre-clinical to a clinical context.

Another way of improving the efficiency of gene transfer is to pack the genetic material into a delivery vehicle that is efficiently taken up by cells, referred to as a gene therapy vector. The type of vector that is selected for gene delivery depends on the specific aim of the therapy. For some purposes, for example, expression of a toxic gene-product in cancer cells, transduction of dividing cells resulting in local transient gene expression is adequate. For other applications, such as metabolic disease, transduction of non-dividing cells in combination with permanent expression of a transgene is desired.

The ideal gene therapy vector should have a reasonable cloning capacity that enables the insertion of advanced expression cassettes with elements that facilitate controlled and sustained transgene expression. The production procedure should be scalable to facilitate generation of high vector concentrations at reasonable expense and should meet the high quality and safety standards required for clinical application. The end product needs to be contamination-free and stable enough for long-term storage. Once applied to the patient, the vector should have low immunogenic potential and should be able to induce efficient and specific gene transfer to the desired target cell-type. After gene transfer, the vector should allow long-term transgene expression and should be safe for both patient and environment. Such an ideal vector does not exist and each vector developed until present has its own specific set of advantages and drawbacks.

Gene therapy vectors fall in two general categories, viral- or non-viral vectors. Non-viral vectors (reviewed by Gao et al. [35]) consist of synthetic or naturally occurring chemicals that form complexes with RNA or DNA. Generally, these molecules are based on either cationic lipids or cationic polymers such as poly-L-lysine, polyethylenimine, cationic dextran or cationic proteins. The function of these additives is to protect the genetic material from degradation by extracellular nucleases and to facilitate entrance to the target cell by endocytosis. After

endocytosis, the vector requires escape from the endosomal compartment to the cytosol in order to prevent lysosomal degradation. Once in the cytosol most functional RNA species are directly active, but therapeutic DNA material needs to be translocated to the nucleus to enable transcription of the transgene.

A method for active nuclear translocation of non-viral vectors has not been described to date. Consequently, these vectors are dependant on cell division to gain access to the nucleus. At the start of mitosis, the nuclear membrane dissociates to enable chromosomal separation and is reconstituted in the two daughter cells. This process provides a chance for foreign genetic material in the cytosol to be incorporated into the newly formed nucleus. Depending on the cell type, *in vitro* gene transfer by non-viral vectors can be highly efficient. In contrast, the low proliferative index of most target cells *in vivo* hamper efficient transfection. Most non-viral vectors give rise to transient transgene expression because of the episomal nature of the delivered transgene. As these transgenes do not integrate into the genome and do not contain centromeres and, they tend to get lost during another round of cell division, hereby diluting the gene therapeutic effect.

Viral vectors

As opposed to non-viral vectors, currently, the most successful gene therapy vectors are based on genetically modified viruses. Viral vector systems take advantage of the evolution of naturally occurring viruses into the most specialized and efficient vehicles for delivery of foreign genetic material to living cells. Various viruses have been modified into gene delivery vehicles and thus the properties of these vectors are as a diverse as the viruses they are derived from.

The vast majority of all cells in the organs that may be targets for gene therapy are non-dividing. Therefore, a serious candidate vector for *in vivo* gene therapy purposes needs to be able to transduce non-dividing cells and most but not all viral vectors are capable of doing this. Another important divergence can be made between viral vectors that deposit their genetic material into the nucleus as episomal DNA and vectors that integrate their DNA into the host genome. Since episomal DNA is lost on cell division, genomic integration is considered to result in more solid gene expression, but is not without risk as will be discussed later. Other variable characteristics include the transgene insert size, the presence of pre-existing immunity against vector-derived viral proteins, the association with acute toxicity againt the vector upon delivery and the ease of vector production. An overview of these characteristics for the most frequently used viral vector types is provided in Table 2 and these vectors will be briefly discussed in the next section.

Table 2. Properties of the most frequently used viral vector types.

Vector type	**Adeno-viral vector**	**Adeno-Associated vector**	**Onco-retroviral vector**	**Lentiviral vector**
Abbreviation	AV	AAV	MLV	LV
Insert size	37 kb	4.4 kb	6 kb	7 kb
Genomic integration	no	sporadic	yes	yes
Pre-existing immunity	yes	variable*	no	no
Transduction non-dividing cells	yes	yes	no	yes
First reported in year	1977	1984	1982	1996
Clinical trials until Sept 2008**	360	60	317	18
Genome composition	dsDNA	ssDNA	ssRNA	ssRNA
Size	43 nm	25 nm	100 nm	110 nm

* serotype dependant, ** source: http://www.wiley.co.uk/genetherapy/clinical/

Adenoviral vectors
Adenoviruses are double stranded DNA viruses that cause common benign respiratory tract infections in humans and for this reason antibodies against this type of virus are widespread within the human population. Adenoviral vectors (AV) are generally derived from serotype Ad2 or Ad5 and can be generated at very high vector titers (up to 10^{13} particles/ml). This vector type is able to transduce non-dividing cells and can realize high- level transgene expression from episomal vector DNA.

Adenoviral vectors (AV) are capable of transducing a wide variety of human cell types but infect epithelial-like cells most efficiently. This gives them possible therapeutic potential to treat lung disease such as cystic fibrosis. However, the main adenovirus receptor, the coxsackievirus and adenovirus receptor (CAR), is situated at the basolateral side of lung epithelium and as a consequence, transduction is only efficient when the epithelium is damaged [36]. Another major target of adenoviral vectors is the liver and the epithelial-like hepatocytes are readily transduced after intravenous vector administration.

The first generation of adenoviral vectors was deleted for the early regions E1 and E3, providing space for the insertion of 6.5 kb foreign DNA. These vectors appeared to elicit very strong immune responses caused by inadvertent expression of the remaining adenoviral genes, which limited the transgene expression to several days. In an attempt to evade this problem, additional early genes E2 and/or E4 were deleted from the adenoviral genome, resulting in a second generation of adenoviral vectors. These vectors showed prolonged expression, but a decline after several weeks due to capsid induced immunogenicity was still apparent. Eventually minimal (or “gutless”) adenoviral vectors were developed lacking all viral sequences except the packaging signal and two inverted terminal repeats (ITRs) on both ends of the genome [37]. These vectors have a relatively high cloning capacity of up to 37 kb and elicit only weak cellular immune responses in transduced cells, allowing long-term *in vivo* expression of the transgene up to several years in mice and rats.

In 1999, the field of gene therapy suffered a major setback with the death of an 18-year-old patient who participated in a liver-directed gene therapy trial with second generation AV for ornithine transcarboxylase deficiency. Four days after vector administration, the patient died from multiple organ failure, believed to be triggered by a severe innate immune response to the adenovirus capsid. Nevertheless, until now, over 350 clinical trials with adenoviral vectors were carried out in humans. In China a p53 expressing AV to treat head and neck squamous cell carcinoma is the first registered commercial gene therapy product [38].

Adeno-associated viral vectors
Adeno-associated virus is a simple, non-pathogenic human parvovirus. It is composed of a single stranded DNA genome that is flanked by two inverted terminal repeats and includes only two genes, *cap* and *rep*. For replication, AAV is dependant on additional viral gene products that are provided by a helper virus, usually adenovirus or herpes simplex virus. Recombinant adeno-associated virus vectors (AAV or rAAV, see Daya et al. for a recent review [39]) are able to transduce non-dividing cells, have a broad tropism for various cell types and can give rise to long-term transgene expression.

The standard vector is based on AAV2 that primarily transduces muscle, liver, brain, retina, and lung. The relatively small particle size makes enables penetration into more compact tissues. Pseudotyping by other AAV serotypes gives the vector different properties regarding tropism, transduction dynamics and immunogenicity. Interestingly, wild-type AAV is the only known mammalian virus that exhibits preferential integration into a specific region of the genome i.e. in the short arm of chromosome nineteen. However, AAV vectors do not share this property and mostly persist in the nucleus as episomal concatamers or randomly integrate at very low frequency.

The limitations of AAV vectors are the relatively small insert size of 4.4 kb and the need for a high multiplicity of infection (MOI) to achieve efficient gene transfer. The vector MOI can be decreased by using the more efficient self-complementary AAV vector (scAAV), which folds into double stranded DNA after transduction without the requirement for DNA synthesis or

base-pairing between multiple vector genomes [40]. The disadvantage is that the amount of free vector space is decreased by half to 2.2 kb.

Another drawback that limits clinical application is the relatively high prevalence of vector neutralizing antibodies in humans due to wild-type AAV2 infection [41]. A recent clinical trial applying AAV2 vectors for liver-directed gene therapy of hemophilia B showed a beneficial effect, but transduced hepatocytes were cleared by a cytotoxic T-cell response directed against the vector capsid [42]. In the future, these problems might be alleviated by the use of different vector pseudotypes with lower immunogenic potential or temporary immunosuppression supplementary to vector administration.

Gamma-retroviral vectors

In 1981 it was demonstrated that simple retroviruses could be used as vehicles to introduce foreign genes into the genome of a mouse cell line. Shortly after, this resulted in the genetic modification of Moloney murine leukemia virus (MuLV) into a vector for gene delivery purposes. In the literature, the nomenclature of this vector type is inconsistent and can be indicated as: retroviral vector, gamma-retroviral vector, or, referring to the fact that the wild-type virus is associated with the development of leukemia in mice, murine leukemia virus vector (MuLV or MLV) or oncoretroviral vector.

The gamma-retroviral genome consists of single stranded RNA that is reverse transcribed into a double stranded DNA counterpart during infection. The proviral DNA genome is flanked by long terminal repeats (LTR) and contains three genes (*gag*, *pol*, and *env*) and a packaging signal. Gamma-retroviral vectors are able to integrate into the host genome and can give rise to long-term transgene expression. Furthermore, they can be produced at high titers of up to 10^{10} particles per ml, exhibit low immunogenicity and can accept about 6 kb of exogenous DNA. The ability to pseudotype the vector with envelope-glycoproteins of different viral origins broadened its tropism to a wide variety of cell types.

Over 300 clinical trials employing gamma-retroviral vectors have been initiated. However, the inability of this vector to transduce non-dividing cells has limited its clinical use. The most successful application involves the *in vitro* modification of hematopoietic stem cells for autologous transplantation purposes [43]). This protocol was successfully applied in several clinical trials to correct several inherited immunodeficiency disorders, including X-linked severe combined immunodeficiency (X-SCID) [44-46], adenosine deaminase deficiency (ADA-SCID) [47, 48] and X-linked chronic granulomatous disease (X-CGD) [49].

In these trials, the majority of treated children showed immune reconstitution, but the enthusiasm was tempered when a patient in a X-SCID trial developed leukemia. To date, 5 of the 23 treated X-SCID patients have developed leukemia as a result of insertional mutagenesis by the retroviral vector (4 in a French and 1 in a British trial). All but one of these children responded well to conventional anti-leukemia treatment. As a result, safety considerations and the inability of gamma-retroviral vectors to transduce non-dividing cells stimulated the emergence of a more sophisticated retroviral vector system based on lentiviruses that will be described in further detail in the next section.

1.3 Lentiviral vectors

Lentiviruses are a subgroup of particularly complex retroviruses that have evolved the capability to infect non-dividing cells [50]. Undoubtedly, the most notorious member of the lentivirus family is human immunodeficiency virus 1 (HIV-1) that was discovered in 1983 as the etiologic agent of AIDS [51]. The combination of extensive HIV-1 research and the experience gathered during the development and clinical application of gammaretroviral-based vector systems were at the basis of the creation of HIV-1 derived lentiviral vectors [52]. Although HIV-1 derived lentiviral vectors are best characterized, vector systems based on several other lentiviruses with less pathogenic origins are under investigation, including HIV-2, simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV) and equine infectious anemia virus (EIAV).

Lentiviral vectors display a unique combination of features that is unequalled by other established vector systems. Lentiviral vectors are able to transduce both dividing and non-dividing cells with high efficiency and can carry approximately 7 kb of foreign DNA that is stably integrated into the genome to facilitate long-term transgene expression. Upon administration, the vector evokes only minimal immune responses in laboratory animals and pre-existing immunity is not a concern, since antibodies against HIV-1 are not widespread in the human population. To overcome the CD4 tropism restriction of wild-type HIV-1, lentiviral vectors can be pseudotyped by a heterologous viral envelope protein, generally derived from the vesicular stomatitis virus glycoprotein (VSV-G). This broadens vector tropism to a wide variety of eukaryotic cells, facilitates the production of high functional vector titers and allows lossless concentration by ultracentrifugation.

Insertional mutagenesis

The most important caveat that impedes broad clinical application of lentiviral vectors is the risk of proto-oncogene activation by insertional mutagenesis. Genomic integration of gammaretroviral vectors was associated with the induction of leukemia after transplantation of gene modified HSCs in two clinical trials for X-SCID. In general, all integrating vectors share the preference to integrate in the open chromatin structure of actively transcribed genes. However, in terms of safety, recent studies have demonstrated that lentiviral vectors display a more favorable integration pattern than gammaretroviral vectors and the infrequently integrating AAV vectors [53, 54]. Where gammaretroviral- and AAV based vectors preferentially integrate in the vicinity of transcriptional start regions, lentiviral vector integration is more randomly distributed over the whole transcriptional unit, which is expected to significantly reduce the risk of proto-oncogene activation.

So far, HIV-1 derived vectors as well as wild type HIV-1 infections have never been linked to increased tumor risks due to insertional mutagenesis. Lentiviral vectors based on EIAV were associated with the induction of liver tumors when administrated to fetal and neonatal mice. However, these results could not be reproduced with HIV-1 derived vectors [55]. The development of self-inactivating vectors described later in this chapter was proven to further reduce the risk of insertional mutagenesis. [56, 57]

Since lentiviral vectors have a preference to integrate into active genes, the pattern of vector integration is also determined by the subset of genes that is active at the time of transduction. Here, transduction of dividing cells may be more hazardous due to the activity of cell cycle regulatory genes. Furthermore, the risk of insertional mutagenesis appears to be proportional to the strength of the internal enhancer-promoter that is used to drive transgene expression [58]. These variables make it extremely difficult to predict the precise risk of lentiviral vector transduction in various mitotic and post-mitotic tissues. Before large-scale clinical application can be considered, elaborate safety studies will be required to assess the risk of insertional mutagenesis for each separate context of application.

The HIV-1 genome and virion

The HIV-1 virion is a spherical, 110 nm diameter particle consisting of a protein core surrounded by a lipid membrane envelope (Fig 1B.). The center of the protein core encompasses the HIV-1 genome, which is present as two identical copies of positive-sense, single-stranded RNA. After reverse transcription and genomic integration, the proviral DNA variant of the genome is 9.18 kilobases (kb) in length and expresses at least nine genes (Fig 1A). The three largest genes, *gag*, *gag-pol* and *env*, encode the major structural proteins of the virus. As opposed to simple gammaretroviruses, the HIV-1 genome encodes six additional proteins, including two regulatory proteins, Tat and Rev and four accessory proteins Nef, Vif, Vpr, Vpu that all play various HIV-1 specific roles in the viral lifecycle.

Gag (p55) and Gag-Pol (p160) are expressed as precursor polyproteins from the same unspliced, full-length RNA strand, which later also acts as viral RNA genome for packaging into the virus particle. Gag-Pol is generated by ribosomal frame shifting during the translation of Gag, which occurs in approximately 5-10% of all translation events. Later, in a process referred to as maturation, which takes place after the newly formed virus particle has been

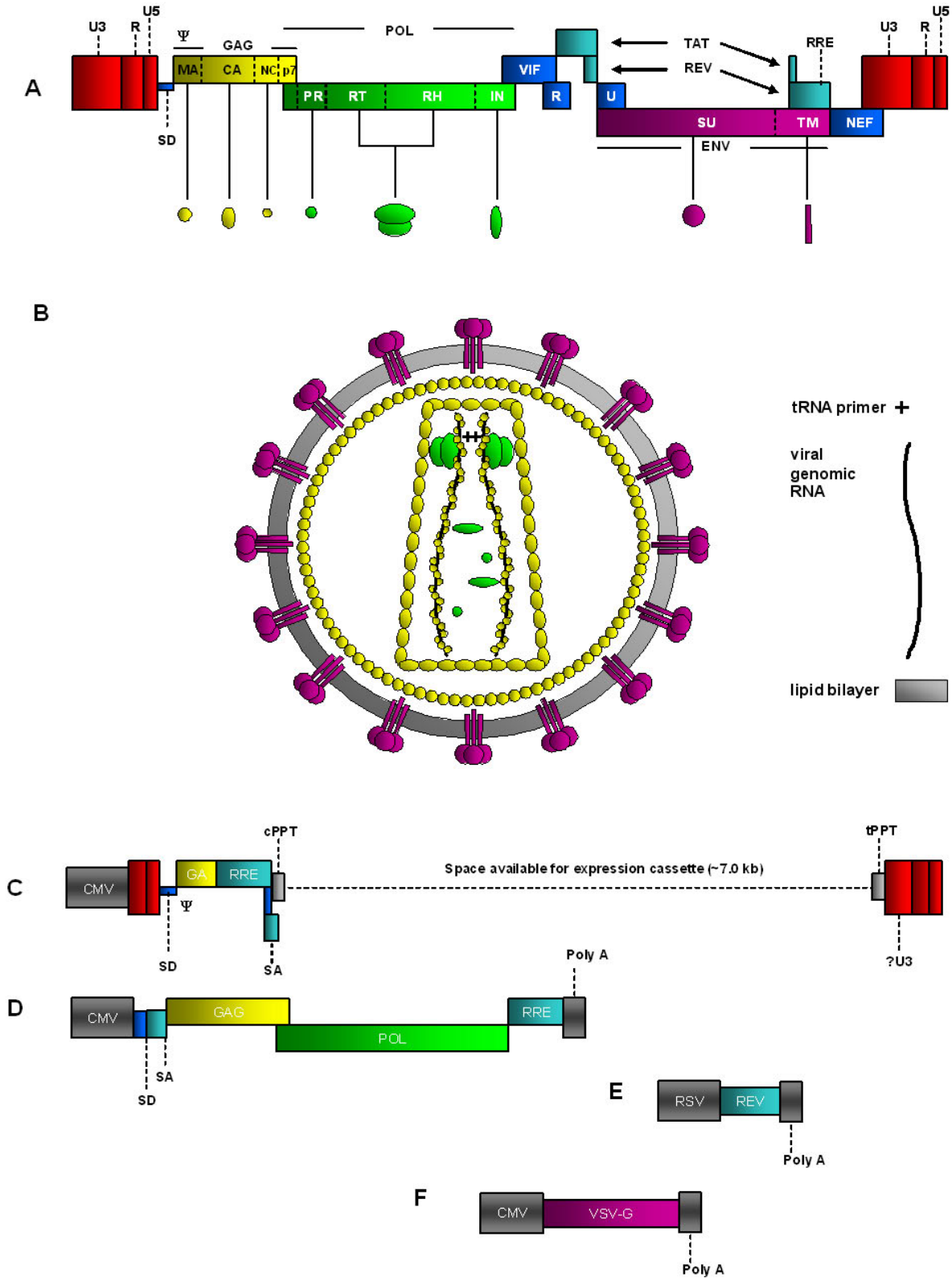

Fig. 1. The HIV1 genome, virion and lentiviral vector production constructs. *(A) The wild-type HIV-1 genome. Indicated are the nine genes, packaging signal (ψ) and the two LTR sequences. Structural genes (gag, pol and env) express precursor polyproteins that are proteolytically processed to yield the mature virion proteins that are indicated under each gene. (B) The mature HIV-1 virion. Represented form the outside to the inside are : the lipid bilayer with envelope spikes, the matrix, the capsid and the nucleocapsid. Inside the capsid, the two genomic RNA copies are present with bound viral enzymes and tRNA primer. (C-F) Schematic representation of the four production constructs for the production of HIV-1 derived SIN VSV-G pseudotyped lentiviral vectors. (C) The transfer construct. Indicated are: the CMV*

promoter that replaces the 5' U3 region, packaging signal (Ψ), RRE, cPPT, the space for the nsertion of a transgene expression cassette and the deletion in the 3'U3 (ΔU3) that renders the vector self-inactivating. (D) The packaging construct under control of the CMV promoter, that expresses Gag and Gag-Pol dependant on the binding of Rev to the RRE. (E) The Rev expression construct under control of the RSV promoter. (F) The pseudotyping construct that expresses the heterologous envelope protein in the form of VSV-G. Figure adapted from [59, 60]

released from its host-cell, the viral protease cleaves the Gag and Gag-Pol polyproteins into their individual subunits.

The cleavage products of Gag including: matrix protein (MA, p17), capsid protein (CA, p24) and nucleocapsid protein (NC, p9) give rise to the three layers that make up the HIV-1 protein core. An additional, non-structural Gag peptide P6, binds Vpr in the Gag protein and facilitates its incorporation into assembling virions. The Pol polyprotein gives rise to the four viral enzymes that are all present in the capsid, including: protease (PR, p10), reverse transcriptase (RT, p50), RNAse-H (RH, p15) and integrase (IN, p31). In addition, the capsid contains some copies of accessory proteins Nef, Vif and Vpr.

The viral membrane envelope is equipped with several viral spikes that facilitate receptor binding and membrane fusion during infection. These spikes consist of the subunits of the Env precursor glycoprotein (gp160) that is expressed from singly spliced viral RNA. Env is expressed in the endoplasmic reticulum, glycosylated in the Golgi and transported to the cell surface where it is cleaved by a cellular protease into transmembrane protein (TM, gp41) and surface glycoprotein (SU, gp120). These two cleavage products form heterodimers and a complex of three heterodimers makes up one viral spike.

In addition, the HIV-1 genome contains multiple cis-acting sequences that are crucial for virus assembly, replication, reverse transcription, integration and expression of viral RNA and proteins. The full-size dsDNA HIV-1 genome is flanked on both sides by long terminal repeats (LTR) that consist of three subregions, designated U3, R and U5. The U3 (unique 3' sequence) region contains the viral promoter for transcription of the viral RNA. The R (repeat) region contains the Tat-activation region (TAR) that acts as a DNA binding site for Tat, which enhances transcription of full-length viral RNA. The R-region also contains a polyadenylation (poly-A) signal to terminate viral transcription. Both the U3 and U5 regions contain the attachment (*aat*) sites for the viral integrase that facilitates genomic integration. The RNA version of the HIV-1 genome is incomplete in the sense that it lacks the 5' U3 and 3' U5 region. During reverse transcription, the RNA forms a circle that facilitates synthesis of the 3'-U5 from the 5'-U5 and the 5'-U3 from the 3'-U3, resulting in the full-sized dsDNA genome.

The remaining cis-acting sequences are all situated between the two LTRs. The primer-binding site (PBS) binds a host-cell t-RNA that is used as primer to initiate reverse transcription. The splice donor and acceptor sites (SD and SA) help to regulate the expression of viral proteins by facilitating alternative splicing. The SD is located upstream of the packaging signal to prevent packaging of spliced RNA. The packaging signal (ψ) is a sequence of which the secondary and tertiary RNA structure forms a complex 'knot' that interacts with the Gag polyprotein and directs the capturing of the two copies of RNA genome into the vector particle. The Rev-response element (RRE), acts as a binding site for Rev, which inhibits splicing and facilitates efficient nuclear export of large unspliced and single-spliced viral RNAs. Finally, the genome contains a central polypurine tract (cPPT) that has been found to be crucial for efficient transduction of non-dividing cells as it leaves a DNA "flap" after reverse transcription that enhances nuclear translocation of the viral pre-integration complex[61].

The HIV-1 life cycle

The primary receptor by which HIV-1 associates with its target cell is CD4, a molecule that normally functions as a co-receptor of the T-cell receptor during T-cell activation. Apart from CD4, HIV-1 infection also depends on the chemokine co-receptors CCR4 or CCR5 that are

present on CD4+ T-lymphocytes or macrophages respectively. The receptors are recognized by the HIV-1 surface glycoprotein SU present in the envelope spikes. Receptor binding triggers a conformational change that exposes HIV-1 transmembrane protein TM to initiate membrane fusion between the viral envelope and the host-cell membrane (Fig. 2A).

The fusion process deposits the viral core in the host-cell cytoplasm where it uncoats to reveal the viral pre-integration complex. The pre-integration complex consists of the homodimer of viral genomic RNA and the proteins RT, IN, NC, p6, Nef, MA and Vpr, the latter two targeting the complex for import via the nuclear pore. During migration to the nucleus, the viral RNA genome is reverse transcribed by RT into linear, double-stranded DNA. After entering the nucleus, the enzyme IN catalyses the integration of the viral DNA into the host genome.

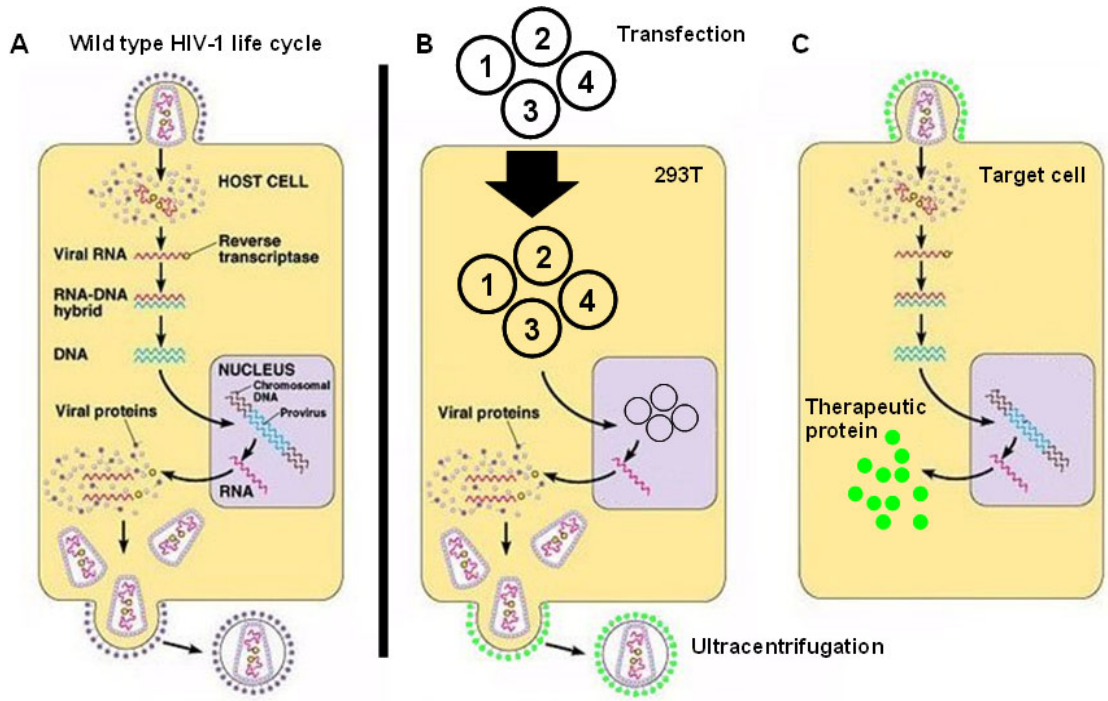

Fig. 2. The continuous wild-type HIV-1 lifecycle compared to dead-ended production and transduction process of lentiviral vectors. *(A) The wild-type HIV-1 lyfecycle is a continuous process. After infection and genomic integration of the viral genome, novel viral particles are generated that leave the cell by budding and are ready to infect another host cell. (B) The transfection of four lentiviral vector production plasmids provides all viral proteins and a modified genome that contains a transgene expression cassette and no viral genes. These components combine to give rise to the production of a lentiviral vector particle. The newly formed vector particles leave the production cell by budding and end up in the production medium. After harvesting of this medium, the vectors can be concentrated by ultracentrifugation. (C) The transduction of a target cell by lentiviral vectors is a dead-end process. No novel vector particles are formed and after genomic integration of the vector genome, only the transgene of interest is expressed.*

After integration, the enhancer-promoter region in the 5' LTR expresses a 9-kb full-length genomic transcript that is processed by an alternative splicing event in an array of more than 40 mRNAs that encode at least 9 different HIV-1 proteins. Early expression consists mainly of multiple-spliced mRNAs, encoding the viral proteins Tat, Rev and Nef. Later, an expression shift is mediated by the binding of Tat to the TAR sequence near the viral promoter, which enhances RNA polymerase II efficiency. The binding of Rev to the RRE sequence in viral

transcripts facilitates nuclear export of full-length unspliced and single spliced mRNAs encoding the Gag, Pol and Env polyproteins and accessory proteins Vif, Vpr and Vpu.

Meanwhile, the viral core starts to assemble, as Gag and Gag-Pol aggregate at the inner surface of the plasma membrane, also attracting two full-length copies of the viral genomic RNA and several copies of the accessory proteins Nef, Vif and Vpr. Subsequently the viral core starts to extrude from the plasma membrane. This process called budding and is enhanced by the presence of Vpr. The particle acquires a membrane lipid coat from the host cell membrane carrying the viral spikes. Shortly after budding, the viral protease is activated and the virion undergoes the morphological changes associated with maturation, which generates the infective form of HIV-1. This completes the viral replication cycle and the newly formed particle is ready to infect another target cell.

Lentiviral vector production and transduction

Compared to the HIV-1 lifecycle, the individual events that occur during the production and transduction of lentiviral vectors involve similar, yet more simplified processes (Fig. 2B,C). Production of third-generation lentiviral vectors is based on transient co-transfection of a human cell line, generally HEK293T, with four production plasmids that will be discussed in more detail later in this chapter. These constructs include: the transfer construct (Fig. 1C), the packaging construct (Fig. 1D), the rev expression construct (Fig. 1E) and a pseudotyping construct (Fig 1F). After transfection, the transfer construct expresses the vector RNA that will be packaged and the packaging construct expresses a Gag/Gag-Pol mRNA. Efficient nuclear export of these unspliced RNAs is facilitated by Rev, which is supplied by the Rev expression construct.

Analogous to wild-type HIV-1, the vector particle assembles at the periphery of the cell, acquiring two copies of the vector RNA by their HIV-1 packaging signal. Eventually, the vector particle buds from the production cell, thereby acquiring a lipid membrane envelope containing the heterologous viral glycoproteins expressed by the pseudotyping construct. After approximately 24 hours, vector particles start to accumulate in the production medium and can be easily harvested in the culture medium. After harvesting, the vector particles can be concentrated one thousand times without loss of titer by ultracentifugation and are frozen until further use.

For the actual gene therapeutic intervention, the vector is administered to cultured target cells *in vitro* or *in vivo* to the organs of live subjects, e.g. intratracheally or via the blood stream. When encountering a target cell, the commonly used VSV-G pseudotype in the vector envelope recognizes an unspecified, yet ubiquitous, membrane phospholipid as its receptor and enters the cell, not by direct membrane fusion like wild-type HIV-1, but by receptor mediated endocytosis of the whole particle, including the lipid membrane. After endocytosis, endosomal acidification triggers a conformational change in VSV-G that allows cytoplasmic transfer of the vector core by facilitating fusion of the vector envelope with the endosomal membrane.

Once in the cytoplasm, the vector undergoes an identical process as wild-type HIV-1. The capsid uncoats to reveal the pre-integration complex and while being translocated to the nucleus, the vector RNA is reverse transcribed into dsDNA. In the nucleus, the vector DNA is integrated into the target cell genome. In contrast to the wild-type provirus, current lentiviral vectors are designed to prevent the generation of RNA copies that can be packaged, *de novo* viral proteins or read through RNA products, by modification of the LTR promoter in integrated vectors as we will describe below. Instead, expression is limited to the gene of interest encoded by the transgene expression cassette present in the vector sequence.

The lentiviral vector production constructs

Since HIV-1 is a dangerous pathogen, the evolution of the production system focused on the implementation of several biosafety features, while maintaining vector titer and transduction efficiency. The first safety feature reduces the chance of homologous recombination by separation of the HIV cis- and trans-acting sequences onto different constructs and

minimization of the degree of overlap between these constructs. Homologous recombination could lead to the inadvertent generation of replicating vector derivates named replication competent retroviruses (RCR). The second safety feature involves the deletion of five of the nine genes present in the wild-type HIV-1 genome and the replacement of the *env* gene by a heterologous glycoprotein for pseudotyping. Finally, the vector transfer construct carries a self-inactivating LTR that does not exhibit transcriptional activity after reverse transcription and vector integration. The functions and characteristics of the four individual production constructs are discussed in further detail below.

The transfer construct

The lentiviral transfer construct serves as the template for transcription of full-length vector RNA for packaging into the assembling vector particle. This construct encodes no viral proteins and contains only the minimal HIV-1 cis-acting sequences required for efficient encapsidation, reverse transcription, nuclear import, and integration into the target cell genome. These include the LTRs (wild-type or modified), PBS, SD, SA, ψ, RRE and cPPT already described earlier in this chapter. The deletion of all viral genes creates approximately 7 kb of free space for the insertion of a custom transgene expression cassette. Interestingly, in contrast to AV, AAV and MLV based vectors, lentiviral vectors do not have a static upper or lower packaging limit [62]. In fact, packaging is generally most efficient when no exogenous sequences are inserted into the transfer construct. As a general rule, the vector titer is inversely proportional to the insert size, with a titer drop of approximately half a log for every 4 kb of extra DNA added to the vector and one log per 4 kb when the size of vector RNA exceeds that of the wild-type HIV-1 genome, i.e. 9,18 kb.

Third generation lentiviral vectors are self-inactivating. Early generations of the transfer construct contained complete wild-type LTRs whose 5' and 3' U3 regions exhibit transcriptional activity that is enhanced by the binding of Tat. Especially in case of a wild-type HIV-1 infection, activation of the 5'LTR promoter could result in unwanted mobilization and packaging of vector RNA. Furthermore, the low-level transcription activity of the 3' LTR in target tissues increases the risk of proto-oncogene activation [56]. Self-inactivating vectors do not share this property, as their 5' U3 region is replaced by a heterologous promoter (generally CMV or RSV) and the parental promoter sequences in the 3´ U3 region are deleted. During reverse transcription, the crippled 3' LTR serves as a template for the 5' LTR, resulting in both LTRs lacking transcriptional activity after vector integration. Two main versions of HIV-1 SIN LTRs exist, one carrying a 133 bp deletion present in transfer constructs designated pCCG from the Verma lab [63] and one with a 400 bp deletion developed by Naldini and Trono et al. present in pRRL transfer constructs [64].

The packaging construct and Rev-expression construct

Except for the envelope glycoprotein, the lentiviral packaging construct provides the vector system with all trans-elements that are required to produce a functional vector particle. The earliest packaging constructs expressed the complete HIV-1 genome, with the exception of Env. Further modification resulted in a first generation packaging construct with improved biosafety [65]. The packaging signal and primer binding site were deleted to prevent packaging and reverse transcription of trans-acting sequences. Also, both LTRs were replaced by a 5' constitutive promoter (CMV or RSV) and a 3' SV40 poly a signal to further minimize sequence homology with the transfer construct.

In the second generation packaging construct, the accessory genes *nef*, *vif*, *vpr* and *vpu* were deleted, while expression of Tat and Rev was preserved [66]. This modification improved vector safety, as the accessory proteins are crucial for HIV-1 pathogenesis and their deletion further reduces the amount of HIV-1 sequences present in the system. Shortly after, a third generation construct was created in which the *tat* gene was deleted [60]. This was made possible by rendering the transfer construct Tat independent. The 5' U3 containing the Tat binding site (TAR) was replaced with a constitutive promoter. Furthermore, the *rev* gene was placed on a separate expression construct. This increases the minimum number of recombination events needed for RCR formation from two to three. In essence, the resulting

third generation packaging construct provides only the Gag and Gag-Pol genes. The envelope glycoprotein and Rev are both provided by separate constructs.

Non-integrating lentiviral vectors

The concern that integrating retroviral vectors may be genotoxic has prompted the development of non- integrating lentiviral vectors. Recently, the efficacy of non-integrating lentiviral vectors for gene therapy purposes has been demonstrated (reviewed by Philpott et al [67]). Lentiviral vectors can be engineered to be integration defective by mutating the viral integrase in the packaging construct, or, with lower efficiency, the integrase attachment (*aat*) sites in the U5 or U3 regions of the transfer construct's 3'LTR. The natural life cycle of HIV gives rise to three variants of dsDNA, a linear version, a 1 LTR circle and a 2 LTR circle[68]. Only the linear version takes part in the integration process. Whether the 1 and 2 LTR circular forms are a product of faulty processing (homologous recombination and non-homologous end-joining of the LTRs respectively) or serve a particular function is unknown.
Like many episomal transgenes, the LTR circles are lost during cell division. They have been shown to be stable in quiescent cells *in vitro* where they can give rise to gene expression levels comparable to integrated lentiviral transgenes. *In vivo*, non-integrating lentiviral vectors are able to realize long-term gene expression in several post-mitotic tissues *in vivo*, including ocular tissue, brain and muscle [69, 70]. In the future, the increased safety profile of these vectors may make them applicable for more routine use such as vaccination against pathogens or tumor antigens. In the field of stem cell research, they could be employed for transient manipulation of cell growth or differentiation. Furthermore, the ongoing research regarding the development of non-integrating lentiviral vectors creates a platform for the design of lentiviral vectors that may be able to integrate at specific sites of the host genome [71].

Transgene expression cassettes for lentiviral vectors

The transgene expression cassette present in the transfer construct is responsible for the generation of the functional RNA species that mediates the gene therapeutic effect after transduction. The properties of this cassette directly determine the level and cell specificity of transgene expression, which are the most important variables when performing gene therapy. The transgene expression level that needs to be realized per vector copy depends on the properties of the target disease. The total amount of transgene expression required to cure a disease can vary from low-level expression in a small number of target cells to high-level expression in the majority of all available target cells. When using lentiviral vectors, the gene therapeutic effect is determined by the product of: 1) the total number of transduced target cells, 2) the expression level per vector copy and 3) the number of vector copies per target cell.

Many gene therapy strategies suffer from limited transduction rates, and beneficial effects can often only be achieved by ensuring high-level gene expression for each vector copy. However, the therapeutic effect is not the only reason why many gene therapists strive to optimize the expression levels from their vectors. Optimization can also improve the biosafety of a gene therapy procedure. First, it minimizes the total number of integrated vector copies required to obtain a desired curative effect, thereby decreasing the risk of insertional mutagenesis. Second, it allows minimization of the applied vector dose, which reduces the chance of an immune response. Another important aspect of minimizing vector dosages is that it encourages the realization of clinical gene therapy protocols by alleviating existing technical and financial hurdles regarding large-scale vector production.

There are various options to improve transgene expression from integrated vector copies. The most straightforward approach is to improve the transcription rate by using stronger transcription regulatory elements. Nevertheless, it has been recently reported that there is a positive correlation between the total amount of transcriptional activity per vector copy and the occurrence of insertional mutagenesis [58]. As a consequence, alternative options to improve transgene expression at a posttranscriptional level are rapidly gaining interest. Furthermore, apart from the expression level, changes in expression cassette design have been shown to

prevent gene silencing and to avoid the induction of immune responses against the introduced transgene.

Self-inactivating lentiviral vectors can be equipped with two types of expression cassettes that are either protein-coding or non-protein-coding. The prototype transgene expression cassette for protein expression has a relatively simple design and consists of a 5' internal enhancer-promoter, a protein-coding sequence (CDS) and a 3' woodchuck hepatitis virus posttranscriptional regulatory element (WPRE). Non-protein-coding expression cassettes direct the expression of various RNA interference (RNAi) molecules, which can be achieved in several ways.

In the next section, the optimal design of lentiviral expression cassettes in terms of transgene expression level, expression sustainability, biosafety, immunogenicity and compatibility with vector production will be discussed. Since the expression of foreign proteins can be highly immunogenic, a promising new strategy to prevent off target transgene expression is reviewed. Finally, we will provide an overview of different expression cassettes for the generation of RNAi molecules for inhibition of gene expression.

The internal promoter

For many research applications it is most convenient to choose an internal promoter that directs high levels of transgene expression in a wide variety of cell types. Constitutive promoter sequences that are frequently used in lentiviral vectors originate from: 1) viruses e.g. CMV (cytomegalovirus), SFFV (spleen focus forming virus), MESV (murine embryonic stem cell virus), GALV (gibbon ape leukemia virus) 2) eukaryotes e.g. PGK (phosphoglycerate kinase), EF1-alpha (elongation factor 1-alpha), UBC (ubiquitin C) or 3) hybrid sources e.g. CAG (CMV immediate-early enhancer/chicken beta-actin promoter). A major disadvantage of using viral enhancer and promoter sequences is their relatively high CpG content which makes them susceptible to gene silencing by DNA methylation, especially *in vivo* [72]. Furthermore, the use of strong viral promoters, such as SFFV, has been associated with enhanced levels of integrational mutagenesis compared to endogenous eukaryotic promoters [58].

Transcriptional targeting by tissue-specific promoters

For most gene therapy purposes, ubiquitous gene expression is not desired, especially when the vector is administrated directly *in vivo*. Most of the current gene therapy vectors lack specificity in the target cells that they transduce, including the commonly used VSV-G pseudotyped lentiviral vectors [73]. When administered via the bloodsteam, the VSV-G pseudotype transduces not only hepatocytes but also endothelial, bone marrow, gonad and professional antigen presenting cells in the form of liver Kupffer cells and splenocytes [74]. The use of constitutive promoters is problematic in this scenario. First, (over)expression of a foreign transgene in non-target cells can deregulate cellular function. Second, off-target expression of neoantigens in antigen presenting cells is associated with the induction of humoral and cellular immune responses against the newly introduced gene product [75].

To compensate for the lack of target specificity displayed by gene therapy vectors at the transduction level, serious efforts are made to restrict transgene expression to a desired target cell-type, a process often referred to as "transcriptional targeting". It was shown that the use of a liver-specific murine albumin enhancer-promoter instead of the ubiquitous PGK promoter prevents immune responses directed against human FIX in the liver of normal immunocompetent mice but not in FIX knock out mice that produce no murine FIX [76].

Regarding promoters, the term "tissue-specific" is relative and the development of true, completely leakage-free, cell-type specific promoters can be challenging and susceptible to inter-species variations. Furthermore, transcription units used in gene therapy vectors are often composed of only minimal enhancer and promoter sequences that lack the subtle genomic context that normally facilitates true tissue-specific expression. Indeed, it has been observed that a promoter such as the murine albumin enhancer-promoter, that was always considered to be liver-specific, gives rise to low-level expression in APCs in mice [77]. On the

other hand, independent of promoter specificity, low-level off-target expression can hardly be avoided when using integrating vectors. In a process named “promoter trapping”, genomic integration occurs in a promoter region in such a manner that the full-length vector sequence is transcribed, resulting in expression of the transgene [78].

Transcriptional de-targeting

Recently, Brown *et. al.* reported an innovative strategy to prevent expression from the ubiquitous PGK promoter in a lentiviral context, specifically in cells of the hematopoietic lineage, including all professional APCs [77]. This de-targeting approach is based on the incorporation of four tandem copies of a 23 bp microRNA target sequence into the 3’ UTR of the transgene expression cassette. This target sequence is recognized by an endogenous microRNA (miRNA) that is only expressed in hematopoietic lineage cells, resulting in cell-type specific degradation of vector transcripts. The sequence of the involved miRNA (mir-142-3p) is conserved from mouse to human, which allows application of this approach in both species.

For gene therapy applications, this procedure can be utilized to further minimize off target expression in APCs caused by both leakage from tissue-specific promoters and promoter trapping. Combined with the synthetic liver-specific ET promoter, this de-targeting approach made it possible to stably express FIX in the liver of FIX knock-out mice without inducing a cellular immune response against FIX expressing hepatocytes, a result that could not be realized before the use of miRNA technology [79]. Furthermore, FIX knock-out mice treated with this vector were irresponsive to FIX immunization, indicating that treatment induced a high degree of tolerance. It is hypothesized that this tolerance is realized by active expression of a neo-antigen in non-professional APCs, such as liver sinusoidal endothelial cells (LSEC) in the liver, in combination with the absence of expression in professionals APCs. By using target sites of other differentially expressed miRNAs or combining them in one vector it has been shown that gene expression can be prevented based on tissue, lineage and differentiation state, facilitating the generation of expression cassettes with completely novel expression patterns [80].

The protein-coding sequence (CDS)

The most basic approach to express a gene of interest from a lentiviral vector is to introduce its cDNA sequence downstream of an internal promoter. The cDNA should always be truncated in the 3’ UTR, upstream of the polyadenlation signal, as the incorporation of premature transcription termination signals in lentiviral vector transfer constructs results in generation of defective vector RNA that lacks the 3’ LTR. Also, when transferring expression cassettes from other eukaryotic expression plasmids to the lentiviral backbone, caution is required not to include the excising poly(A) signal from the donor construct. Another frequent modification is the removal of the cDNA 5’ and 3’ UTR, leaving only the Kozak sequence and the CDS. Removal of the UTRs can be beneficial, not only because it reduces the insert size, which improves lentiviral vector titers in general, but also, UTRs may contain various unidentified RNA regulatory sequences that can interfere with efficient vector production and/or transgene expression.

Removal of the UTRs is generally performed by PCR amplification of only the relevant sequence from the cDNA, followed by cloning and sequencing of the new PCR product. To improve the ease of subcloning into the lentiviral vector backbone, custom restriction sites can be added to both PCR primers. Another improvement that can be easily realized while performing PCR is the replacement of the existing Kozak sequence by the consensus sequence (GCC)GCCA/GCCATGG that can be introduced via the forward PCR primer. PCR primers that force inclusion of custom sequences into the end-product exhibit only partial template homology at the start of the reaction. Accordingly, the annealing temperature in the first few cycles should be chosen relatively low and can be gradually increased as the reaction proceeds. Furthermore, protein-coding sequences can be best amplified by using a high fidelity DNA polymerase to prevent the introduction of mutations into the PCR product as much as possible.

Another strategy to improve the expression-level per vector copy is to perform codon optimization. The occurrence of infrequently used codons in the CDS hampers protein synthesis by the limited availability of their corresponding cellular tRNAs [81, 82]. Codon optimization involves *de novo* synthesis of a CDS in which the codon bias has been adapted with respect to non-limiting tRNAs pools, hereby maintaining the original amino acid sequence of the gene product. The generation of a synthetic CDS provides an opportunity to optimize several additional sequence parameters that favor sustained, high-level gene expression. This process is often referred to as sequence or transgene optimization. The changes can include e.g. G/C content adaptation to improve mRNA stability [83], elimination of immunostimulatory CpG motifs, avoidance of direct DNA repeats to prevent vector recombination, removal of internal splice sites and premature polyadenylation signals, preventing the formation of stable RNA secondary structures, deletion of translational silencers, avoidance of dsRNAs with host transcriptome, avoidance of a-specific host miRNA interactions, addition of RNA stabilizing and nuclear translocation supporting sequence elements and optimization of translation initiation regions.

Many of these adaptations not only improve transgene expression levels but also vector titers by improving the packaging efficiency or the number of vector genomes available for packaging. [84-86]. Considering the decreasing costs of this procedure, performing codon optimization has become an advisable option when constructing novel gene therapy vectors, especially for pre-clinical evaluation.

The WPRE

An extra regulatory element that is routinely included in lentiviral expression cassettes is the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) [87]. The inclusion of WPRE in the 3' UTR downstream of the transgene, increases expression levels several fold in a transgene, promoter and vector independent manner. Since its application, it was always assumed that WPRE acts by improving RNA nuclear export or stability [88]. However, recently it was reported that the WPRE mainly functions by reducing the amount of transcriptional read-through in retroviral vectors, thereby improving not only vector efficiency, but potentially also biosafety [89].

High degrees of transcriptional read-through are observed in all retroviral vector types. Retroviruses have evolved weak poly(A) sites to prevent premature termination and polyadenylation in the R region of the 5′ LTR. In case of self-inactivating lentiviral vectors, deletion of the U3 further increases the probability of read-through, suggesting that U3 contains termination enhancer motifs in addition to enhancer-promoter sequences. The incorporation of additional viral upstream sequence elements (USE) that are known to improve transcription termination are currently being evaluated for incorporation into self-inactivating lentiviral vectors [90]. Finally, the WPRE encodes the first 60 amino acids of the woodchuck hepatitis X protein, the full-sized version of which was associated with the generation of liver cancers. Recently an improved version of WPRE has been generally adopted in which all transcription start codons have been mutated [91].

Expression cassettes expressing noncoding RNAs

Apart from protein-coding sequences, lentiviral vectors can also be utilized to express noncoding RNAs. The expression of ribozymes or RNA decoys has been described [92]. However, the most used application is post-transcriptional gene silencing by means of RNA interference (RNAi). RNAi is performed via small interfering RNAs (siRNA) that are identical to the miRNA molecules mentioned earlier in the de-targeting strategy. Both RNA species associate with the RNA-induced silencing complex (RISC) to degrade homologous RNA target sequences. Whereas miRNAs are derived from cellular primary-miRNA (pri-miRNA) genes, siRNAs exist in the form of synthetic exogenous RNA duplexes that are generally delivered by non-viral gene delivery methods.

Apart from synthetic molecules, siRNA sequences can also be indirectly generated from lentiviral vectors by transcription. The first option to generate siRNAs is to express an antisense RNA directed against a desired target sequence. Hybridization of the antisense

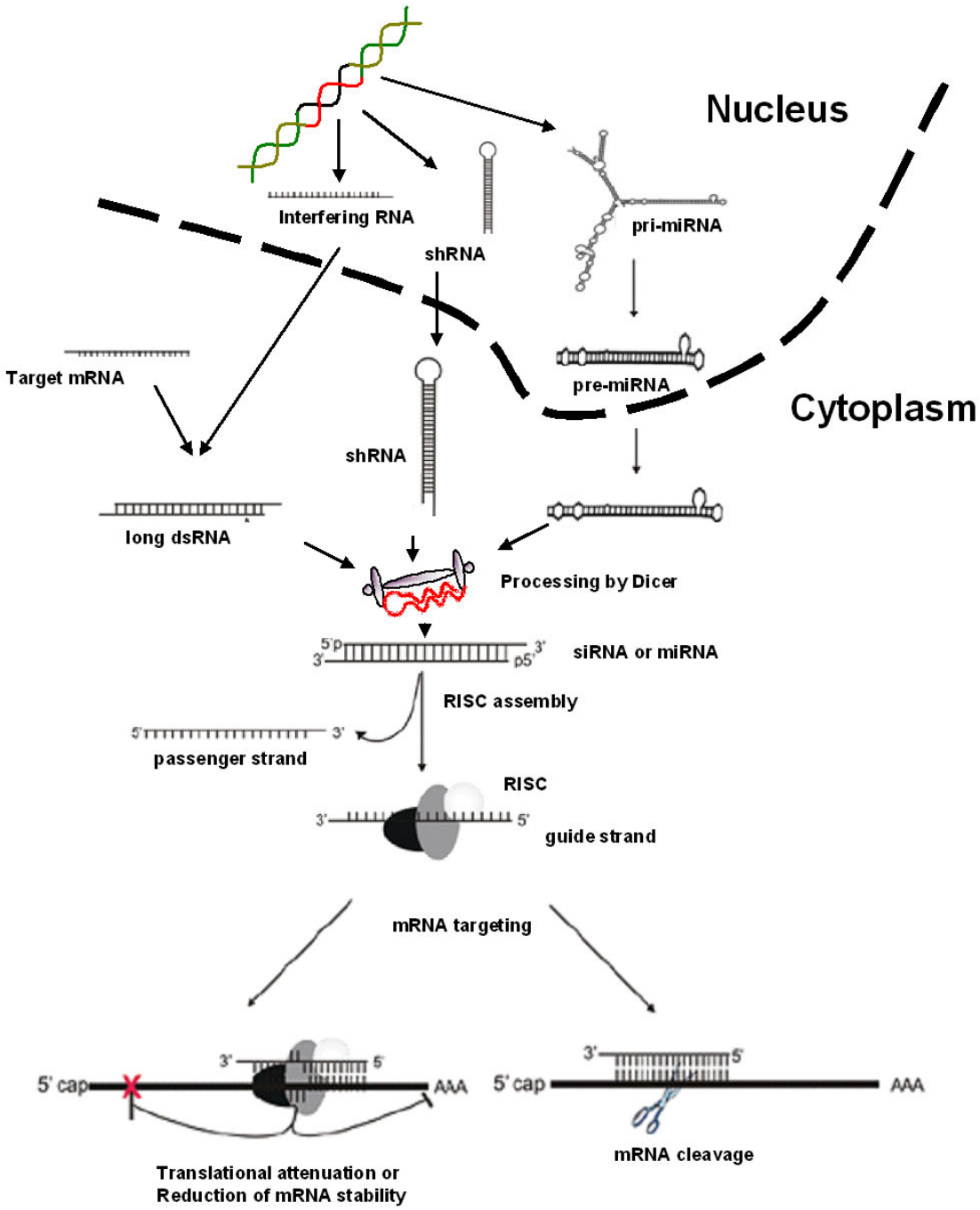

Fig. 3. The iRNA, shRNA and miRNA pathways for downregulation of gene expression. *Small interfering RNA (siRNA) and microRNA (miRNA) are small, noncoding RNAs of 20-25 nucleotides in size that act as a guide for the RNA-induced silencing complex (RISC) to repress the translation of homologous target transcripts. The only difference between siRNA and miRNA is their origin. Mature functional miRNAs are generated from larger primary microRNA (pri-miRNA) transcripts by excision of a ~70 nucleotide stem-loop precursor miRNA (pre-miRNA). siRNAs are derived from interfering RNA (iRNA) or short hairpin RNA (shRNA). After nuclear export, pre-miRNA and shRNA are directly processed by Dicer into a small RNA duplex, the functional strand of which is then loaded into RISC. The iRNA first needs to hybridize to its homologous target mRNA to form a long dsRNA before being processed by Dicer into smaller siRNA duplexes that are transferred to RISC. Finally, the binding of RISC to homologous targets directs complete degradation, whereas slightly mismatched targets are translationally repressed.*

RNA and its complementary target sequence results in the formation of a dsRNA strand that is recognized by the cell as a possible viral threat or RNA processing error. Therefore, dsRNA is recognized and processed by the enzyme DICER which cuts the duplex into double stranded 20-25 nt siRNAs that are loaded onto RISC to realize further expression inhibition of the target RNA.

The second option is to express short hairpin RNA (shRNA), which are small, 60 nt sequences that mimic the stem-loop structure of pre-miRNAs. These sequences are typically transcribed from minimal RNA polymerase III (polIII) promoters such as H1 and U6 that produce non-polyadenylated RNAs. By the action of DICER, the loop is cleaved, resulting in the generation of fully functional 21 nt siRNA sequences that are ready for loading onto RISC. These shRNA expression cassettes can be custom constructed by ligation of a synthetic oligo-nucleotide duplex into an empty expression cassette. Alternatively, lentiviral vector transfer construct libraries have been created that can be used to generate vectors expressing computer designed shRNA sequences for specific inhibition of almost every known murine or human gene [93].

Others and we have demonstrated that it is possible to express multiple shRNAs from a single lentiviral vector by placing different shRNA expression cassettes in tandem. (Chapter 4) [94]. However, repetitive polIII promoter sequences should be avoided as it was reported that they promote recombination of the vector RNA during reverse transcription resulting in the excision or duplication of vector elements. These recombination events can be prevented by using different polIII promoter sequences or by taking advantage of endogenous multicistronic microRNAs and to exploit them by exchanging their active sequences by custom sequences of interest [95, 96]. An advantage of this approach is that these RNAs are transcribed by conventional polII promoters so that tissue-specific promoters can be used to prevent off-target expression.

1.4 Scope of this thesis

The primary aim of the project described here was to establish lentiviral vector technology and large-scale vector production in the context of several pre-clinical animal and cellular models of human disease. The work presented in this thesis focuses on gene therapy for liver disease but we have also contributed to other research projects. In collaboration with Prof. Dr. G. Wagemaker (ErasmusMC, Department of Hematology) we took part in a study that evaluates the effect of *ex vivo* gene therapy in combination with hematopoietic stem cell transplantation in acid alpha-glucosidase knockout mice, a model for the lysosomal storage disorder, Pompe's disease (Publication in preparation). Also experiments in the context of cystic fibrosis gene therapy will be described elsewhere. We have evaluated the effect of naphthalene induced lung damage on the efficacy of lentiviral vector administration to the lungs of mice in vivo [97]. Furthermore, we have developed an efficient method to transduce human airway epithelial cells in air liquid interface cultures *in vitro*, and evaluated the efficacy of lentiviral vector derived short hairpin RNAs targeting the sodium channel ENAC, a potential therapeutic target in CF (Publication in preparation).

In this thesis, Chapters 2 and 3 describe the successful treatment of the high plasma bilirubin levels observed in the Gunn rat after lentiviral vector administration. The Gunn rat is a model for the human condition of Cirgler-Najjar disease type I, a heritable form of bilirubin conjugation deficiency in the liver. In **Chapter 2** we evaluated the efficacy of direct systemic vector administration via the blood stream in the Gunn rat. **Chapter 3** describes the efforts to establish a more clinically relevant and safer method for liver directed lentiviral vector application. For this, we investigated the efficacy of lentiviral vector administration to the liver by means of isolated hepatic perfusion.

Chapters 4 and 5 focus on a gene therapy strategy for infection of the liver with hepatitis C virus. This research, in collaboration with Dr. L van der Laan, (ErasmusMC, Department of Surgery), focuses on the prevention of HCV re-infection after liver transplantation. **Chapter 4** describes the effect of a lentiviral vector expressing multiple short-hairpin RNA sequences directed against conserved regions of the HCV genome and a cellular HCV co-receptor in an *in vitro* model of HCV replication. In **Chapter 5**, we investigate the efficiency of lentiviral vector transduction under conditions that resemble liver transplantation. Here, lentiviral vector was administered to human hepatoma cells *in vitro*, under cooled conditions in various organ graft preservation solutions used in liver transplantation. Under similar conditions, a start was made to evaluate the possibility of vector deposition in the rat liver during isolated hepatic perfusion, hereby mimicking the procedure of liver transplantation.

Chapter 6 provides a summary and discussion regarding the described experiments and will discuss the hurdles that will need to be overcome before lentiviral gene therapy for liver disease can become a clinical reality.

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Chapter Ⅱ

Successful Treatment of UGT1A1 Deficiency in a Rat Model of Crigler-Najjar Disease by Intravenous Administration of a Liver-Specific Lentiviral Vector

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Successful Treatment of UGT1A1 Deficiency in a Rat Model of Crigler–Najjar Disease by Intravenous Administration of a Liver-Specific Lentiviral Vector

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Treatment of congenital and acquired liver disease is one of the main issues in the field of gene therapy. Self-inactivating lentiviral vectors have several potential advantages over alternative systems. We have constructed a self-inactivating lentiviral vector (LV-ALBUGT) that expresses the human bilirubin UDP-glucuronosyltransferase (UGT1A1) from a liver-specific promoter. UGT1A1 is involved in the clearance of heme metabolites in the liver. This enzyme is deficient in Crigler–Najjar disease, a recessive inherited disorder in humans characterized by chronic severe jaundice, i.e., high plasma bilirubin levels. Gunn rats suffer from the same defect and are used as an animal model of this disease. We have treated juvenile Gunn rats by single intravenous injection with the LV-ALBUGT vector. Over 1 year after treatment with the highest dose (5×10^8 transducing units), we observed a stable reduction of bilirubin levels to near normal levels and normal secretion of bilirubin conjugates in the bile, in contrast to untreated animals. *In situ* hybridization showed expression of the therapeutic gene in more than 30% of liver parenchymal cells. Thus, we demonstrate stable and complete clinical remission of a congenital metabolic liver disease in an animal model, after systemic administration of a therapeutic lentiviral vector.

Key Words: gene therapy, liver, lentivirus, Crigler–Najjar syndrome, UGT1A1, glucuronyltransferase, Gunn rat, bilirubin

INTRODUCTION

Crigler–Najjar disease is a recessive inherited disorder characterized by chronic severe jaundice. Clinical manifestations include progressive damage to the central nervous system (kernicterus) [1,2]. This condition is caused by mutations in the *UGT1A1* gene, which encodes the enzyme required for the conjugation of glucuronyl moieties to bilirubin. The enzyme is primarily expressed in the endoplasmic reticulum of liver parenchymal cells. Conjugated bilirubin is subsequently transported to the biliary tract via the canalicular membrane. Current treatment is to enhance biliary clearance of photoactivated bilirubin by extensive light therapy [2]. This treatment is cumbersome, associated with skin abnormalities, and in the long run insufficient to prevent brain damage. Liver transplantation is the only effective remedy [3]. However, this is hampered by a lack of donor organs, and it requires high-risk surgery and life-long immune suppression to avoid rejection of the transplant [4].

The alternative would be a form of gene therapy. Indeed, Crigler–Najjar disease is one of the model diseases for liver-targeted gene transfer studies. This is due mainly to the availability of an animal model of UGT1A1 deficiency, the Gunn rat. This strain carries a spontaneous single-base-pair deletion of the *UGT1A1* gene, resulting in a complete loss of UGT1A1 function and a clinical phenotype very similar to the human condition [5]. Compared to normal littermates, Gunn rats have very high plasma bilirubin concentrations. They do not secrete any bilirubin conjugates in the bile, which provides an extremely sensitive parameter of complementation in this model. Successful complementation was achieved with adenoviral vectors [6–10] and MLV-based vectors [11]. Delivery of second-generation adenoviral vectors in adult immunocompetent animals resulted in short-term expression, probably due to a CTL response induced by the vector [12]. After submission of this article it was shown that a minimal adenoviral vector with a

liver-specific promoter results in complete normalization of plasma bilirubin levels for at least 2 years in the Gunn rat [13]. However, delivery of a high dose of adenoviral vector is associated with possibly fatal acute toxicity [12,14]. Further, the episomal nature of the adenoviral vector does not guarantee vector stability during the lifetime of a patient. Therefore the development of alternative vector systems is still desirable.

Third-generation lentiviral vectors may have significant advantages over alternative viral vector systems, as they do not produce viral antigens and most prospective recipients will not be immunized to the vector capsid or envelope. The ability to integrate into the genome of quiescent stem/progenitor cells is expected to add to the stability of their expression [15]. The vector can contain up to 8 kb of foreign DNA. Pseudotyping of the vector with a heterologous envelope glycoprotein from the vesicular stomatitis virus (VSV-G) results in a broad tropism and allows vector concentration to titers sufficient for *in vivo* applications. The latest generation of HIV-1-derived vectors is "self-inactivating" (SIN), due to a modified LTR that allows integration of the vector but prohibits LTR-driven transcription (Fig. 1). This modification reduces the risk of aberrant transcription of nearby genes and prevents production of full-length vector RNA in the presence of wild-type virus [16].

Efficient delivery of lentiviral vectors expressing therapeutic or marker genes to the liver in animal models has been reported by several authors [17–20]. A major challenge remains to avoid an immune response against the therapeutic gene. Recent data suggest that expression of the therapeutic gene from a liver-specific promoter contributes to the stability of expression in immunocompetent animals, possibly by avoiding expression in professional antigen-presenting cells [19,21]. Further, expression in hepatocytes could result in tolerance toward the therapeutic gene [22]. Intrauterine delivery of a ubiquitously expressed lentiviral UGT1A1 vector has been shown to result in significant reduction (45% on average) of serum bilirubin levels in Gunn rats [23]. After submission of this article, it was shown that intravenous administration of a liver-specific lentiviral vector in 2-day-old Gunn rats resulted in complete normalization of serum bilirubin levels [24]. Our data significantly extend these studies by showing that intravenous administration of a liver-specific self-inactivating lentiviral human UGT1A1 vector to juvenile immunocompetent Gunn rats results in complete clinical and long-term remission of UGT1A1 deficiency. These results support the assumption that clinical application of self-inactivating lentiviral vectors to treat Crigler–Najjar disease and other monogenetic or acquired liver disease is feasible.

Results and Discussion

Expression of Functional Human UGT1A1 by LV-ALBUGT *in Vitro*

We produced a liver-specific SIN lentiviral vector encoding the human UGT1A1 as described under Materials and Methods (Fig. 1). We infected BNCL2 mouse hepatoma cells *in vitro* with VSV-G-pseudotyped LV-ALBUGT at an effective multiplicity of infection (m.o.i.) of 10 and 100. Southern and Northern analysis using a probe specific for the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequence showed the presence of vector DNA and expression of mRNA of the predicted size. Western blots of infected cells revealed a band of the correct length, compared to human liver microsomes. (*In vitro* data are shown in Fig. 6, supplementary data) Further, immortalized Gunn rat cells [25] infected with LV-ALBUGT at an m.o.i. of 100, but not control cells infected with a GFP-expressing lentiviral vector, showed bilirubin conjugation activity in culture (data not shown). In summary, these *in vitro* data confirm the expression of functional human UGT1A1 mRNA and protein from the LV-ALBUGT vector.

Intravenous Administration of LV-ALBUGT Results in Complete Remission of Chronic Jaundice in Gunn Rats

We treated juvenile female Gunn rats (23 ± 2 days of age) by tongue vein injection with the VSV-G-pseudotyped LV-ALBUGT vector. We calculated the number of infec-

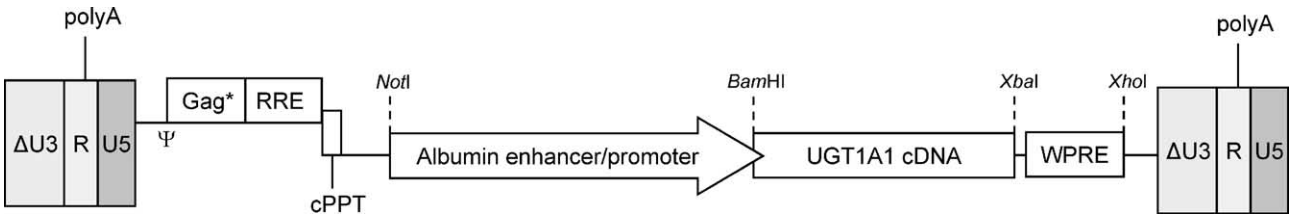

FIG. 1. Schematic representation of the LV-ALBUGT provector construct. After genomic integration of the provector, the internal albumin enhancer/promoter combination drives transcription of the human *UGT1A1* cDNA. Also indicated are the disrupted HIV-1 gag gene (Gag*) including the packaging signal (Ψ), Rev-responsive element (RRE), central polypurine tract (cPPT), and woodchuck hepatitis virus posttranscriptional regulatory element (WPRE). The 5′ and 3′ long terminal repeat (LTR) sequences (consisting of U3, R, and U5) are rendered self-inactivating by a deletion (Δ) in the U3 region. The R region of the LTR contains the HIV-1 polyadenylation signal (polyA).

TABLE 1: Average plasma bilirubin level in Gunn rats treated with 5×10^8 TU LV-ALBUGT

Animal	Mean plasma bilirubin level (μmol/L) ± SEM	Number of data points
1	32.2 ± 1.8	6
2	40.4 ± 4.2	6
3	48.0 ± 1.8	7
4	44.7 ± 2.5	7
5	36.0 ± 3.1	4
6	14.6 ± 2.0	4
7	25.6 ± 2.2	4
8	31.5 ± 3.2	4
Gunn rat	158.9 ± 5.2	24
Normal rat	18.2 ± 1.0	12

Juvenile female Gunn rats (23 ± 2 days of age, $n = 8$) were treated by tongue vein injection with 5×10^8 TU of LV-ALBUGT. At different time points after injection plasma bilirubin levels were measured as described. Data represent average values of multiple measurements ± SEM obtained between days 50 and 200 after injection in each animal. Data from untreated normal and Gunn rats were obtained from 6 and 12 age-matched animals, respectively (compare Fig. 2A). The plasma bilirubin level was significantly ($P < 0.0001$, Student's t test) reduced in all treated animals compared to untreated Gunn rats.

tious particles in the dose (TU, transducing units) by real-time PCR analysis of transduced 293T cells, as described under Materials and Methods. In all treated animals we observed a marked reduction of plasma bilirubin concentration compared to untreated or PBS-injected Gunn rats of the same age and gender (Table 1). In the eight animals treated with the highest vector dose, bilirubin levels were close to normal, and below the level that is considered pathological in humans, until at least 1 year after treatment (Fig. 2A). The number of viral vector copies in the liver of LV-ALBUGT-treated animals treated with different dosages ranged from 0.1 to 3 copies per cell, as shown by quantitative PCR analysis (Fig. 2B and Table 2). Quantitative Southern blot analysis of liver DNA digested with *Eco*RI plus *Xba*I from animals 56 days after treatment with a high vector dose gave similar results using dilutions of vector plasmid for calibration (not shown). Southern blot analysis of liver DNA using *Xba*I alone, which cuts only once inside the vector, did not reveal a discrete band of episomal vector, confirming that a majority of vector copies are integrated.

The number of vector copies in the liver showed a clear relationship with the plasma bilirubin levels, illustrating that one vector copy per liver cell is required for clinical remission (Fig. 2B). Our data further show that vector deposition with tongue vein injection is most substantial in liver and spleen and considerably lower in lung, gonads, and kidney (Table 2). This is consistent with retrograde perfusion of the organs through the venous circulation, bypassing the heart–lung circulation. The intravenous injection volume used here (1% body wt), is considerably less than required for successful hydrodynamic plasmid transfection (8% body wt) and was shown not to cause detectable liver damage in rats [26]. The use of juvenile animals raises the question whether mitotic activity of the growing organ is required for efficient lentiviral vector delivery [27]. Published data show that mitosis is not required for efficient vector delivery and that strong activation of

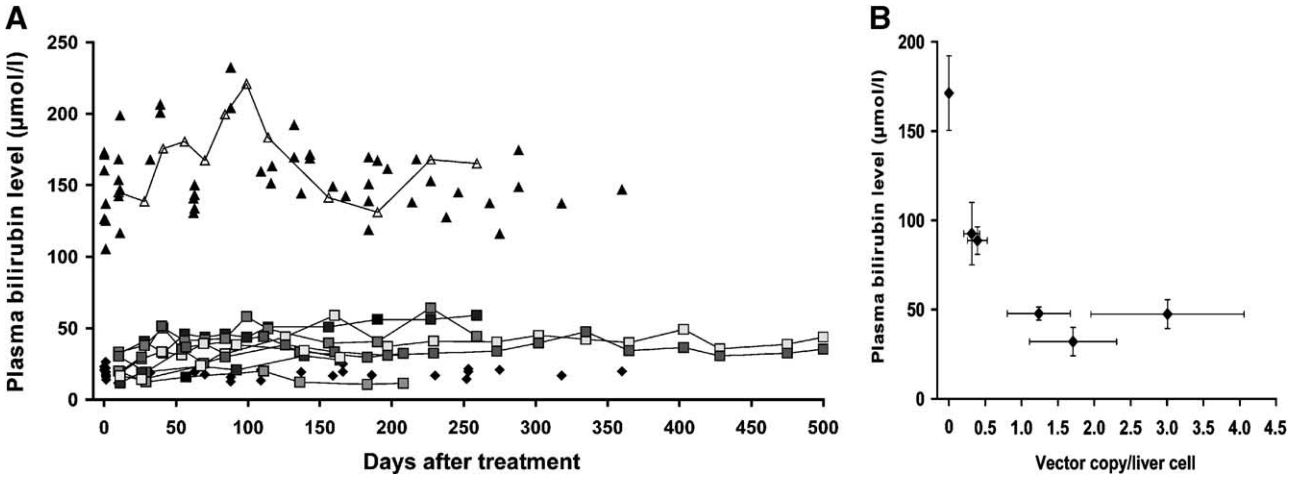

FIG. 2. The effect of a single intravenous injection of LV-ALBUGT on plasma total bilirubin levels in female Gunn rats. (A) Plasma bilirubin levels in individual Gunn rats treated with 5×10^8 TU LV-ALBUGT (filled squares) by tongue vein injection (23 ± 2 days of age, $N = 8$) were significantly reduced for over 1 year compared to untreated age-and sex-matched Gunn rats (filled triangles, multiple individuals) and a saline-injected Gunn rat (open triangles). Also indicated are plasma bilirubin data from normal animals (filled diamonds, multiple individuals). (B) The relationship between vector copy number per liver cell and plasma bilirubin level after treatment with LV-ALBUGT. Female Gunn rats were treated with different dosages of LV-ALBUGT and bilirubin levels were monitored for up to 259 days, as described for A. DNA was isolated from total liver and the number of vector copies per cell was determined by quantitative TaqMan PCR as described under Materials and Methods. The graph displays average values of multiple bilirubin measurements stable for at least 3 months (±SD) and triplicate measurements of vector copy levels (±SD).

TABLE 2: Vector copies per diploid genome in different organs after tongue vein injection in juvenile Gunn rats

		Days after treatment				
	—	56	259	56	259	259
Dosage (TU):	Untreated	1.25×10^8	2.5×10^8	5×10^8	5×10^8	5×10^8
Liver	0	0.126	0.393	1.708	1.237	3.007
Spleen	0	0.112	0.128	0.493	0.259	0.421
Kidney	0	0	0.003	0.328	0.005	0.013
Lung	0	0.014	0.027	0.016	0.013	0.019
Gonad	0	0	0.023	0.033	0.005	0.008

Juvenile female Gunn rats (23 ± 2 days of age) were treated by tongue vein injection with dosages of LV-ALBUGT ranging from 1.25×10^8 to 5×10^8 TU. At different time points after injection, DNA was isolated from different organs, and the number of vector copies per cell (SD ± 50% for all data points) was determined by quantitative PCR as described.

mitotic activity by partial hepatectomy prior to transduction resulted in less than a 50% increase in vector integration in the liver [20,27–29]. This suggests that the high vector deposition we observe in the liver of juvenile rats cannot be attributed to mitotic activity or tissue remodeling.

Gunn rats do not show any bilirubin conjugation activity, due to a frameshift mutation in the *UGT1A1* gene [5]. Consequently, no bilirubin conjugates are found in the bile of Gunn rats, in contrast to normal animals (Figs. 3A and 3B). In bile collected 8 weeks after treatment with LV-ALBUGT, we observed high levels of bilirubin mono- and diconjugates (Fig. 3C). Peak areas ranged from 30 to 100% of normal values, at vector dosages of 1.25×10^8 and 5×10^8, respectively. Further, expression of human UGT1A1 antigen could be readily detected by Western blotting in the liver of the treated Gunn rats (Fig. 4A). This is further evidence for significant expression of human UGT1A1 from the LV-ALBUGT vector.

In situ hybridization with a vector-specific probe on cryosections of Gunn rat liver treated with a high dose of LV-ALBUGT showed a clear LV-ALBUGT mRNA stain in 34% (±11, $N = 4$) of the cells (Figs. 5A and 5B). This positive signal is absent in untreated animals or in vector-treated animals hybridized with a nonspecific probe (Figs. 5C and 5D). This result is consistent with the data obtained by quantitative PCR, considering the fact that not all vector copies are delivered to hepatocytes and that not all copies in hepatocytes are expected to be active enough to generate a positive signal. The location, size, and nuclear shape of the UGT1A1 signal are consistent with expression in liver parenchymal cells, as expected with an albumin enhancer/promoter cassette. Specificity of expression is further shown by the observation that vector deposition in the spleen was comparable to that in the liver (Fig. 4B, Table 2), whereas human UGT1A1 antigen expression was undetectable in spleen (Fig. 4A, lanes 5 and 6). These results

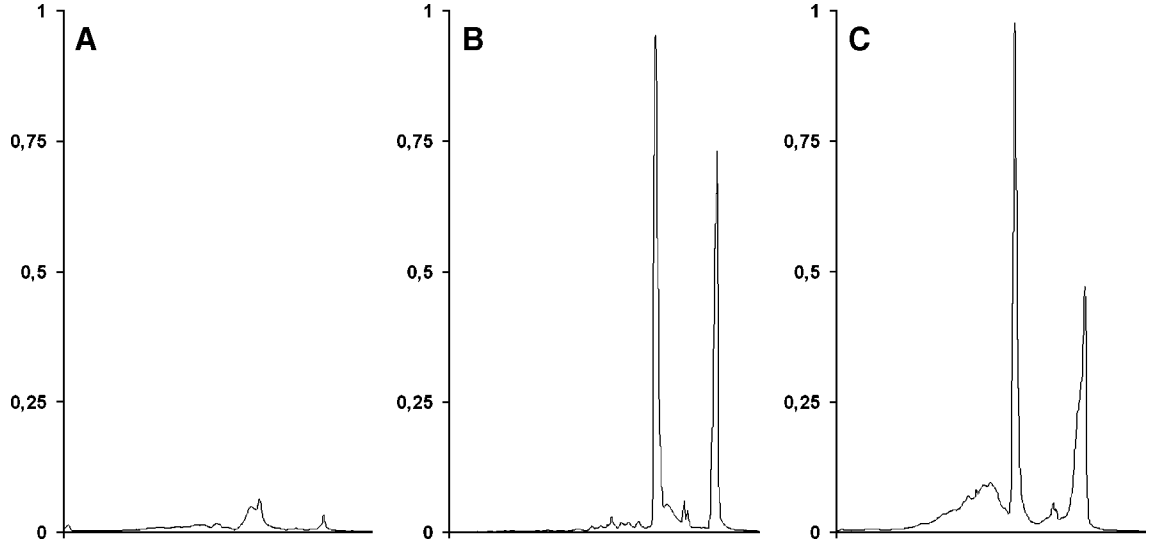

FIG. 3. Bilirubin conjugates in the bile of LV-ALBUGT-treated rats. FPLC analysis of bilirubin mono-and diconjugates in bile of (A) an untreated Gunn rat, (B) a Gunn rat 56 days after treatment with 5×10^8 TU of LV-ALBUGT, and (C) a normal rat. The first peak indicates the presence of bilirubin monoconjugates and the second diconjugates. Quantitative data are presented in Table 2.

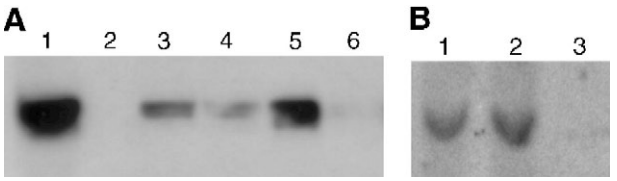

FIG. 4. Vector deposition and expression of human UGT1A1 antigen in Gunn rats after intravenous administration of LV-ALBUGT. (A) Western blot analysis of human UGT1A1 antigen in rats treated with LV-ALBUGT was performed as described. Lane 1, 1 μg concentrated human liver microsomes; lane 2, 20 μg Gunn rat liver microsomes; lanes 3, 4, and 5, 20 μg Gunn rat liver microsomes treated with 1.5×10^8, 0.75×10^8. and 5×10^8 TU LV-ALBUGT, respectively; lane 6, 20 μg microsomes from the spleen of a Gunn rat treated with 5×10^8 TU LV-ALBUGT. (B) Southern blot analysis of 15 μg DNA isolated from liver (lane 1) and spleen (lane 2) from a Gunn rat treated with 5×10^8 TU LV-ALBUGT. Lane 3 is DNA from an untreated Gunn rat. DNA was digested with *Eco*RI and probed with a ^{32}P-labeled WPRE fragment from the vector (Fig. 1).

confirm that the well-characterized mouse albumin enhancer/promoter that we used directs expression of the LV-ALBUGT vector primarily to the liver parenchyma [21]. This is the major site of expression of UGT1A1 in normal individuals and most effective in view of the clearance of bilirubin conjugates via the canalicular membrane to the bile.

Stability of Therapeutic Gene Expression

Stability of expression is one of the most important requirements for a gene therapy vector that is intended to cure a congenital deficiency. One of the factors that strongly determine expression stability is the immune response to vector administration, in particular a cytotoxic T lymphocyte response to the therapeutic gene [30]. Whether a CTL response occurs depends not only on the expression of "nonself" epitopes, either the therapeutic gene or the vector-specific proteins, but also on the acute responses to vector administration that may include the generation of "danger signals" [30]. Another important issue is expression of the therapeutic gene by professional antigen-presenting cells, such as Kupffer cells and dendritic cells in the liver, which limits the stability of the vector by eliciting a CTL response against the therapeutic gene product [12,29–31]. For this reason we chose to use a liver-specific promoter, which should effectively suppress this problem. The validity of this approach was shown previously using adenoviral vectors [32,33] and lentiviral vectors [21]. Our plasma bilirubin data show stable expression for at least 1 year at the highest dose (Fig. 2A, Table 1). We did not observe significantly increased lymphocyte infiltration by CD45, CD3, and CD8 immunohistochemistry in the liver of four animals 56 and 259 days after treatment with a high dose of LV-ALBUGT, compared to control (not shown). From this, in combination with the apparent stability of UGT1A1 activity in vector-treated rats (Fig. 2A), we conclude that delivery of a lentiviral (LV) vector and subsequent expression of human UGT1A1 from a liver-specific promoter do not elicit a significant CTL response against the therapeutic gene in Gunn rats under these conditions. This is remarkable since human UGT1A1 and the truncated Gunn rat gene product show substantial sequence differences.

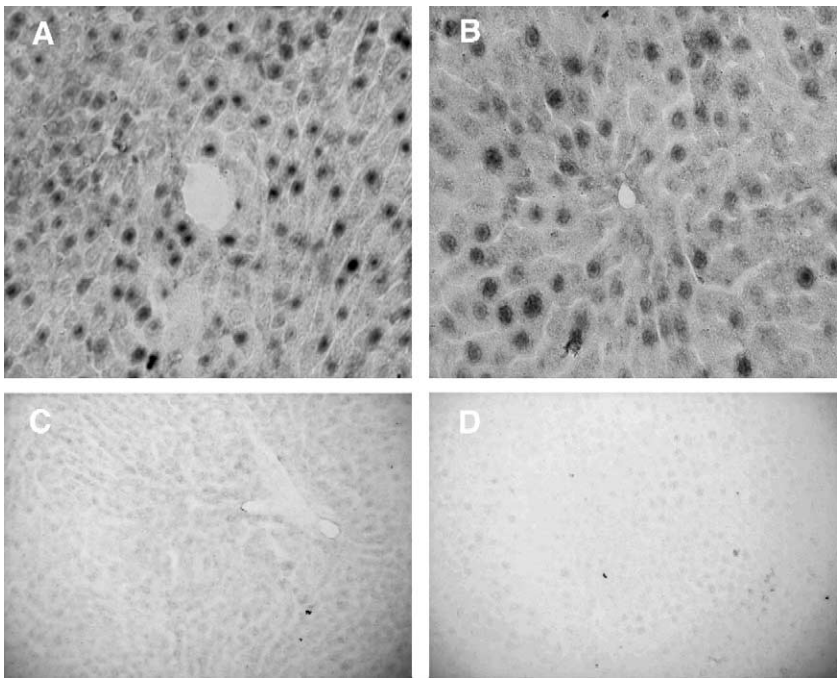

FIG. 5. *In situ* hybridization of liver sections from Gunn rats treated with LV-ALBUGT. Expression of human UGT1A1 mRNA in the liver of Gunn rats 259 days after treatment with LV-ALBUGT is shown by *in situ* hybridization on cryosections using human UGT1A1-specific probes. (A) Gunn rat treated with 5×10^8 TU LV-ALBUGT, (B) Gunn rat treated with 2.5×10^8 TU LV-ALBUGT, (C) saline-injected Gunn rat, (D) Gunn rat treated with 5×10^8 TU LV-ALBUGT, nonspecific probe.

Toward Clinical Application of Lentiviral Vectors for the Treatment of Liver Disease

Our data clearly show that the LV-ALBUGT vector can completely and stably correct the clinical phenotype of the Gunn rat after intravenous administration. While this is a most encouraging result, clinical application of this vector will require further study. Though we did not observe a significant CTL response in the context of our experiments we cannot completely rule out a response at later time points. Most Crigler–Najjar patients produce a mutant form of human UGT1A1 [34], which could potentially lead to a CTL response toward the therapeutic gene product.

The present LV-ALBUGT vector is of relatively simple design (Fig. 1), carrying a liver-specific enhancer/promoter combination that has been shown to be specific for liver parenchymal cells *in vivo* [35,36]. It can be easily adapted to accommodate other therapeutic genes. Considering the effective titer of the applied vector dose (10^8–10^9 TU), the systemic administration method, and the estimated number of target cells in the liver of juvenile rats (10^8), the number of liver parenchymal cells targeted with this VSV-G-pseudotyped lentiviral vector seems quite satisfactory (Fig. 5, Table 2). In similar studies in adult mice, comparable percentages of liver cells targeted with LV were reported, ranging from approximately 0.3 to 2 copies/cell [21,27,37]. Experiments to establish the optimal conditions for delivery to adult liver in the rat model are ongoing. Further optimization of the vector in terms of large-scale production, transcription activity, pseudotype [38–40], and boundary elements that reduce the potential risk of oncogene activation is expected to improve further the performance of the system in future [41].

Materials and Methods

Lentiviral vector construction and production. The third-generation lentiviral vector backbone used to create LV-ALBUGT (Fig. 1) is derived from pCS-CG [42]. The LTR sequences are rendered self-inactivating by substitution of the U3 region in the 5′ LTR with the CMV promoter and a 133-bp deletion in the U3 region of the 3′ LTR. In addition, the HIV-1 central polypurine tract was reintroduced to maintain efficient nuclear import of the preintegration complex [43]. The WPRE was included 3′ of the transgene to improve mRNA processing and stability [44]. LV-ALBUGT makes use of a liver-specific transcription unit consisting of the 1.9-kb distal enhancer of the mouse albumin gene fused to 0.3 kb of the mouse albumin promoter [35]. This unit drives expression of the human *UGT1A1* complementary DNA (terminated at the *Xba*I site in the 3′ UTR).

Lentiviral vector particles were produced with a split-genome packaging system [45]. Transfer construct pLV-ALBUGT and the three packaging plasmids pMDLg/RRE, pRSV-REV, and pMD.G were transferred to HEK293T cells by polyethylenimine-mediated transfection in a 4:2:1:1 ratio [46]. Transfections were carried out on confluent 500-cm^2 dishes (1.5×10^8 cells/dish) using a total amount of 400 μg DNA plasmids purified free from bacterial endotoxins using a kit (EndoFree Plasmid Kit; Qiagen, Hilden, Germany). Vector particles were harvested 48 and 72 h posttransfection and concentrated by ultracentrifugation. The vector preparation was resuspended in Hanks' balanced salt solution and stored at −80°C until further use.

Animal procedures. All animal experiments were approved by the local ethics committee and performed under strict biological containment. Animal procedures were performed under isoflurane anesthesia.

Gunn rats (HsdBlu:Gunn; Harlan CPB, Austerlitz, The Netherlands) were bred by crossing homozygous males with heterozygous females and were housed under standard conditions on normal chow and water *ad lib*. Pups were genotyped by PCR analysis on tail DNA using the primers forward, 5′-GTCATCCAAAGGCTCGGGC-3′, and reverse, 5′-AGGGCGTTTTCCAATCATCG-3′, followed by digestion of the PCR fragment with *Bst*NI. Homozygous Gunn rats were identified by the double loss of a *Bst*NI restriction site [5].

Homozygous Gunn rats, 23 ± 2 days of age, 56 ± 6 g body wt, received different doses of LV-ALBUGT by tongue vein injection. Untreated and PBS-injected animals were used as controls. The administered vector volumes were adjusted to 600 μl in PBS and injections were carried out using a 1-ml insulin syringe (Micro-Fine Plus; BD, Franklin Lakes, NJ, USA).

For determination of plasma bilirubin levels, blood samples were collected by retro-orbital bleeding in heparin tubes. Plasma bilirubin concentrations were determined with BIL-T bilirubin DPD reagents (Roche Diagnostics GmbH, Mannheim, Germany).

For analysis of bilirubin conjugates, bile was collected by cannulation of the bile duct for approximately 30 min, after which animals were sacrificed by bleeding. Tissues (liver, spleen, kidney, lung, ovary) were harvested and snap-frozen in liquid nitrogen for further analysis.

Bilirubin conjugates were extracted from bile by alkaline methanolysis and analyzed by FPLC using a 200-μl reverse-phase column μRPC C2/C18 PC 3.2/3 fitted on a SMART FPLC system (Amersham Biosciences, Roosendaal, The Netherlands) [47]. Bilirubin mono-and diconjugate peaks were detected at 436 nm (Fig. 3).

In situ hybridization. *In situ* hybridization on liver cryosections was carried out as described previously [48]. Oligonucleotide probes were obtained from Eurogentec (Seraing, Belgium), labeled with digitonin (DIG) or dinitrophenol (DNP) at positions 1, 25, and 50. DIG-labeled human *UGT1A1*-specific sequences were from the 3′ untranslated region of the mRNA including the WPRE, to avoid interaction with rat *UGT1A* sequences: UGT1, 5′-TTGGAAATGACTAGGGAATGGTTCAAAATTTTACCTTATTTCCCACCCAC-3′; UGT2, 5′-TGCAAAGTATTTCCTTAATAAGAATAAAATGAATTTAACACTGATTCTG-3′; UGT3, 5′-CCACATAGCGTAAAAGGAGCAACATAGTTAAGAATACCAGTCAATCTTTC-3′. As a positive control, rat albumin mRNA-specific probes were DNP-labeled: ALB1, 5′-GGGCTCTTGTTTTGCACAGCAGTCAGCCAGTTCACCGTAGTTGTCACGAA-3′; ALB2, 5′-GGCTGCTTTGTCAGACTCTGTGCAGCACTGGGTCAGAACCTCATTGTATT-3′; ALB3, 5′-TGTTGCCAATTTGGTCATTTCTGCGAACTCAGCATTGGGGAATCTCTGGG-3′.

After hybridization, DIG-and DNP-labeled probes were detected by incubation with either alkaline phosphatase-conjugated polyclonal rabbit anti-DIG (Roche Diagnostics GmbH) or rabbit anti-DNP antiserum (Molecular Probes, Eugene, OR, USA) followed by an alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit IgG; DakoCytomation, Glostrup, Denmark). Alkaline phosphatase activity was visualized using NBT/BCIP staining (Roche Diagnostics GmbH).

Southern blot analysis. For Southern analysis, genomic DNA was isolated from transduced cells or tissue and digested with the desired restriction enzyme and the fragments were resolved by electrophoresis in a 1% TAE agarose gel. Southern blots were generated by capillary transfer to Hybond-N+ nylon membrane (Amersham Biosciences). The human UGT1A1 expression cassette of LV-ALBUGT was detected by hybridizing the blots to the ^{32}P-labeled 592-bp *Xba*I/*Xho*I WPRE fragment from the vector plasmid (Fig. 1). After hybridization, the probe was visualized by a phosphoimager (Typhoon 9410; Molecular Dynamics, Sunnyvale, CA, USA).

Real-time PCR for genomic vector integration. For determination of vector deposition in rat tissue, quantitative TaqMan PCR was performed in a 25-μl volume containing 50 ng of genomic DNA, 12.5 μl 2× TaqMan PCR master mix (Applied Biosystems, Foster City, CA, USA), 300 nmol/L each primer (Invitrogen, Groningen, The Netherlands), and 100 nmol/L probe (Eurogentec). The primer set and probe used to detect the residual GAG

sequence present in the LV backbone were forward primer, 5′-GGAGCTAGAACGATTCGCAGTTA-3′; reverse primer, 5′-GGTTGTAGCTGTCCCAGTATTTGTC-3′; probe, 5′-FAM-ACAGCCTTCTGATGTTTCTAACAGGCCAGG-TAMRA-3′ (sequences from Dr. P. Salmon, Department of Microbiology, Geneva School of Medicine, Geneva, Switzerland). Amplification of the X-chromosomal rat HPRT sequence [49] in separate reactions was used for normalization of PCR efficiency and amount of DNA present in the sample: forward primer, 5′-GCGAAAGTGGAAAAGCCAAGT-3′; reverse primer, 5′-GCCACATCAACAGGACTCTTGTAG-3′; probe, 5′-FAM-CAAAGCCTAAAAGACAGCGGCAAGTTGAAT-TAMRA-3′.

For vector titration on 293T cells, quantitative SYBR green PCR was performed using the qPCR Core kit for SYBR Green I No ROX (Eurogentec) according to the manufacturer's instructions. Reactions were carried out in a 25-μl volume containing 50 ng of genomic DNA and 300 nmol/L each primer. The primer set used to detect the residual GAG sequence is identical to the one mentioned above. For normalization a part of the human albumin locus was amplified using primers forward, 5′-AGGGTAAAGAGTCGTCGATATGCT-3′; reverse, 5′-CAATCTCAACCCACTGTCAGCTA-3′.

All quantitative PCRs were analyzed in duplicate on a DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA) using the following reaction parameters: 10 min at 95°C, 40 cycles of 30 s at 95°C, 60 s at 60°C. Vector copy numbers were calculated by interpolating C_t(GAG) − C_t(HPRT) or C_t(GAG) − C_t(albumin) values to a dilution series of plasmid DNA containing the LV backbone in Gunn rat DNA or 293T cell DNA, respectively, simulating the presence of 100 to 0.001 vector integrations per cell.

Western blot analysis. Microsomes were isolated by homogenizing 100 mg of tissue in homogenization buffer (250 mmol/L sucrose, 1 mmol/L EDTA, 50 mmol/L Tris, pH 7.5), supplemented with protease inhibitor cocktail (Roche Diagnostics GmbH). The homogenate was centrifuged for 10 min at 16,000*g* to remove nuclei and debris and subsequently the supernatant was centrifuged at 170,000*g*. The microsome pellet was resuspended in homogenization buffer and protein concentration was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Microsomal protein (20 μg/lane) was resolved on a 12% SDS–polyacrylamide gel and electroblotted to a nitrocellulose membrane (Protran; Schleicher & Schuell, Dassel, Germany). The membrane was preincubated in blocking buffer (5% nonfat milk powder, 0.1% (w/v) Tween 20 in PBS) and incubated with a 1:1000 dilution of monoclonal antibody WP1, which is specific for the human UGT1A isoforms [50]. Subsequently, the membrane was incubated with a 1:1000 diluted horseradish peroxidase-conjugated goat anti-mouse IgG antiserum (DakoCytomation). Immunoreactive protein bands were visualized on X-ray film using a bioluminescence assay kit (ECL Plus; Amersham Biosciences). For reference, a commercial preparation of pooled human liver microsomes was used (BD Biosciences, San Jose, CA).

Statistical analysis. Student's two-tailed *t* test was used for statistical analysis. *P* values less than 0.05 were considered significant.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymthe.2005.09.022.

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Chapter III

Liver-Restricted Administration of Lentiviral Vector Results in Significant Reduction of Plasma Bilirubin Levels in the Adult Gunn Rat

Submitted for publication

Liver-restricted administration of lentiviral vector results in significant reduction of plasma bilirubin levels in the adult Gunn rat

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Abstract

Lentiviral vectors have allow efficient gene delivery to the liver by intravenous administration in rodent models of human disease. However, systemic delivery of high lentiviral vector dosages in a clinical setting is associated with several possible drawbacks, including vector inactivation by human complement and transduction of non-target cells, which reduces efficacy and safety. To reduce these risks, we investigated the possibility to restrict lentiviral vector deposition to the liver by isolated hepatic perfusion (IHP). IHP is currently used in humans to treat liver carcinomas with a high dose of cytostatics, and thus could in principle be applied for gene therapy purposes. Furthermore, we assessed the power of this approach to treat inherited metabolic liver disease in the hyperbilirubinemic Gunn rat, an animal model for Crigler-Najjar disease type 1. IHP was carried out in adult male Gunn rats weighing between 280 and 380 grams, using a previously described lentiviral vector expressing UGT1A1 by a liver-specific murine albumin enhancer-promoter. Application of the highest vector dose tested (1.5×10^9 transducing units, TU), resulted in a significant decrease in the plasma bilirubin level of 36 % for at least eighteen weeks. Prior to IHP, a subgroup of animals was depleted from Kupffer cells by intravenous administration of clodronate liposomes. This pre-treatment reduced the vector dose needed to achieve a similar effect on plasma bilirubin levels (34 % decrease) by a factor of three to 5×10^8 TU. In both cases, the liver of treated animals contained approximately 0.25 vector copies per cell, while no vector copies were detected in other organs, in contrast to intravenous delivery. We conclude that transduction of the liver with lentiviral vectors during IHP can be an appealing alternative for systemic vector administration.

Introduction

Treatment of congenital and acquired liver disease is a key issue in the field of gene therapy. Because of the physical properties of the liver, most commonly used viral gene therapy vectors, including lentiviral vectors, display a certain degree of liver-preference after intravenous administration. It is hypothesized that intravenously administered gene therapy vectors gain access to hepatocytes by passing through the endothelial fenestrae, which are 100 nm pores in hepatic sinusoids which together form the liver sieve. Furthermore, as compared to other organs, hepatocytes are not separated from the blood vasculature by a basement membrane, providing free exchange of large complexes from the blood plasma to the liver and vice versa.

Lentiviral vectors are able to transduce non-dividing cells and can accommodate up to 8 kilobases of foreign DNA that is stably integrated into the host genome to facilitate long-term transgene expression. Transduction of the liver via systemic intravenous HIV-1 derived vector administration has been confirmed in various species, including mouse, rat, rabbit, dog and fetal rhesus monkey [1-5]. In many of these experiments, vector administration is accomplished via injection into a suitable peripheral vein, such as the tail vein, not directly afferent to the liver. We have previously demonstrated efficient lentiviral vector transduction of the liver in juvenile rats after tongue vein injection, (Chapter 2)[6]. Other protocols deliver vector by infusion of vector via the portal vein or hepatic artery. Recently, highly efficient

lentiviral vector administration in rats via a stop-flow ischemic clamping method has been reported [7].

Crigler-Najjar disease type I is a rare form of severe hereditary unconjugated hyperbilirubinaemia caused by a mutation in the gene encoding uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1). This disease is associated with accumulation of toxic bilirubin accumulating in the blood causing severe jaundice and progressive and irreversible neurological damage (kernicterus). Current therapy in the form of phototherapy can only partially ameliorate the disease until liver transplantation is required.

Due to the availability of a representative animal model for this condition in the form of the Gunn rat, this disease has become an important model for pre-clinical gene therapy. In this rat strain, approximately five percent of the normal expression level is required to correct the hyperbilirubinemic phenotype and therapeutic effects can be easily determined in the blood. Recently, others and we reported that intravenous administration of a lentiviral vector expressing UGT1A1 from a liver specific promoter stably normalizes plasma bilirubin levels in the hyperbilirubinemic Gunn rat [6, 8, 9]. We reported that intravenous injection with a vector dose of 5×10^8 TU in three-week-old juvenile female Gunn rats resulted in correction of the hyperbilirubinemic phenotype to near baseline levels for a period of at least 500 days.

Although results obtained after intravenous delivery of lentiviral vectors are encouraging, systemic delivery is associated with several potential drawbacks that impair clinical application. First, VSV-G pseudotyped lentiviral vectors are efficiently inactivated by human complement and circulating antibodies [10, 11]. Second, an acute inflammatory response upon delivery as observed with adenoviral vectors cannot be excluded [12]. Also, high systemic vector dosages increase the risk of germ line transmission of integrated lentiviral vector copies when gonadal cells are transduced. Finally, genotoxicity can occur by transduction of inappropriate target cells, such as hematopoietic stem cells, which are readily transduced by lentiviral vectors [13, 14].

To partially overcome these problems we have studied lentiviral vector deposition to the liver by isolated hepatic perfusion (IHP) for the treatment of hyperbilirubinemia in the Gunn rat. The surgical IHP procedure has been developed to treat liver metastases in humans with high local dosages of chemotherapy that would not be systemically tolerated. The technique has been well established in the clinic and was demonstrated to reduce the rate of tumor progression [15, 16]. Currently, the surgical procedure is being optimized to minimize leakage, blood loss and mean operation time to further improve patient survival [17].

Apart from cancer treatment, another appealing application for IHP is local delivery of gene therapy vectors to the liver. The main potential advantages of IHP over systemic vector administration include improved transduction efficiency and the capability to control complement and macrophage activity during deposition. Furthermore, the vector can be washed out of the liver at the end of the procedure, which may reduce systemic toxicity and attenuate inflammatory and immune responses. In addition, genotoxic and undesired integrations in gonad and other stem cell populations can be minimized.

Various isolated hepatic perfusion approaches have been studied in rat, sheep and pig for administration of different vector types, including adenoviral vector [18-21], gamma-retroviral vector (MuLV) in combination with partial hepatectomy [22-25], Herpes Simplex Virus vector [26] and Sendai virus vector [27]. Here we report that the rat liver can be efficiently transduced by VSVG pseudotyped lentiviral vectors during IHP, and that application of a liver-specific, UGT1A1 expressing lentiviral vector results in partial correction of the high plasma bilirubin levels observed in the Gunn rat.

Results

Vector construction and production

For these studies we used the HIV-1 based, self-inactivating, VSV-G pseudotyped lentiviral vector LV-ALB-UGT that expresses human UGT1A1 under control of a well characterized murine albumin enhancer-promoter combination. The construction an functionality testing of LV-ALB-UGT has been described in previous work and it was found that an average of two vector copies per cell is required to reduce the plasma bilirubin levels in the Gunn rat to near baseline levels (Chapter 2) [6]. In addition, a firefly luciferase expressing variant of LV-ALB-UGT was constructed, designated LV-ALB-LUC, for vector deposition studies in combination with *in vivo* bioluminescence imaging. Lentiviral vectors were produced in HEK293T cells by polyethylenimine (PEI) mediated transfection of a standard third generation four-plasmid production system and concentrated by ultracentrifugation, gaining typical titers of 10^9 TU/ml. Batches of the size required for *in vivo* application in adult animals were produced using a modification of previously described methods (see supplementary data to chapter 3 for full details).

The isolated hepatic perfusion procedure in Gunn rats

Isolated hepatic perfusions were performed under conditions standardized by Ten Hagen et al [28]. This perfusion protocol is well tolerated in normal male WAG/RIJ or Brown-Norway (BN) rats, with a survival rate of over 95%, provided the rats weigh at least 280 gram. However, the Gunn rat strain (HsdBlu:Gunn) did not tolerate the routinely used phenobarbital (PB) anesthesia in combination with IHP, and animals typically died during narcosis (n=4), possibly because PB displaces bilirubin from binding sites causing acute toxicity, or because PB metabolism is abnormal in Gunn rats. Isoflurane gas anesthetics proved not to have this detrimental effect, and this was chosen as the standard regimen.

Further, the isolated hepatic perfusion procedure proved to be particularly challenging in the Gunn rat strain. The most critical moment during surgery is the installation of a temporary ligature around the suprahepatic inferior vena cava, efferent of the site where the hepatic veins connect. This involves resection of connective tissue at the site close to where the vena cava penetrates the diaphragm. This procedure resulted in frequent rupture of the vena cava or perforation of the diaphragm in approximately 25% of all sessions. This failure rate is at least 5 times higher than in operations performed in the context of the tumor model, probably due to anatomic nuances between the rat strains used. Nevertheless, the perfusion procedure itself once established correctly was well tolerated in both control heterozygous (100% survival, n=3) as homozygous jaundiced Gunn rats (80% survival, n=5).

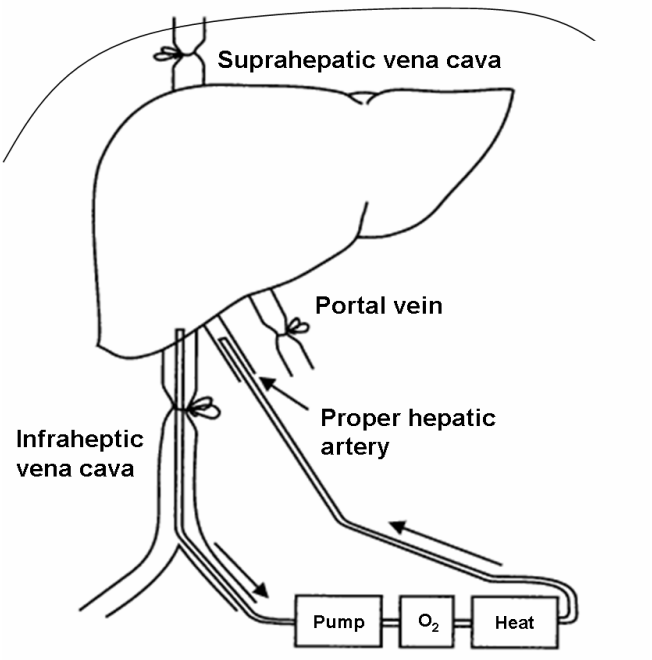

Fig. 1. Schematic representation of the isolated hepatic perfusion set-up.
Adult male Gunn rats 280-380 g were subjected to oxygenated IHP by cannulation of the hepatic artery and inferior vena cava. At an orthograde perfusion rate of 5 ml/min, a solution containing lentiviral vector, diluted in blood plasma substitute (Gelofusin) was circulated for 10 minutes, followed by a 5 ml wash out with fresh plasma substitute. Prior to IHP, a subgroup of animals was depleted from Kupffer cells by intravenous administration of clodronate liposomes.

Sham IHP has a significant effect on plasma bilirubin levels in the Gunn rat
In a series of sham operations (no vector deposition), the effect of the isolated hepatic perfusion procedure on the plasma bilirubin level was determined in both homozygous mutant Gunn rats and non-jaundiced heterozygous rats. Compared to non-treated rats, IHP in heterozygous animals had no significant effect on plasma bilirubin levels (data not shown, n=3) but the bilirubin levels in jaundiced homozygous Gunn rats were affected by the procedure alone (Fig. 2A, n=3, rat #1-3). Within the first month after operation, the plasma bilirubin levels in these animals showed a transient reduction of 57% ± 10 SD on average, compared to pre-operation values. After this period, plasma bilirubin levels gradually increased to pre-operational levels that were reached at day 75 in all animals.

IHP with a vector dose of 5×10^8 TU LV-ALB-UGT has no effect on plasma bilirubin levels
The post-operational decrease in plasma bilirubin levels in sham treated homozygous Gunn rats made the interpretation of experiments possible only after a period of at least 75 days. The first series of perfusion experiments was performed with a lentiviral vector dose of 5×10^8 TU LV-ALB-UGT (n=2). It was previously demonstrated that this vector dosage reduced the plasma bilirubin levels after intravenous administration in 3 week old (56 g) Gunn rats to near baseline levels, with a transduction rate of 2 copies per liver cell on average (Chapter 2) [6]. The application of this vector dose during IHP to adult male Gunn rats, weighing between 280 and 380 grams, did not result in a significant reduction of plasma bilirubin levels (Fig. 2B, #6,7). However, quantitative real time PCR (qPCR) analysis of liver tissue, 94 or 198 days after IHP, indicated low but detectable transduction rates of 0.1 vector genome per cell (Table 1). No vector copies were detected in the spleen, an organ that is significantly transduced upon intravenous lentiviral vector administration [1].

IHP with the highest vector dose of 1.5×10^9 TU significantly reduces plasma bilirubin levels
In an attempt to increase the transduction rate, the perfusion procedure was performed with a three times higher vector dose of 1.5×10^9 TU in a homozygous Gunn rat. Indeed, between day 91 and 198 after perfusion, a significant reduction of 36% ± 10 SD ($p<0.01$) in the average plasma bilirubin level was evident (Fig. 2B, #8). QPCR analysis demonstrated the presence of 0.27 vector copies per cell in the liver and no copies were detected in the spleen or kidney (Table 1). However, by using this higher vector dose (n=7), the success rate of the operation was reduced due to obstruction of the smallest perfusion cannulae (n=2) and clot formation in the perfusion vessel (n=2). In addition, the post operational mortality was high, and after autopsy the liver of diseased animals (n=2) appeared to have suffered from ischemia, consistent with obstruction of blood vessels.

Effect of Kupffer cell depletion on vector deposition during IHP
Is has been demonstrated in the literature that Kupffer cell depletion enhances the transduction efficiency of both adenoviral and lentiviral vectors in the liver by preventing sequestration of vector particles in liver macrophages [29, 30]. To investigate the effect of Kupffer cell depletion on the efficiency of vector deposition in the IHP setting, clodronate liposomes were intravenously administrated, 3-4 days before the perfusion procedure. Clodronate liposomes are efficiently and specifically taken up by macrophages, including the liver Kupffer cells [31, 32]. After their endocytosis, the lipid component is digested and clodronate accumulates intracellularly, resulting in killing of the macrophage. Clodronate displays only intracellular cytotoxicity, extracellular clodronate is not able to pass cell membranes and has an extremely short half-life in the circulation and other body fluids.

The intravenous administration of clodronate liposomes alone did not have a significant effect on the plasma bilirubin levels of jaundiced Gunn rats (n=3, data not shown). It was also excluded that clodronate pre-treatment in combination with the administration of a non-UGT1A1 expressing lentiviral vector during IHP influences the plasma bilirubin level in the Gunn rat. For this purpose, IHP experiments were performed in clodronate liposome pre-treated animals using 5×10^8 TU of luciferase expression vector LV-ALB-LUC (n=2). The

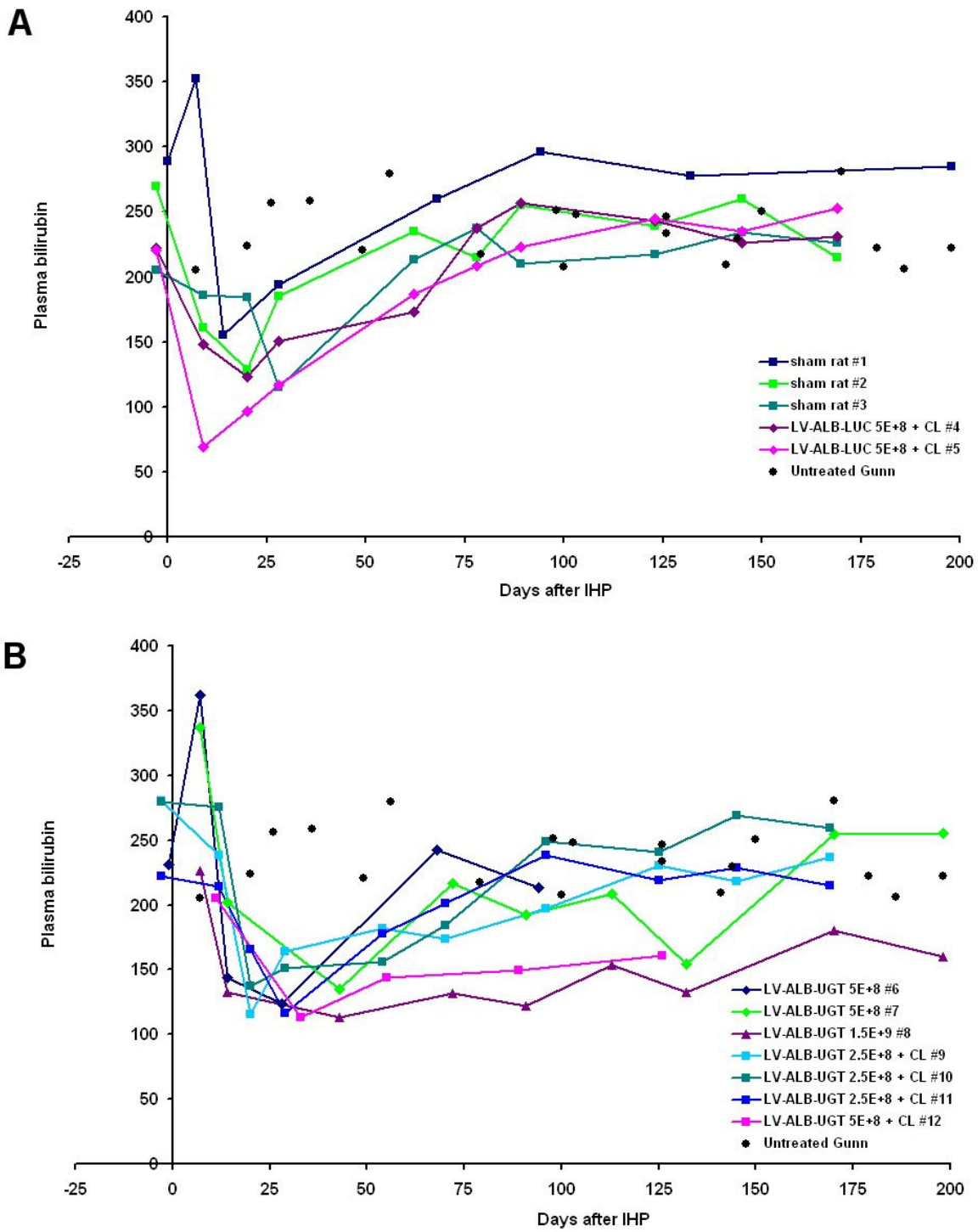

Fig. 2. Effect of IHP on the plasma bilirubin level of adult male Gunn rats. *In both figures, the plasma bilirubin data of age-matched untreated Gunn rats are indicated by black circles. +CL in the legend indicates that the rats received clodronate liposome pre-treatment before IHP. (A) As a control, Gunn rats were subjected to sham IHP operations with no vector added (squares, #1-3) or received clodronate pre-treatment in combination with IHP with a vector dose of $5x10^8$ TU of luciferase expression vector LV-ALB-LUC (diamonds #4,5). (B) The effect of administration of different dosages of UGT1A1 expression vector LV-ALB-UGT to the perfusion circulation. Two animals were treated with a vector dose of $5x10^8$ TU (diamonds #6,7) and one animal with a vector dose of $1.5x10^9$ TU (triangles #8). Three Gunn rats received clodronate liposome pre-treatment in combination with a vector dose of $2.5x10^8$ TU (squares #9-11) and one with $5x10^8$ TU LV-ALB-UGT (squares #12).*

plasma bilirubin levels and dynamics in these animals were comparable to those observed in sham treated Gunn rats (Fig. 2A, #4,5). At day 169 post perfusion, qPCR analysis indicated the presence of an average of 0.2 vector copies per cell in the livers of these animals (Table 1).

When IHP was performed in Kupffer cell depleted Gunn rats with a vector dose of 2.5×10^8 TU LV-ALB-UGT (n=3), this treatment did not result in a significant effect on the plasma bilirubin levels (Fig. 2B, #9-11), with an average of 0.1 vector copies per cell in the liver (Table 1). The treatment was repeated with a two times higher vector dose of 5×10^8 TU LV-ALB-UGT. In contrast to animals treated with an identical vector dose, but without clodronate pre-treatment (#6,7), the mean plasma bilirubin level in this animal was significantly reduced with 34% ± 7,6 SD ($p<0.05$, Fig. 2B #12) after 89 and 126 days. This result is similar to the effect achieved with 1.5×10^9 TU LV-ALB-UGT in the non-clodronate liposome pre-treated animal described earlier (Fig. 2B, #8). Consistently, 126 days post IHP, qPCR indicated the presence of 0.23 vector copies per cell in the liver, double the frequency found in the absence of clodronate with this vector dosage and comparable to the frequency observed at a three times higher dosage without clodronate (Table 1). Similar transduction efficiencies were found upon delivery of the luciferase expressing vector LV-ALB-LUC after clodronate pretreatment. This result indicates that Kupffer cell depletion by intravenous administration of clodronate liposomes has a positive effect on vector transduction efficiency during perfusion, and can induce similar beneficial effects on the plasma bilirubin level with a three times lower vector dose.

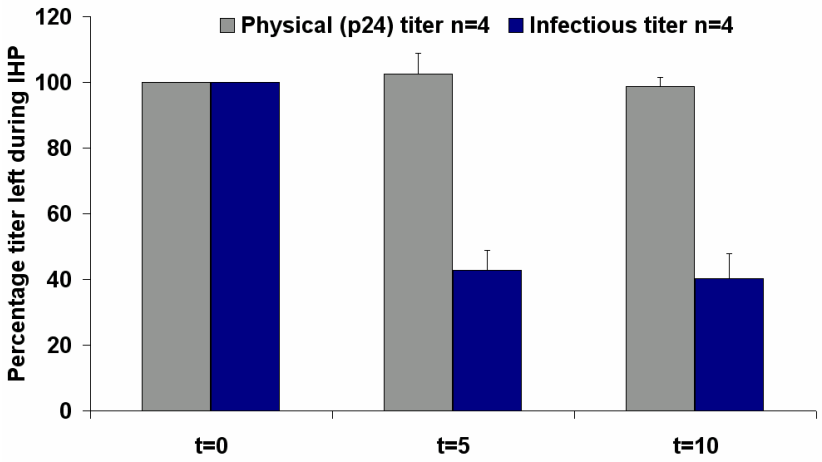

Fig. 3. Changes in the total amount of vector antigen (p24) and infectious titer in perfusate samples. *The perfusate reservoir was sampled at t=0, t=5 and t=10 min during IHP with $5x10^8$ TU LV-ALB-LUC. The total amount of vector antigen (P24 ELISA) in the perfusate did not decrease significantly during perfusion. The remaining infectious titer (LV-ALB-LUC, luciferase titration on 293T cells) decreased on average 57% (n=4) the first five minutes and remained constant until the end of the perfusion time.*

Vector clearance during IHP with LV-ALB-LUC

The luciferase marker gene in vector LV-ALB-LUC enables determination of reproducibility, efficiency and specificity of vector deposition during IHP. The treated animals (#4,5) were subjected to *in vivo* bioluminescence imaging (Fig. 4). Vector expression was detected in the region where the liver is situated and showed similar distribution patterns and comparable luciferase expression levels between the two individual animals.

To determine the efficiency of lentiviral vector clearance during perfusion, samples were withdrawn from the perfusate reservoir at different time points and analyzed for changes in vector concentration and titer (Fig. 3). The total amount of vector antigen as determined by p24 ELISA, did not show a significant decrease throughout perfusion time. However, the

Table 1. Post mortem quantitative PCR data for vector copy numbers in in liver of IHP treated animals compared to intravenously injected juvenile Gunn rats and adult FVB mice.

Target animal, delivery route and vector used	rat #	Vector dose (TU)	Vector copies in liver*	Vector copies/ liver cell	Vector copies/TU
Gunn rat, IHP					
LV-ALB-UGT	6	5 E+08	1,43E+08	0,10	0,29
LV-ALB-UGT	7	5 E+08	1,43E+08	0,10	0,29
LV-ALB-UGT	8	1,5 E+09	3,86E+08	0,27	0,26
LV-ALB-UGT +CL	9	2,5 E+08	---	ND	---
LV-ALB-UGT +CL	10	2,5 E+08	1,72E+08	0,12	0,69
LV-ALB-UGT +CL	11	2,5 E+08	1,43E+08	0,10	0,57
LV-ALB-UGT +CL	12	5 E+08	3,29E+08	0,23	0,66
LV-ALB-LUC +CL	4	5 E+08	3,00E+08	0,21	0,60
LV-ALB-LUC +CL	5	5 E+08	2,72E+08	0,19	0,54
Juvenile Gunn rat, iv					
LV-ALB-UGT		1,25 E+08	4,10E+07	0,13	0,33
LV-ALB-UGT		2,5 E+08	1,28E+08	0,39	0,51
LV-ALB-UGT		5 E+08	5,56E+08	1,71	1,11
LV-ALB-UGT		5 E+08	4,02E+08	1,24	0,80
LV-ALB-UGT		5 E+08	9,75E+08	3,00	1,95
Adult FVB mouse, iv					
LV-ALB-LUC (n=5)		2 E+08	1,25E+07	0,08	0,06

** Assuming a relative liver mass of 3.35 % body weight in adult rats, 4,5 % in juvenile rats and 5.3 % in adult mice and a mean hepatocyte density in the liver of 1.3×10^8 cells per gram. + CL, clodronate liposome pre-treatment, ND, not determined, iv, intravenous. No vector integrations could be detected in spleen and kidney of animals # 6,7, 8 and 12, with a detection limit of 0.05 vector copies per cell.*

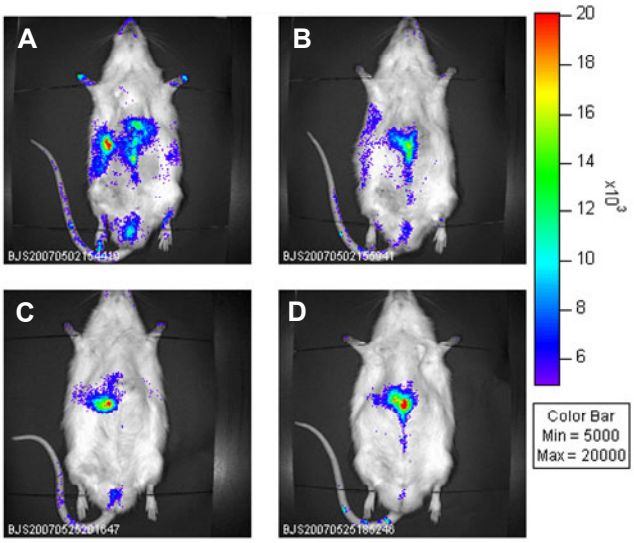

***Fig. 4. Bioluminescence imaging of clodronate pre-treated Gunn rats after IHP with $5x10^8$ TU LV-ALB-LUC**. Animals were imaged at three (A,B) and six (C,D) weeks after perfusion.*

infectious vector titer decreased more than 57% in the first five minutes of perfusion and remained constant thereafter until the end of the perfusion procedure.

The perfusion set-up includes a pre-heating step of the perfusate to 50 ºC before entering the liver (see materials and methods for further details). Furthermore, during perfusion, the blood content in the perfusate increases from near zero to approximately ten percent, due to leakage into the isolated liver vasculature. To rule out that the decrease in vector titer after five minutes of perfusion was caused by these factors, a separate simulation experiment was performed with vector added to 10% total rat blood in perfusion solution (n=2) or fresh blood cell depleted perfusate samples withdrawn at t=0, t=5 and t=10 from sham-treated animals (n=2). These samples were subjected to three repeated cycles of 1 min at 50ºC and 5 min at 37ºC. Subsequent titration based on luciferase expression in 293T cells did not show any detrimental effects of these procedures on the infectious vector titer (data not shown)

Discussion

In these experiments, we demonstrate that for liver directed gene therapy applications, lentiviral vector administration via isolated hepatic perfusion can be an attractive alternative to intravenous vector delivery. As opposed to systemic vector administration, this delivery protocol confines vector deposition to the liver.

Effect of IHP alone on the plasma bilirubin levels in the Gunn rat

The post-operational transient decrease in plasma bilirubin levels observed in jaundiced Gunn rats treated by IHP without vector or in combination with a non-UGT1A1 expressing vector cannot be readily explained. Possibly, the perfusion procedure or blood loss affects the production or turnover rate of erythrocytes, resulting in reduced bilirubin production during recovery. Furthermore, IHP in rats is known to be associated with mild ischemia in the intestine. The process of damage and repair could also have a transient positive effect on the clearance of bilirubin via the intestine, a pathway that is relatively efficient in rats compared to humans. In two animals (#1,6), a peak in the plasma bilirubin level ranging from 120 to 150% of the pre-operational values was present approximately one week after IHP. While this did not appear to affect the survival of the Gunn rats, this phenomenon would be a concern causing acute bilirubin toxicity in human Crigler-Najjar patients. The incidence and cause of these high peak bilirubin levels remains to be further investigated.

Kupffer cell depletion increases vector efficacy

We investigated the effect of Kupffer cell depletion on vector deposition during IHP. Kupffer cell depletion was performed by intravenous administration of clodronate liposomes before IHP, and this had a positive effect on transduction efficiency (Table 1). Two clodronate liposome pre-treated Gunn rats subjected to IHP with a vector dose of 5×10^8 TU LV-ALB-LUC (#4,5), showed twice the amount of vector integrations in the liver (0.2 versus 0.1 copies/cell), as compared to non-pretreated animals subjected to IHP with an identical dose of 5×10^8 TU LV-ALB-UGT (#6,7). Furthermore, a clodronate liposome pre-treated animal subjected to IHP with 5×10^8 TU LV-ALB-UGT (#12) showed a significant reduction in mean plasma bilirubin levels and a vector copy number of 0.23 copies/cell. In this case, both the beneficial effect on the plasma bilirubin level (34% versus 36.4%) and the vector copy number (0.23 versus 0.27) are comparable to an animal treated with a three times higher vector dose of 1.5×10^9 without clodronate pretreatment. Overall, we conclude that Kupffer cell depletion increases the efficiency of vector deposition during IHP with a factor of two to three.

Vector clearance from the perfusate during IHP

The perfusion time was set to ten minutes, since longer perfusion increases the risk of tissue damage in the liver, but is also associated with mild ischemia in the intestine and kidneys. Within this timeframe, the data on vector clearance during perfusion show an apparent

discrepancy. The total amount of vector antigen as determined by p24 ELISA did not decrease during the procedure. However, the infectious vector titer (TU) decreased by more than half in the first five minutes of perfusion and remained stable afterwards (Fig. 3). It was excluded that the drop in vector titer was caused by vector inactivation due to the 50 ºC pre-heating step that is part of the perfusion procedure or due to contact of vector with total rat blood or fresh perfusate samples from a sham treated animal. The reason for the absence of a decrease in p24 concentration in the perfusate may be that p24 is not a representative quantitative measure for the total number of infectious vector particles due to considerable amounts of free p24 antigen in lentiviral vector concentrates [33]. However, the decrease in vector titer in the first five minutes of perfusion suggests that vector adsorption to liver tissue during IHP is a rapid process. The observation that the decrease in vector titer takes place only in the first five minutes of the ten-minute perfusion period is not readily explained.

The mechanism of hepatocyte transduction by lentiviral vectors is relatively unknown. It is hypothesized that lentiviral vectors applied to the bloodstream gain access to hepatocytes via the fenestrations present in the liver sinusoids. Vector particles then travel through the space of Disse, a plasma filled compartment between the endothelial wall and the target hepatocyte. While equilibration by diffusion and adsorption will be relatively fast, uptake of viral particles is likely a slower process. Possibly, during IHP with lentiviral vector, a form of saturation takes place, where the fenestrae become blocked with vector particles or impurities in the vector preparation. However, this seems less likely, since the addition of a higher vector dosage does result in higher liver transduction rates. Another possibility is that an equilibrium forms rapidly, where vector particles enter and leave the space of Disse at equal rate, after depositing a maximum amount of vector particles. The precise dynamics of vector clearance during perfusion need to be further investigated and the results may lead to significant shortening of the perfusion time. Hereby, it would be particularly interesting to directly compare vector clearance data from animals that underwent Kupffer cell depletion and ones that did not to demonstrate potential differences in vector uptake.

Transduction efficiency during IHP

We hypothesized that isolated perfusion with a high-titer lentiviral vector preparation in the rat would allow effective vector deposition in the liver, despite the short exposure time. This would minimize the amount of vector used and improve the safety and feasibility of lentiviral vector delivery. Our data show that we can indeed achieve stable transduction of liver cells and significant and stable reduction in bilirubin levels after a ten minute perfusion, followed by a washout with five milliliter of fresh perfusion solution, with improved transduction efficiency in clodronate treated rats.

When applying identical vector dosages, Kupffer cell depletion prior to IHP resulted in a mean increase of the average number of vector integrations per TU with a factor of two, from 0.3 to 0.6 (Table 1). The number of vector copies per TU is independent of the vector dosage in this system. It should be noted that the vector titer (TU/ml) is determined in cultured cells *in vitro*, therefore we can not assume that the maximum efficiency in IHP delivery equals 1.0 integration per TU. In juvenile Gunn rats transduced by systemic intravenous injection in a previous experiment (Chapter 2) [1], the number of vector copies per TU depends on the vector dosage, and appears to be two times higher than in IHP delivery to clodronate treated adult rats.

However, comparing these two conditions is not straightforward. First, the vector exposure time and the conditions of exposure, which are both crucial for efficacy, are different. Further, comparison of the effective target size is complicated. The lentiviral vectors have a preference for the liver after intravenous injection. This means that the effective target is better represented by the liver weight than the total body weight. Nevertheless a substantial number of integrations is detected in other organs. Here we have used the number of integrations in liver cells as a measure of efficacy, because these are both most numerous and most relevant to our purpose. Further, juvenile rats have a significantly higher mitotic index of 2.4 in the liver, as compared to 0.1 in adult animals. Mitotic activity has been shown to improve the transduction efficacy (integrations/TU) of lentiviral vectors. In mice, significantly higher transduction efficiencies were achieved in adolescent animals as compared to adults [34]. In

another study, vector administration after partial hepatectomy in adult mice resulted in an increase of 1.5 in terms of vector copy number in the liver and a four times increase in the number of transgene expressing hepatocytes [35]. In juvenile animals, other factors, such as an increased relative liver mass and differences in liver architecture may play additional roles. The relative liver mass in juvenile rats is approximately 4.5 % of the body weight, whereas in adult rats this is approximately 3.35 %. Indeed, intravenous injection of the vector preparation used in Gunn rats by IHP in adult mice (20-25 gram body weight, liver weight 1-1.4 gram), yields an average of 0,06 integrations per TU in the liver (Table 1). This suggests that in adult animals IHP is more effective in liver cell transduction than intravenous delivery.

Specificity of vector deposition during IHP

After systemic lentiviral vector administration in a previous study in juvenile Gunn rats, qPCR detected off-target vector deposition primarily in the spleen and in one case in the kidney (Chapter 2) [6]. In contrast, vector deposition after IHP was only detectable in the liver (Table 1), and regarding a detection limit of the qPCR procedure of 0.05 copies/cell, no vector copies were detected in spleen and kidney of animals . Bioluminescence imaging, three and six weeks after perfusion, shows luciferase expression confined to the liver area (Fig. 4). In this figure, the non-specific signals observed in the genital area were caused by light scatter in urine. Other sources of scatter included fur and the naked skin of tail and paws. When the liver area was covered, these non-specific signals were absent.

Future developments: Administration of immunomodulatory drugs via the perfusate

It is known that human, and to a lesser extend rodent, complement inactivates VSV-G pseudotyped lentiviral vectors via the classical pathway. The used IHP technique is not free from blood, as during the procedure blood still leaks into the perfusion circulation. Also, acute inflammatory responses upon vector delivery are undesired in gene therapy applications, since these “danger” signals may alert the immune system and can lead immune responses against the introduced therapeutic protein later in time [36]. However, the isolated setup facilitates the addition of complement and inflammation inhibitory drugs.

Ideal candidate molecules would be synthetic low molecular weight serine protease inhibitors, such as nafamostat mesilate (FUT-175) that combine the inhibition of complement and inflammation and function as anti-coagulant as well. FUT-175 was shown to prevent classical pathway complement inactivation of retroviral vectors by human serum [37]. Furthermore, this molecule inhibits the production of proinflammatory cytokines IL-6 and IL-8 in human monocytes and protects against lipopolysaccharide-induced hepatoxicity by decreasing the production of TNF-alpha, IL-1beta, and IFN-gamma in the liver [38, 39].

Improvement of the IHP procedure

Recently, Ren et al. 2007 described that a repeated full flow occlusion (FFO) approach results in highly efficient lentiviral transduction rates in the liver. Here, the liver vasculature was clamped and lentiviral vector (5×10^8 TU) was infused via the portal vein. During this procedure, the blood supply to the liver was stopped for 5 min and then recommenced for 2 min. This procedure was repeated three times, using 1.5×10^9 TU lentiviral vector in total, similar to the highest vector dose used in our IHP experiments. The vector used, pHR’-CMV-GFP, is a second-generation vector without central polypurine tract, packaged by a packaging system that expresses all HIV-1 accessory proteins. This primitive vector lacks the cPPT, which negatively affects lentiviral vector transduction efficiency *in vivo* by a factor of approximately 2.5, by limiting nuclear translocation of pre-integration complexes [40]. Nevertheless, flow cytometric analysis of GFP expression in hepatocytes showed transduction rates of approximately 60% after both two weeks and two months. The vector copy number in the liver after FFO was not determined by QPCR, however, we can assume that this number exceeds the number of GFP positive cells by at least a factor of two. Therefore, this procedure appears to be more effective than IHP. However, this approach does not have the advantage of liver restricted deposition. Further, the procedure involves high pressure and ischemia, which may be prohibitive in human patients.

When compared to IHP, in the described FFO experiment, several factors could have contributed to the high transduction levels in the liver. These include: the temporary absence

of flow, the presence of a high vector concentration in the liver and the induction of moderate liver damage by ischemia. Also, in the FFO approach, vector administration was carried out via the portal vein instead of the hepatic artery and the stop flow in combination with the infused vector volume (not specified in the publication) and ischemia resulted in a significant swelling of the liver (personal communication, Sir R.Y. Calne, Department of Surgery, Douglas House Annexe, Cambridge, United Kingdom).

Systematic validation of these individual parameters in the IHP approach is likely to improve the transduction rate while keeping the advantages of prevention of systemic vector spreading and the local administration of immunomodulatory drugs. The IHP can be carried out non-oxygenated to induce ischemia and/or with a smaller perfusion volume to increase the vector concentration in the liver. Alternatively, the perfusion system can be modified to incorporate most characteristics of the FFO approach. In principle, vector can be administered multiple times via a syringe coupled to a side cannula that is connected to the main cannula of the inflow limb (either portal vein or hepatic artery or both) and the outflow can be stopped for five minutes. Vector is then washed out for two minutes and the procedure repeated.

Curing hyperbilirubinemia by IHP of lentiviral vectors in humans, a perspective

Here we demonstrate that lentiviral vector deposition to the rat liver can be efficiently achieved during IHP. When we extrapolate these IHP data using the dose-response rates of LV-ALB-UGT reported in previous work, an increase of vector deposition with a factor of three could realize a reduction of plasma bilirubin to near normal values (Chapter 2) [6]. However, application of a higher vector dose proved to be problematic in the perfusion system. During pre-concentration of vector supernatants by hollow fiber tangential flow filtration, we observed that serum and cellular contaminants were co-concentrated with vector particles, which resulted in difficult to resuspend pellets after ultracentrifugation. Attempts to reduce the amount of small debris in the thawed vector preparations by centrifugation of the vector preps resulted in a titer drop of up to sixty percent, which indicated that the vector particles were not fully resuspended, or had aggregated after thawing. Indeed, vector preparations appeared less translucent after thawing than directly after concentration and resuspension. Known procedures to purify lentiviral vectors from crude stocks are associated with considerable loss of titer, which basically defeats its purpose [41].

For the treatment of humans, the scale of vector production becomes problematic and by far exceeds the capacity of standard laboratory facilities. The lentiviral production system is relatively inefficient, and it can be stated that as many vector producing cells are needed as the number of target cells that are to be transduced *in vivo*. This indicates that for clinical applications *in vivo* we need an alternative procedure that can yield larger, more effective, and clinical grade preparations. Despite high expectations in the previous years, such a production system is still elusive. The application of serum free production media and could help to reduce serum components in concentrated vector preps. The concentration procedure should be adapted to allow handling of large volumes and considerable purification. A cell line that allows continuous or induced vector production, without the need for plasmid co-transfection, would be a tremendous asset. However, such a system has not been presented.

Future work aimed at lentiviral gene therapy application for Crigler-Najjar disease will include the use of more efficient, codon optimized, expression cassettes with stronger liver-specific promoters, combined with a micro RNA target site to prevent off-target expression in leukocytes to prevent cellular immune responses [42]. An increase in stable expression can have a similar effect as the administration of higher vector dosages. The capacity to metabolize bilirubin of a hepatocyte is very high and not rate limiting using liver specific promoters. Nguyen et al published that 1% transduction in the Gunn rat with a highly expressing retroviral promoter can achieve full normalization of plasma bilirubin levels [9]. The downside of this approach is that a highly active enhancer/promoter can cause genotoxicity, a risk that has to be assessed in future studies [43].

Materials and Methods

Construction of LV-ALB-LUC

The liver-specific luciferase expression construct LV-ALB-LUC was constructed by replacing the UGT1A1 cDNA sequence from the previously described LV-ALB-UGT (Chapter 2) [6] for the luciferase coding sequence derived from pGL3-Basic (Promega).

Large-scale lentiviral vector production

The protocol used to produce LV vectors (Chapter 2) [6] was modified and optimized to generate the titers required for multiple IHP experiments. Briefly, VSV-G psudotyped lentiviral vectors were produced in HEK-293T cells, seeded 1:4, 72 hours prior to transfection and grown confluent in CellSTACK culture chambers (10 stack, 6360 cm^2 cell growth area, Corning) counting a total of ~2×10^9 cells per chamber.

A three-plasmid split-genome packaging system combining plasmids pMDLg/RRE, pRSV-REV, and pMD.G [45] together with transfer construct LV-ALB-UGT of LV-ALB-LUC was introduced by polyethylenimine (PEI)-mediated transfection in a 2:1:1:4 ratio respectively. Plasmid DNA was purified using a commercial kit (Jetstar 2.0 plasmid mega kit, Genomed). PEI transfection reagent was prepared by diluting PEI (Ave. Mw ~25.000 LS, Aldrich) in water (10 g in 100 ml) followed by extensive dialysis to remove toxic low molecular weight polymers. Dialysis was performed using dialysis tubing (Visking, Mol. Wt. cut-off ~14kD) for a period of one week at room temperature in water (1.5 L) which was refreshed every 24 hours. The dialyzed PEI reagent was aliquotted and stored at 4 ºC until further use.

For the transfection of one CellSTACK culture chamber, the transfection mixture was prepared by slowly (one drop per second) adding 15,24 mg dialyzed PEI in 63,5 ml HBS (20 mM HEPES, 150 mM NaCl) to an equal volume of 5,08 mg total plasmid DNA in HBS. The mixture was incubated for 15 minutes at room temperature and added directly to 1143 ml DMEM containing pen/strep and 1% FCS and transferred to the cells. After 24 hours, the transfection medium was changed for 560 ml DMEM containing 1% FCS and vector was harvested 5 times at 12 hour intervals by changing the medium. Immediately after harvesting, the vector supernatant was filtered through a 0,45 um bottle top filter (ZapCap-S PLUS, Whatman/Schleicher & Schuell) and stored at 4 ºC.

The vector containing medium (2.8 L per culture vessel) was concentrated 15.5 times by passing through a hollow fiber tangential flow filtration unit (Spectrumlabs MiniKros sampler, PS, 0.05um, part no: M2-500S-600-01P). The pre-concentrate was concentrated another 65 times by ultracentrifugation (Beckman SW28 rotor in ultraclear tubes), resuspended in a total volume of 2,8 ml PBS, snap frozen in liquid nitrogen and stored at -80ºC until further use. Three 10-stack culture chambers could be processed by one person in one week gaining, yielding a total vector quantity of approximately 8×10^9 TU.

Isolated hepatic perfusion procedure

All animal experiments were approved by the local ethics committee (DEC consult) and performed under strict biological containment. Gunn rats were bred by crossing homozygous animals and housed in ventilated cages on normal chow and water ad lib. The isolated hepatic perfusion procedure was performed as described earlier in normal rats [44]. Male Gunn rats, 280-380 g body weight and 25-50 weeks old where anesthetized by isoflurane gas, and the hepatic ligament was exposed by mid-line laparotomy. For the arterial inflow limb, the gastro-duodenal side branch of the common hepatic artery was cannulated, positioning the tip of the cannula in the proper hepatic artery. To collect hepatic venous outflow, the femoral vein was exposed through a small inguinal incision and a cannula was introduced and moved up into the caval vein positioning the tip of the cannula at the level of the hepatic veins. Subsequently, the hepatic vascular bed was isolated by temporarily ligating the common hepatic artery and the portal vein. The venous outflow limb was isolated by temporarily clamping the supra-hepatic caval vein and by applying a temporary ligature around the infra-hepatic caval vein containing the cannula, cranial to the right adrenal vein. The mesenteric artery was temporarily clamped in order to reduce splanchnic blood pressure.

The perfusion solution in the reservoir contained 10 ml heparinized (50 IU, Heparine Leo) colloid plasma volume substitute (Gelofusin, B. Braun) which was oxygenated with a mixture of O2:CO2 (95:5%). The circuit was primed with perfusion solution and lentiviral vector was added to the reservoir. Subsequently, rats were perfused for 10 min at an arterial flow of 5 ml/min, maintained with a low-flow peristaltic pump. Before entering the liver, the perfusate was pre-heated by a short passage of the afferent cannula through a 50ºC heat exchanger coupled to a water bath. After heating, the length of the downstream cannula is such that the perfusate has cooled down to 38-39 ºC when entering the liver.

During perfusion, 500 ul samples were withdrawn from the perfusion reservoir at t=0, t=5 and t=10 min and the total wash-out was collected. Samples were immediately centrifuged to remove blood cells and the supernatant was snap frozen in liquid nitrogen. After 10 minutes, unbound vector particles were washed out of the liver with 5 ml of fresh heparinized plasma substitute. At the end of the procedure, the blood flow was restored by removing the clamps on caval vein, portal vein, hepatic artery and mesenteric artery. The gastroduodenal artery and femoral vein were ligated, the gastroduodenal and femoral cannulas were removed and the operation wounds sutured. The complete procedure took approximately one hour.

Clodronate liposome pre-treatment of rats
Clodronate liposomes were purchased from Dr. N. van Rooijen et al, Department of Molecular Cell Biology, Free University Medical Center, Amsterdam, The Netherlands. For Kupffer cell depletion, rats received clodronate liposomes (5 ml/kg body weight) via penile vein injection, 3-4 days prior to undergoing IHP.

In vivo bioluminescence imaging
For real time *in vivo* bioluminescence imaging, rats were anesthetized with isoflurane anesthesia and intraperitoneally injected with D-Luciferin potassium salt in PBS (30 g/L, 75 mg/kg body weight, Caliper Life Sciences). Ten minutes later, luciferase expression was quantified for 5 minutes from the ventral side of the animal using an IVIS Imaging System 200 Series (Caliper Life Sciences).

Luciferase assay for vector titer
The titer of LV-ALB-LUC in perfusion samples (Fig. 3) was determined by serial dilution on 293T cells, seeded in black 96 well tissue culture pates. After 4 days, cells were lysed and luciferase activity was determined by using the Luciferase Assay System (Promega) according to the manufacturer's protocol and a Top Count-NXT scintillation/luminescence counter (Packard BioSciences).

Determination of plasma bilirubin levels
For the determination of the plasma bilirubin level in experimental animals, blood samples were withdrawn in heparinized tubes by retro-orbital bleeding under isoflurane anesthesia. Plasma bilirubin levels were quantified by using BIL-T bilirubin DPD reagents (Roche) as described earlier (Chapter 2) [6].

Quantitiative real time PCR to determine vector copy number
The procedure for the determination of the average vector copy number per cell using a vector specific Taqman^{TM} probe was performed as described earlier (Chapter 2) [6]. The detection limit of the qPCR procedure was 0.05 vector copies per cell.

P24 ELISA
The total amount of vector particles in perfusate samples was determined by using the p24 Capture Elisa Kit (ImmunoDiagnostics, Inc) according to the manufacturer's instructions and absorbance was measured on a SpectraMAX EIA reader (Molecular Devices).

Statistical analysis
Student's two-tailed t test was used for statistical analysis. P-values less than 0.05 were considered significant.

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Supplementary data

Optimization of large scale LV vector production

The production procedure for lentiviral vectors was optimized to enable a more efficient generation of sufficient vector in shorter periods of time. The main bottleneck in the processing of large volumes of lentiviral vector supernatant is the ultracentrifugation concentration step. It was determined that reducing the volume of the production medium from 0.180 ml to 0.090 ml/cm^2 of tissue culture surface area did not affect the total amount of transducing units produced and, as a consequence, this reduced the time required for ultracentrifugation to produce a vector batch by half. The half-life time of lentiviral vectors at 37 ºC is approximately 10 hours and probably shorter in 293T production cell cultures [45]. Consistent with this, we determined that there was no significant difference in vector titer per milliliter in production supernatants harvested at 12 hour intervals compared to the 24 hour interval which is standard procedure in many protocols (Fig. S1). We concluded that harvesting at two 12 hour intervals yields twice the amount of active vector particles.

Previously, PEI mediated transfections were carried out in serum free medium for 3,5 hours, followed by replacement of the transfection medium with fresh medium containing 10% FCS. After 20.5 hours, the medium was again refreshed and production was started in medium containing 10% FCS. We observed however that transfection for 24 hrs in medium containing 1% FCS resulted in superior transfection efficiencies and vector titers, while the extra medium refreshment step between transfection and the actual start of production can be omitted. Furthermore, it was determined that vector production could also be performed in 1% FCS containing medium without drastic reductions in vector titer, hereby decreasing the amount of contaminating serum proteins in concentrated vector preparations.

Reduction of the amount of plasmid DNA used for transfection by half, resulted in 50-60 percent lower vector titers. It was reported in the literature that in combination with calcium phosphate based transfection protocols, the addition of sodium butyrate had a beneficial effect on the amount of vector produced by upregulating expression from the transfected production plasmids [46]. In our hands, however, in combination with the PEI based transfection procedure, the addition of 4 mM sodium butyrate to the production medium after transfection did not have a significant effect on the efficiency of vector production. It was also reported that reducing the temperature during production from 37 to 32 ºC increased the half life of gamma-retroviral vectors during production, which resulted in a higher total vector yield [47]. However, for lentiviral vectors in our system this resulted in forty percent lower vector titers.

A standard procedure during LV vector production is the low-speed centrifugation and subsequent filtration of production medium trough a 0.45 µm filter prior to concentration, in order to remove detached production cells and cell debris. The common use of disposable 50 ml tubes and filtration through standard 25 mm diameter syringe filters that typically clog after filtration of approximately 40-50 ml production medium is time-consuming and inefficient with large volumes. Therefore we omitted the centrifugation step and applied 76 mm diameter vacuum operated 0.45 um cellulose acetate membrane bottle-top filters fitted with a glass fiber pre-filter (ZapCap-S PLUS, Whatman/Schleicher & Schuell). These units enable direct processing of 500 ml vector supernatant without loss of titer.

To avoid unnecessary waste of lentiviral vector titer, it was verified that concentrated vector supernatants can resist at least four freeze-thaw cycles without significant reduction in vector titer as determined by using preparations of LV-ALB-LUC in combination with *in vitro* transduction of 293T cells and a luciferase assay (data not shown). During these experiments, vector preparations were snap-frozen in liquid nitrogen.

Implementation of multi layer culture vessels and pre-concentration by hollow fiber tangential flow filtration

We anticipated that in order to obtain a true clinically relevant effect on the plasma bilirubin level in the Gunn rat, the vector dose per experiment had to be increased by at least a factor

of three, from 5×10^8 to 1.5×10^9. To facilitate production of these high vector quantities, the vector production procedure was scaled up by carrying out transfections in stacked 6500 cm2 tissue culture chambers as opposed to the previously used 500 cm2 tissue culture plates. Further, after harvesting, we introduced a pre-concentration step based on hollow fiber tangential flow filtration using a Spectrumlabs MiniKros sampler filtration unit (part no: M2-500S-600-01P) and a Watson-Marlow peristaltic pump (Sci-Q 323) (Fig. S2). This procedure enables the exclusion of liquid from the vector supernatant while preventing the mechanical vector inactivation observed with dead-end filtration systems. The production in multi-layer tissue culture chambers combined with hollow fiber tangential flow filtration made it possible to process up to nine liters of vector containing medium by a single person in one week without affecting reproducibility, relative vector yield or vector titer as compared to low scale production without pre-concentration.

Supplementary figures

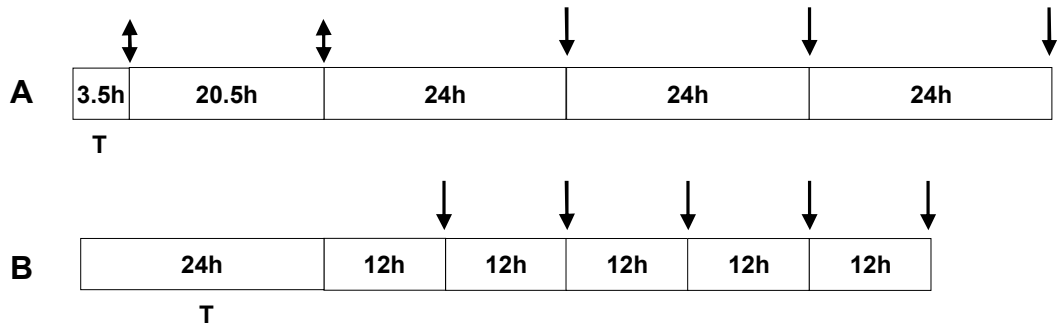
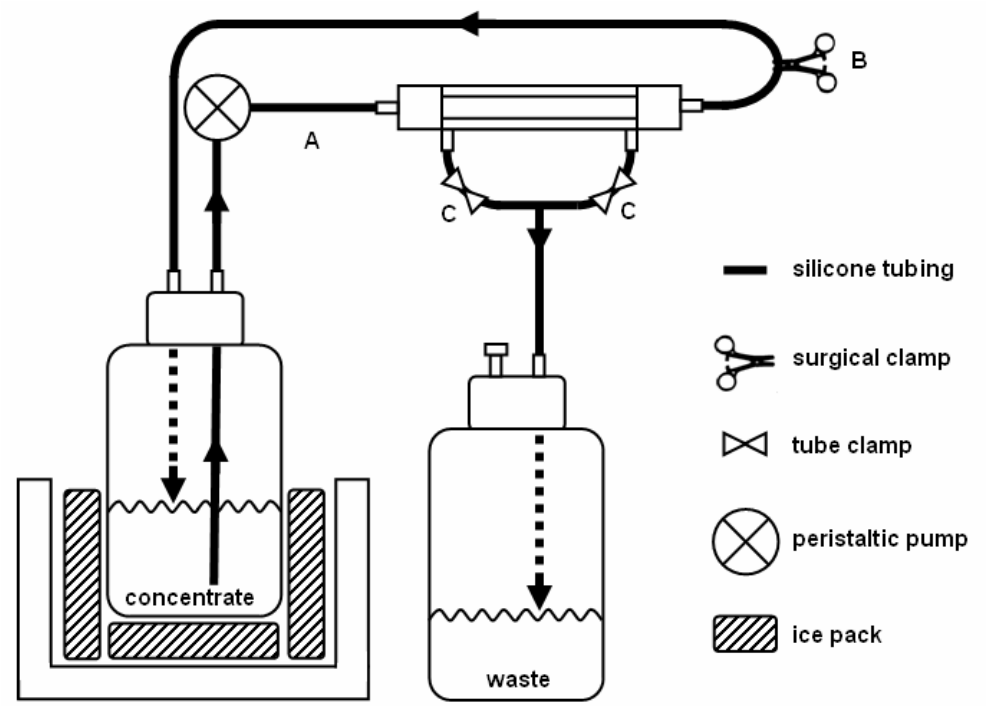


Fig. S1. An improved transfection and harvesting schedule for the production of lentiviral vectors in medium with lower serum content. *(A) The standard transfection and harvesting procedure consist of a transfection period (T) of 3.5 hours in medium without FCS. After the transfection period, the medium was changed (double arrow) for medium containing 10% FCS followed by an incubation period of 20.5 hours. After this time, the medium was changed again and vector production was started in medium containing 10% FCS, which was collected and refreshed (arrow) three times with 24 hour intervals. (B) With the improved method, transfection was performed for a period of 24 hours in medium containing 1% FCS. After removal of the transfection medium, vector production was immediately started in medium containing 1% FCS and harvested five times with 12 hour intervals. These modifications resulted in better transfection efficiencies, harvesting of more active vector particles and a reduction in FCS content in the harvested vector supernatant.*

Fig. S2. Pre-concentration of lentiviral vector supernatant by hollow fiber tangential flow filtration. *This simple filtration set-up does not require the use of pressure detectors and can be set-up with minimal costs in a flow hood. The harvested vector medium is present in the concentrate tank and will be concentrated approximately two times by each cycle through the filter unit. Excess fluid flows through the pores of the filter fibers to the waste tank. After approximately 1.5 hours the volume of the concentrate has decreased from 2.8 L to 180 ml, which can be further concentrated in one SW28 rotor. Pressure is regulated on sight by the surgical clamp at location B. The flow into the two flasks (concentrate and waste) was kept at an identical rate, making sure the pressure at point A did not build up. The pressure could be further regulated by adjusting the rotation speed of the pump. Normally, the pump was set between rotation speeds 20 and 40. At the end of the procedure, the clamps at C were closed and the pump was set to reverse (pump rotation speed 10) to remove the dead volume form the tubing. The filtration units could be re-used for concentration of at least three 2.8L vector batches to facilitate concentration of up to nine liters of vector supernatant in total.*


B
A
C
C
silicone tubing
surgical clamp
tube clamp
peristaltic pump
ice pack
concentrate
waste

Chapter IV

Simultaneous Targeting of HCV Replication and Viral Binding with a Single Lentiviral Vector Containing Multiple RNA Interference Expression Cassettes

Molecular Therapy, 2006, 14: 485-493

Simultaneous Targeting of HCV Replication and Viral Binding with a Single Lentiviral Vector Containing Multiple RNA Interference Expression Cassettes

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Chronic hepatitis C virus (HCV) infection has a major medical impact and current treatments are often unsuccessful. RNA interference represents a promising new approach to tackling this problem. The current study details the design and testing of self-inactivating lentiviral vectors (LV) delivering RNA interference to prevent HCV replication and infection. Vectors were constructed with single, double, and triple cassettes expressing short hairpin RNAs (shRNAs) simultaneously targeting two regions of the HCV 1b genome and the host cell receptor, CD81. The shRNAs directed against HCV IRES or NS5b regions were shown to be effective in inhibiting HCV replication *in vitro* (82 and 98%, respectively). No evidence of shRNA-related interferon production was observed. Vectors containing CD81 shRNA reduced cell surface expression up to 83% and reduced cell binding of HCV surface protein E2 up to 82% while not affecting levels of unrelated surface protein (Ber-EP4) or HCV replication. Double or triple shRNA vectors were independently effective in simultaneously reducing HCV replication, CD81 expression, and E2 binding. This study demonstrates lentiviral delivery of multiple shRNA, inhibiting HCV in a specific, IFN-independent, manner. The targeting of multiple viral and host cell elements simultaneously by RNAi could increase the potency of antiviral gene therapies.

Key Words: gene therapy, shRNA, RNAi, hepatitis, CD81, lentivirus

INTRODUCTION

The hepatitis C virus (HCV), together with the hepatitis B virus, now accounts for 75% of all cases of liver disease around the world. HCV is an enveloped positive-strand RNA virus classified as the sole member of a distinct genus called hepacivirus in the family *Flaviviridae* [1]. The genome of HCV encompasses a single ~9600-nucleotide RNA molecule carrying one large open reading frame (ORF) that is flanked by nontranslated regions [2]. The polyprotein created from this ORF is cleaved co- and post-translationally by cellular and viral proteases into 10 different products, with structural proteins located in the amino-terminal one-third and the nonstructural replicative proteins in the remainder [3,4]. The structural proteins include two envelope proteins, E1 and E2, which determine HCV tropism. E2 binding to cell-surface tetraspanin CD81 is instrumental to viral entry [5–7].

RNAi-based antiviral therapies have begun to show great promise for HCV and other viral infections. RNAi is a process of sequence-specific, posttranscriptional gene silencing in plants and animals triggered by double-stranded RNA [8,9]. Many viruses produce a transitory double-stranded RNA intermediate during replication that can be processed by an enzyme termed DICER into 21-nucleotide short interfering RNAs (siRNAs) [10], starting the RNAi antiviral mechanism. One strand of the siRNA associates with the RNA-induced silencing complex [10,11], which guides the siRNA to its homologous mRNA target resulting in cleavage of the target sequence [8,11]. To date RNAi has been used effectively to reduce infection in many *in vitro* viral systems including human immunodeficiency virus (HIV) [12,13], poliovirus [14], influenza virus [15], and hepatitis B virus [16], with recent studies showing that RNAi can be effective in blocking HCV replication [17–19]. In addition to down-

regulating viral RNAs, RNAi can be an effective tool to downregulate host cell genes involved in viral infection such as CD4 and CXCR4 in HIV [13,20]. In analogy, downregulation of CD81 was shown to be effective in preventing HCV envelope binding [21].

If RNAi therapies are to be utilized as an effective treatment or preventer of disease, long-term, stable siRNA expression needs to be achieved. Raw or plasmid siRNA transfections elicit only short-term silencing, whereas integrating self-inactivating LV [22,23] that encode short hairpin RNAs (shRNAs) can produce long-term, continuous silencing [24,25]. Vector-derived shRNAs are single-stranded RNAs containing complementary sequences separated by a loop sequence that can fold into a predicted double-stranded hairpin. These structures are recognized by DICER and cleaved into biologically active siRNA.

Many viruses mutate at high rates [14,26] and can therefore rapidly develop resistance to monotherapies. HCV has been shown to generate mutants with monotherapy of ribavirin or RNAi [27–29]. Host cell factors involved in infection are not prone to mutation and are therefore good therapeutic targets for RNAi to help prevent resistance [13]. We present here the construction of lentiviral vectors producing single and multiple shRNAs that are capable of inferring protection by downregulating the host cell receptor CD81 as well as simultaneously targeting multiple HCV sequences. Efficacy of these therapeutic vectors was demonstrated in an HCV replication model [30–32].

Results and Discussion

Effects of Single shRNA Vectors

We constructed lentiviral vectors containing shRNA sequences (Table 1) directed against HCV and CD81 by insertion of shRNA oligonucleotides (Fig. 1A) downstream of the H1 promoter in the pSP72 plasmid (Fig. 1B). We removed the H1-shRNA cassettes and ligated them in the direction opposite that of the CAG promoter within the lentiviral packaging plasmid (Fig. 1C). To determine the effects of shRNA constructs on HCV replication and CD81 expression, we incubated Huh-7 replicon cells for 24 h with concentrated LV at similar titers. The level of transduction at 3 days, as determined by flow cytometry for enhanced green fluorescent protein (GFP) expression, ranged from 85 to 97% for all vectors. Single HCV shRNA vectors very significantly reduced HCV replication compared to mock transductions with empty GFP vector (Fig. 2A). The NS5b shRNA reduced HCV replication by 97 ± 2% (SEM) ($P < 0.001$) and IRES shRNA by 82 ± 8% ($P < 0.05$). The Con (10 ± 3%) and CD81 (20 ± 3%) shRNAs showed a slight reduction in HCV replication but it was not significant compared to mock control. Results obtained with luciferase activity correlated with significant reductions in HCV mRNA (IRES, 72 ± 2%, $P < 0.05$; NS5b, 97 ± 0.1% ($P < 0.01$)) (Fig. 2B) as well as a clear reduction in nonstructural protein 3 (NS3) protein levels with both vectors compared to Con vector (Fig. 2C).

CD81 shRNA vector reduced CD81 mean fluorescence intensity by 83 ± 3% ($P < 0.05$) compared to mock, while other non-CD81 vectors had no effect on the expression levels of CD81 (IRES, 8 ± 3%, NS5b 12 ± 2%) (Fig. 2A). CD81 mRNA levels were not significantly affected by non-CD81 shRNA vectors, whereas they were significantly reduced by CD81 vector (91 ± 1%, $P < 0.01$) (Fig. 2B). We saw a reduction in cell surface CD81 expression in only the CD81 shRNA-containing vector as shown by immunocytochemistry (Fig. 2D). CD81 shRNA was specific, with no effect on an unrelated surface protein Ber-EP4, as confirmed by FACS profiles of cells treated with CD81 vector (Fig. 2E). Together, these results demonstrate the effectiveness of single shRNA expression vectors to silence HCV replication and CD81 surface expression.

Double and Triple shRNA Vectors Inhibit CD81 and E2 Binding

We created plasmids containing double and triple shRNA cassettes by sequential insertion of H1-shRNA cassettes. Each insertion results in a unique downstream restriction site that is available for additional H1-shRNA insertions. Based on the packaging limitation of lentiviral vectors (~7000 bp), this method could potentially be used to insert up to 21 self-expressing shRNA cassettes within a single vector. As shown in Fig. 3, double and triple vectors containing CD81 shRNA created with this method were effective in reducing CD81 expression levels (NS5b-CD81, 77 ± 5%; IRES-NS5b-CD81, 78 ± 1%). CD81 mRNA was reduced by all these vectors (NS5b-CD81, 71 ± 1%; IRES-NS5b-CD81, 80 ± 1%) but not with double HCV shRNA (IRES-NS5b, 10 ± 4%) (Fig. 3B). Multiple target vector NS5b-CD81 downregulated NS3 protein expression (Fig. 3C) as well as CD81 surface expression (Fig. 3D).

TABLE 1: shRNA sequences, targets, and their references

shRNA	Sequence	Target	Reference
Con	GCGCGCUUUGUAGGAUUCG	None (scrambled)	[16]
IRES	AGGUCUCGUAGACCGUGCA	Region IV (321–340) HCV IRES	[17]
NS5b	GACACUGAGACACCAAUUGAC	HCV NS5B (6367–6388)	[18]
CD81	GGAUGUGAAGCAGUUCUAU	Human CD81 (594–614)	Ambion siRNA 14501

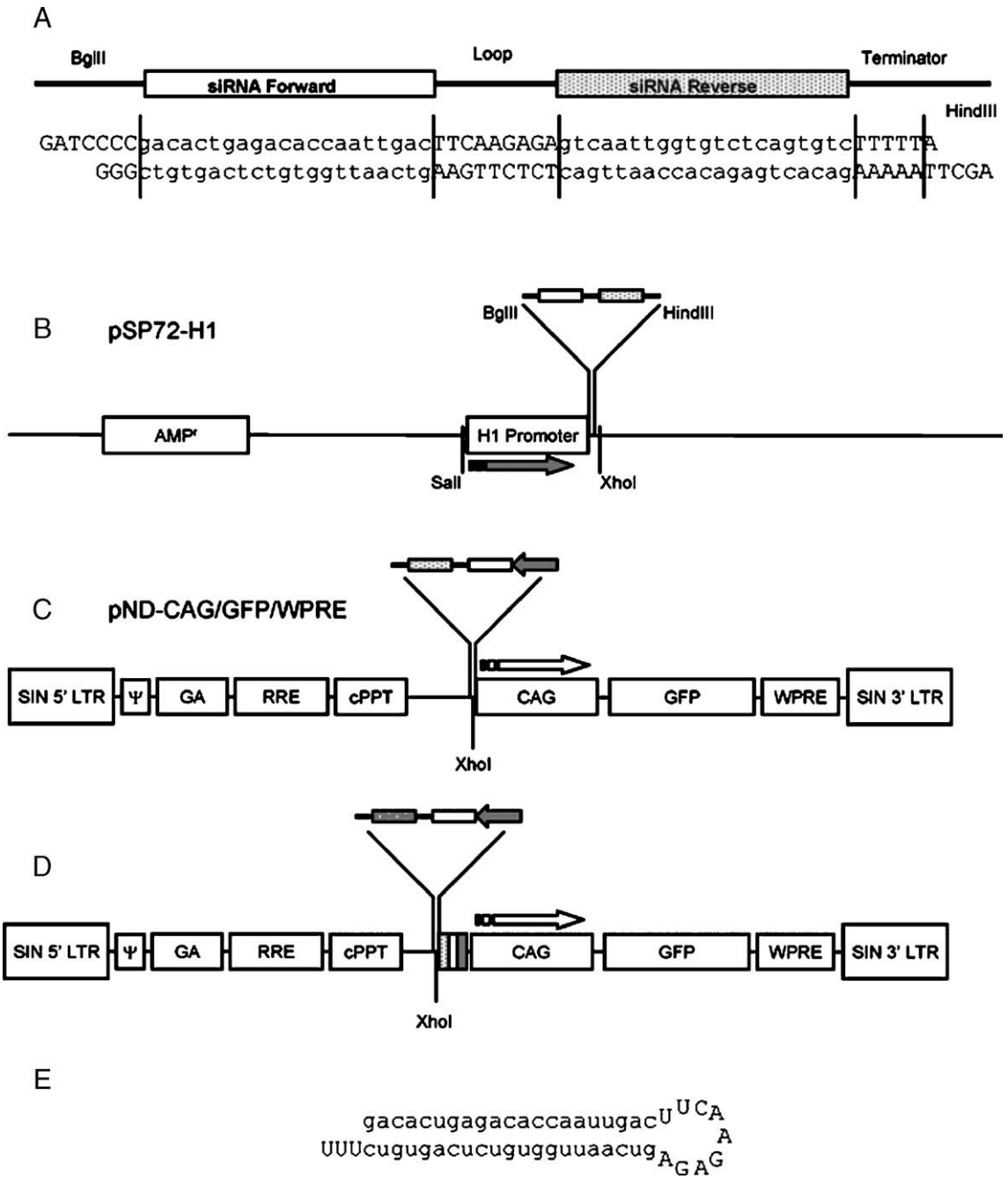

FIG. 1. shRNA vector design. (A) Each shRNA is composed of both a forward and a reverse sequence, interrupted by a hairpin loop, flanked by a downstream termination sequence and restriction enzyme overhangs. (B) shRNA sequences were ligated into *Bgl*II/*Hin*dIII-cut sites downstream of the H1 promoter of the generated pSP72-H1 plasmid, leaving the upstream *Sal*I and downstream *Xho*I sites intact. (C) The H1-shRNA cassettes were removed by *Sal*I/*Xho*I and placed into the *Xho*I site upstream and in opposing direction to the CAG promoter within the packaging plasmid pND-CAG/GFP/WPRE, destroying the *Sal*I site while leaving an intact upstream *Xho*I site. (D) Multiple shRNA pND plasmids were created by opening the newly formed *Xho*I site and inserting additional shRNA cassettes, each time leaving an intact *Xho*I site allowing further insertions of shRNA cassettes. (E) Predicted hairpin structure of fully formed NS5b shRNA.

A possible complication of the presence of multiple shRNAs is the potential for competition for RNAi resources. However, we and others [24,41,42] have found no significant competitive inhibition by the inclusion of multiple shRNAs. The presence of other shRNAs in the same vector did not clearly interfere with the inhibitory

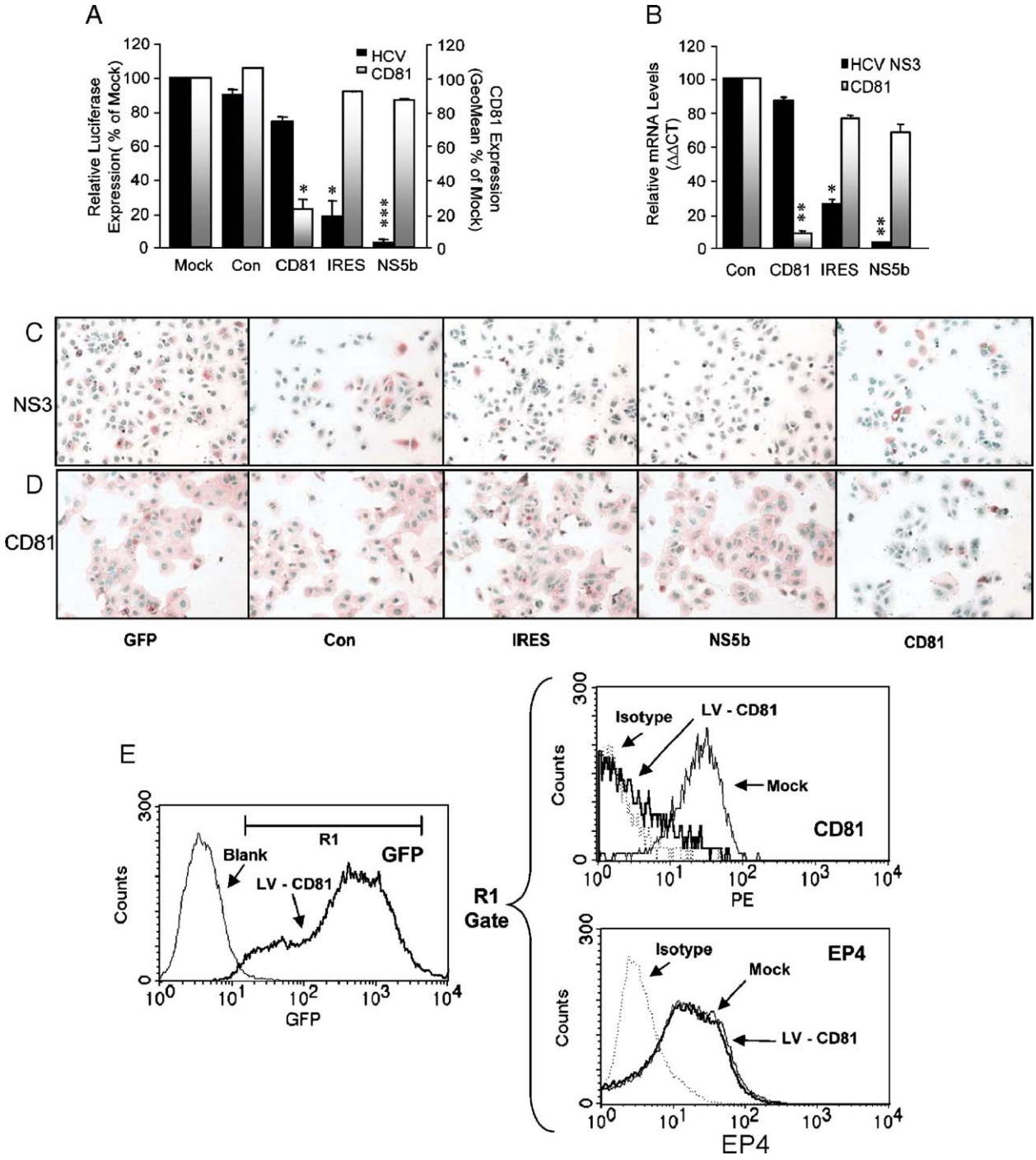

FIG. 2. Single shRNA vectors effectively inhibit HCV replication and CD81 expression. (A) Specific shRNAs significantly reduced HCV replication ($n = 5$, left axis) and significantly reduced surface expression of CD81 ($n = 4$, right axis) at 3 days posttransduction ($P < 0.05$, compared to mock). Control shRNA (Con) showed no inhibitory effect. (B) Semiquantitation of HCV (NS3) and CD81 mRNA normalized to GAPDH ($\Delta\Delta C_T$ calculation). Specific shRNAs significantly reduced HCV and CD81 mRNA ($n = 3$) ($P < 0.01$, compared to Con). (C) HCV NS3 and (D) CD81 staining (red) in Huh-7 replicon cell line after 3-day transduction with single shRNA vectors. Clear reduction of protein expression was seen with the corresponding shRNA and not with the unrelated shRNAs (one of three representative experiments). (E) Representative FACS profile of total GFP expression and surface expression of CD81 and EP4 in Huh-7 replicon cells after transduction with CD81 shRNA LV. Isotype-matched controls are included to demonstrate specificity of staining. CD81 and EP4 expression is shown for GFP-positive cells only (R1 gate). Specific reduction was seen in CD81 expression levels but no effect on EP4 surface staining was seen in transduced (GFP-positive) cells.

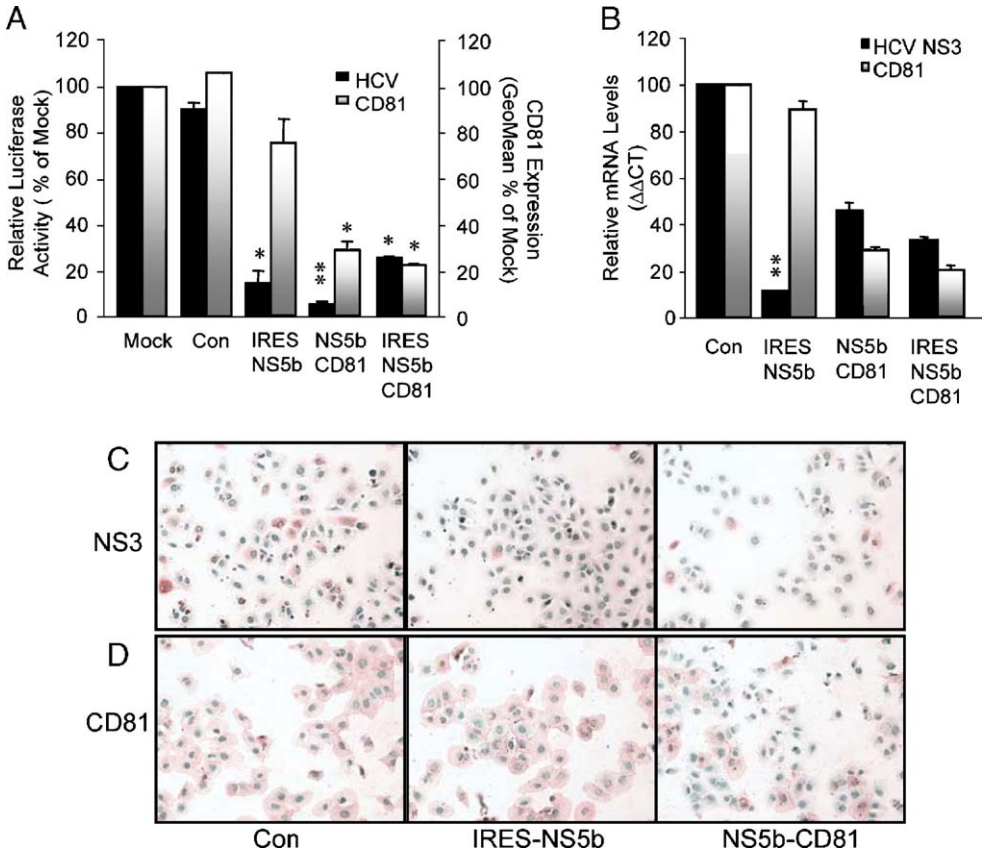

FIG. 3. Double and triple shRNA vectors effectively inhibit HCV replication and CD81 expression. (A) Double and triple shRNA vectors significantly reduced HCV replication ($n = 5$, left axis) and significantly reduced surface expression of CD81 ($n = 5$, right axis) at 3 days post-transduction ($P < 0.001$, compared to mock). Control shRNA (Con) showed no inhibitory effect. (B) Semiquantitation of HCV (NS3) and CD81 mRNA normalized to GAPDH ($\Delta\Delta C_T$ calculation). Double and triple shRNAs significantly reduced HCV and CD81 mRNA ($n = 3$) ($P < 0.001$, compared to Con). (C) HCV NS3 and (D) CD81 staining (red) in Huh-7 replicon cell line after 3-day transduction with double shRNA vectors. Clear reduction of protein expression was seen with the corresponding shRNA and not with the unrelated shRNAs (one of three representative experiments).

activity of the first. Single shRNA CD81 reduced surface expression by 83 ± 3% ($P < 0.05$), while double and triple vectors reduced CD81 by 77 ± 5 (NS5b-CD81) and 78 ± 1% (IRES-NS5b-CD81), respectively. This confirms that it is possible to introduce several shRNAs to silence many genes simultaneously with little or no loss in efficacy. Recent reports have suggested that nonspecific effects can occur from siRNAs, at the levels of both mRNA and protein [43]. In the current study we did not observe this. The Con, IRES, and NS5b shRNAs had no effect on CD81 (Fig. 2) or Ber-EP4 surface expression (data not shown). Conversely, Con and CD81 shRNAs had no significant effect on HCV replication or EP4 surface expression (Fig. 2).

To determine the biological relevance of reducing cell surface CD81 by shRNA vectors we performed an E2 binding assay. As shown in Fig. 4, the reduction of bound E2 was seen with all vectors containing CD81 shRNA within the transduced cell population. As expected, the nontransduced cells (GFP negative) showed no reduction in E2 binding (data not shown). The reduced binding of E2 was correlated directly with a reduction in the surface expression of CD81 (Fig. 4C). This suggests that even if CD81 cell surface density is reduced; low levels of bound HCV E2 can be observed.

Double and Triple shRNA Vectors Inhibit HCV Replication

Double and triple vectors containing HCV-specific shRNA significantly inhibited HCV replication (IRES-NS5b, 85 ± 5%, $P < 0.05$; NS5b-CD81, 90 ± 4%, $P < 0.01$; IRES-NS5b-CD81, 74 ± 1%) (Fig. 3A). As with the single vectors, we also saw reduction in HCV mRNA levels when cells were treated with any of the double vectors (Fig. 3B) (IRES-NS5b, 97 ± 1%, $P < 0.01$; NS5b-CD81, 54 ± 3%; IRES-NS5b-CD81, 66 ± 2%). Double HCV vector IRES-NS5b showed similar downregulation of NS3 intracellular staining compared to single HCV vectors (Fig. 3C).

There are several advantages to using single vectors containing multiple shRNAs. The probability of hitting a single hepatocyte with more than one vector, in the setting of clinical gene therapy, is very small. With the inclusion of several different shRNAs in one vector, the probability of one cell receiving the appropriate combination of shRNAs is ensured. Our design theoretically overcomes the problem of mutational escape by targeting two or more regions within the viral genome. HCV is particularly mutational due to the low fidelity of its RNA polymerase. Treating HCV with monotherapies like ribavirin has been shown to select rapidly for resistant mutants, making therapy impotent [27,28]. Even the

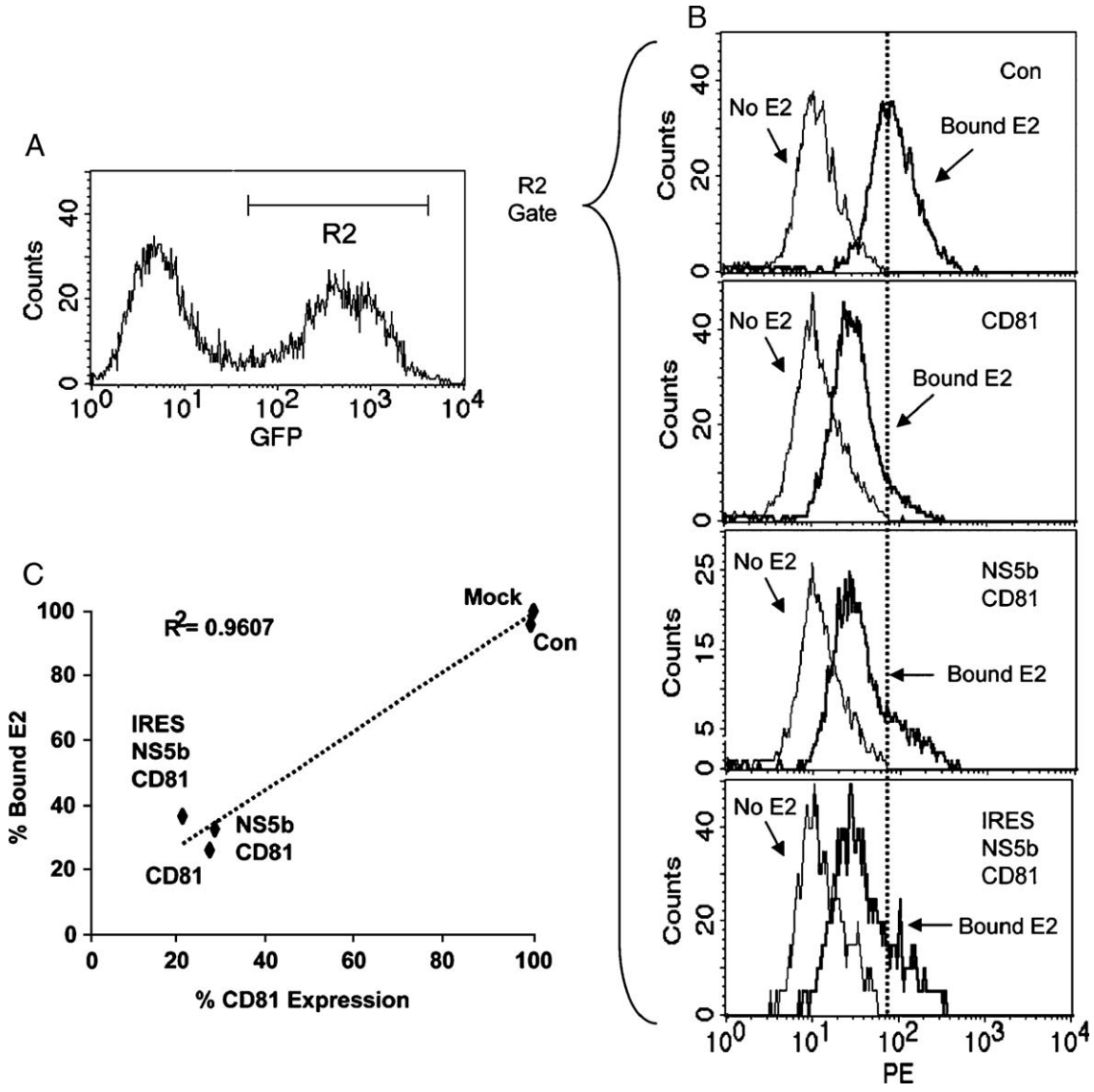

FIG. 4. shRNA-mediated reduction of CD81 inhibits HCV E2 binding. (A) E2 binding was measured in GFP-positive cells (R2 gate), 7 days after transduction (left). (B) Single, double, and triple vectors containing CD81 shRNA effectively reduced binding of E2 (black line). Specificity of detection was confirmed with no-E2-protein control (gray line). Peak channel (bound E2) in control cells is indicated by dotted line. (C) A reduction in the percentage of cells binding E2 was directly proportional to the percentage of cell surface CD81 expression, as determined by flow cytometry. One of three representative experiments is shown.

most powerful siRNAs, given singly, will not prevent selection of viral mutants, rapidly reducing efficacy and finally rendering the treatment obsolete. A mathematical model by Leonard and Schafter predicts that prevention of viral escape can be achieved when a combination of four different suboptimal siRNAs are used [44]. The model also predicts that if more individually effective siRNAs are used in combination, fewer are needed. We calculated that, based on the individual efficiencies, a combination of IRES and NS5b shRNAs could, theoretically, eliminate HCV viral escape.

Long-term Effects of Double and Triple shRNA Vectors

To be effective as a therapy, RNAi needs to have long-term expression. Transfection of raw siRNAs is not a viable gene therapy for persistent viral infection, as they lose their effectiveness within approximately 5 days [45]. We show that by using integrating lentiviral vectors to provide sustained shRNA-mediated RNAi; our therapy is effective for at least 2 weeks. We treated replicon cells with shRNA vectors and kept them in culture without selection for up to 17 days. Integrated lentiviral shRNA constructs continued to reduce HCV replicon activity 17

days post-transduction (longest culture period). We found that in cells transduced with vectors containing a single HCV shRNA (NS5b-CD81), efficacy was reduced at later time points. With vectors containing double HCV shRNA (IRES-NS5b or IRES-NS5b-CD81), efficacy was sustained over the same time points (Fig. 5).

Lack of Interferon (IFN) Response to shRNA Vectors

Some evidence suggests that the use of siRNA under certain circumstances can invoke a host cell interferon response. To address the production of IFN by replicon cells containing shRNA in our model, we used an IFN bioassay with Huh-7 replicon cells as a sensitive indicator of secreted IFN [40]. No IFN was produced in response to any shRNA, regardless of sequence (Fig. 6). In fact, a recent paper has shown that Huh-7 cells containing HCV replicons have a strongly reduced capacity to produce IFN-α [46]. These findings support the notion that a host cell IFN response does not contribute to the antiviral effect of these shRNAs.

In conclusion, RNA interference offers new opportunities to control and eliminate viral infections. In the present study we developed lentiviral vectors that express multiple shRNAs. To our knowledge, this is the first report of a triple shRNA expression vector being used to simultaneously downregulate independent targets involved in viral replication and viral binding. Integration of lentiviral vectors ensures long-term stable expression of RNAi-based therapy. These vectors could be used to transduce hepatocytes to attack existing HCV infections and to make cells resistant to infection by downregulating the viral-binding coreceptor CD81. The shRNAs were shown to be independently active, without clear evidence of cross-interference. Specifically, we showed that vectors can be used to target multiple regions of the HCV genome as well as the cellular coreceptor CD81. This simultaneous targeting theoretically reduces the risk of viruses developing RNAi resistance. Though these vectors are effective in reducing viral replication and infection, the use of multiple shRNAs could be effective in treating many multifactorial diseases, such as diabetes or cancer, in which several genes are involved in disease progression.

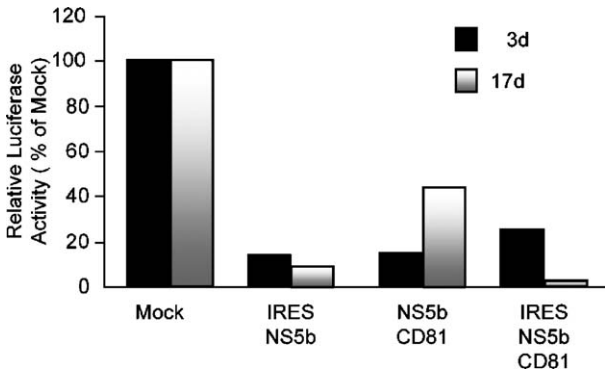

FIG. 5. Long-term downregulation of HCV replication by double and triple shRNA vectors. Double vectors were effective at reducing HCV replication at 3 days posttransduction. Long-term cultures showed that the double vector, containing a single HCV shRNA (NS5b-CD81), showed a decrease in effectiveness over the course of 17 days. Vectors containing both HCV shRNAs (IRES-NS5b and IRES-NS5b-CD81) continued to reduce HCV replication to 17 days (one of three representative experiments shown).

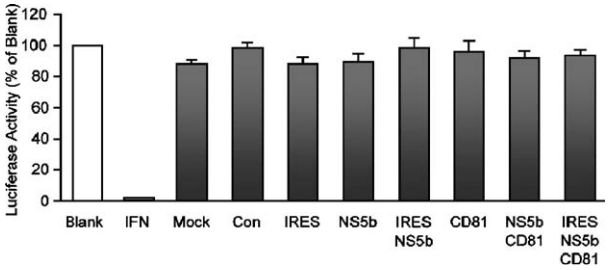

FIG. 6. No interferon production in response to shRNA. IFN bioassay was performed using the Huh-7 replicon cell line as a sensitive indicator of secreted IFN. Supernatants from Huh-7 replicon cells 3 days posttransduction had no effect on luciferase activity, indicating an absence of interferon response ($n = 3$). 10 IU/ml IFN-α completely inhibited HCV replication.

Methods and Materials

Cell culture. Cell monolayers of the human embryonic kidney epithelial cell line 293T and human hepatoma cell line Huh-7 [33] were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen–Gibco, Breda, The Netherlands) and complemented with 10% v/v fetal calf serum (PerBio Science), 100 IU/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (Invitrogen–Gibco) (cDMEM). Huh-7 cells containing a subgenomic HCV monocistronic replicon (I_{389}/NS3-3′/HygUbi/5.1) or bicistronic replicon (I_{389}/NS3-3′/LucUbiNeo-ET) [34] were maintained with 25 μg/ml hygromycin (Sigma, Zwijndrecht, The Netherlands) or 250 μg/ml G418 (Sigma), respectively.

shRNA annealing and plasmid construction. shRNA oligo sequences used were derived from various sources (Table 1). All were purchased as 60-nt ssDNA oligomers (Invitrogen–Gibco) composed of both forward and reverse sequences with 9-bp loop structures[35] and 3′ *Bgl*II and 5′ *Hin*dIII self-inactivating overhangs (Fig. 1A). Sense and antisense oligomers (both at 20μM) were incubated in annealing buffer (as previously described) [8] for 3 min at 90°C, then temperatures were lowered in 2°C/min increments until 5°C above their respective T_m and then dropped to 4°C at maximum ramp rates.

pSuper (Oligoengine, Seattle, WA, USA) was digested with *Pst*I and *Hin*dIII, and the fragment including the human H1 promoter and a portion of the multiple cloning site (MCS) was gel extracted and ligated into pSP72 (Promega, Leiden, The Netherlands). This new pSP72-H1 plasmid was digested with *Sma*I and *Hpa*I to remove the second *Bgl*II site and 83 bp of the newly inserted MCS, in preparation for later ligations. Annealed shRNA oligomers were inserted into pSP72-H1 digested with *Bgl*II and *Hin*dIII via unidirectional insertion (Fig. 1B). The H1-shRNA fragments were then removed with either *Pst*I/*Xho*I or *Sal*I/*Xho*I and cleaned via gel extraction. This study used the self-inactivating lentiviral transfer construct pND-CAG/GFP/WPRE, containing the composite CAG promoter (consisting of the cytomegalovirus immediate early enhancer, chicken β-actin promoter, and rabbit β-globin intron) that drives transcription of the enhanced GFP cDNA and woodchuck hepatitis virus posttranscriptional regulatory element (WPRE). The HIV-1 central polypurine tract was reintroduced to streamline nuclear import of the

preintegration complex as well as to ensure efficient infection of hepatocytes [36]. The HI-shRNA cassettes were ligated into the *Xho*I site of pND-CAG/GFP/WPRE in opposition to the CAG reading direction (Fig. 1C). New shRNA plasmids were checked for insertion and direction with restriction analysis using *Nde*I/*Xho*I. The resulting plasmids left one downstream *Xho*I site available for multiple cassette insertions. Plasmids containing double and triple shRNA cassettes were created by repeating this process for each additional shRNA cassette (Fig. 1D). Final pND plasmid constructions included singles, mock (no shRNA GFP lentivector), Con (scrambled HBV shRNA), IRES, NS5b, CD81; doubles, IRES-NS5b, IRES-CD81, NS5b-CD81; and triple IRES-NS5b-CD81. These plasmids each express a GFP reporter gene as well as all of the inserted self-expressing shRNA cassettes, each under the control of its own H1 promoter (Fig. 1E). All plasmid constructs were sequenced (Baseclear, Leiden, The Netherlands) in the region of the shRNA cassettes to ensure direction and that no mutations occurred during cloning steps.

Lentiviral production. A third-generation lentiviral packaging system originally described by Dull *et al.* [22] in combination with pND-CAG/GFP/WPRE containing single, double, or triple shRNA expression cassettes was used to produce high-titer VSV-G-pseudotyped shRNA-GFP expression vectors, as previously described [37]. Briefly, 293T cells were grown in cDMEM to 80% confluency in 500-cm^2 flasks and transfected with pND, VSV-G, GagPol, and Rev plasmids in a 4:1:2:1 ratio using 3:1 (264 μg) polyethylamine in serum free DMEM, which was replaced with cDMEM after 6 h. Vector supernatants were removed 36 and 48 h posttransfection, passed through a 0.45-μm filter, and concentrated 1000-fold by ultracentrifugation. Concentrated virus stocks were titrated using 293T cells 24 h after infection, with transduction efficiency based on the number of GFP-positive cells as determined by flow cytometry (FACSCalibur; BD BioSciences, Mountain View, CA, USA). Vector titers ranged from 7×10^6 to 3×10^7 transducing units per milliliter.

Real-time RT-PCR. Confluent monolayers of replicon cells were lysed by Trizol (Invitrogen–Gibco) and RNA was precipitated with 75% EtOH and captured with a Micro RNeasy silica column (Qiagen, Venlo, The Netherlands). RNA was quantitated using a Nanodrop ND-1000 (Wilmington, DE, USA) and adjusted to 1 μg for each sample. cDNA was prepared using Promega's AMV reverse transcriptase following standard protocols. Real-time PCR (MJ Research Opticon, Hercules, CA, USA) was performed using primers NS3 (F, 5′-GGTTCTGTGCGAGTGCTATG-3′, R, 5′-TCTCCTGCCTGCTTAGTCTG-3′), CD81 (F, 5′-AGTGGAGGGCTG-CACCAAGT-3′, R, 5′-GTGAGCGGGTCTCTGAGTCGAA-3′), and GAPDH (F, 5′-CCATGGAGACTGGGG-3′, R, 5′-CAAAGTTGTCATGGATGACC-3′). Semiquantitation of sample amplicons was performed with Sybr-Green (Sigma) using *Taq* DNA polymerase (Invitrogen–Gibco) as previously described [38].

Luciferase assay. Luciferase activity in cells was quantified using a Top Count scintillation/luminescence counter (Packard BioSciences Top Count-NXT, Milan, Italy). Cells were lysed and luciferin substrate was added using the Steady Glo Luciferase System (Promega) according to the manufacturer's protocols.

Flow cytometry and immunocytochemistry. Surface expression of CD81 and Ber-EP4 was determined via FACS after incubation with antibody conjugated to each, mouse α-human CD81 monoclonal IgG1 (BD Pharmingen, San Diego, CA, USA) followed by a fluorescently labeled rat α-mouse r-phycoerythrin (PE) (BD Pharmingen) and mouse α-human Ber-EP4/FITC (Dako Cytomation, Fort Collins, CO, USA) using standard immunofluorescence staining techniques. Mouse monoclonal IgG1 (Dako Cytomation) was used for negative isotype controls.

Cells for immunocytochemistry were fixed in 2% paraformaldehyde and labeled with antibodies conjugated to HCV NS3 (Novo Castra, New Castle, UK) and CD81 (BD Biosciences). Secondary antibody binding and amplification of signal was accomplished with EnVision horseradish peroxidase and visualized with 3′-aminoethylcarbozole (Sigma). Images were created on a Zeiss Axioskop microscope (Sliedrecht, The Netherlands) fitted with Nikon's (USA) Digital Sight DS-U1 imaging system. Images were captured and formatted using Eclipse Net digital software.

E2 binding assay. To determine whether CD81 downregulation prevents binding of HCV E2, an HCV E2 binding assay was performed [5,39]. Huh-7 cells were transduced with CD81 shRNA-containing vectors (CD81, NS5b-CD81, and IRES-NS5b-CD81) or control vector (Con). At 7 days, transduced Huh-7 cells were trypsinized and incubated with recombinant E2 protein (Austral Biologicals, San Ramon, CA, USA) at a concentration of 8 μg/ml for 30 min at 4°C in PBS/0.1% BSA. Cells were washed and incubated with 1 μg/ml E2 detection antibody HuMAb108 (Genmab, Utrecht, The Netherlands) for 45 min at 4°C. Cells were washed and incubated with a donkey anti-human PE (BD Pharmingen)-labeled secondary antibody, and binding was detected by flow cytometry.

IFN bioassay. To determine the IFN response to shRNA, HygUbi replicon cells were transduced with shRNA constructs and incubated for 72 h. One hundred microliters of supernatant was harvested and added to LucUbi-Neo replicon cells. After 24 h incubation, luciferase activity was determined as described above. IFN levels were calculated using the method described by Vrolijk *et al.* [40]. IFN-α (10 IU/ml (40 pg/ml)) was used as a positive control.

Statistical analysis. Statistical analysis was performed using a non-parametric ANOVA with Dunn multiple comparison posttest using GraphPad InStat software.

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Chapter V

Hydroxyethyl Starch–Based Preservation Solutions Enhance Gene Therapy Vector Delivery Under Hypothermic Conditions

Liver transplantation, 2008, 14: 1708-1717

Hydroxyethyl Starch–Based Preservation Solutions Enhance Gene Therapy Vector Delivery Under Hypothermic Conditions

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Isolated liver perfusion offers a unique prospect for safe, effective targeting of gene therapies that can be directed against allograft rejection or recurrent diseases such as reinfection by hepatitis C virus (HCV). We aimed to examine the effect of organ preservation solutions on vector-based gene therapy delivery under hypothermic conditions. University of Wisconsin (UW) solution, histidine tryptophan ketoglutarate (HTK), EloHaes, sodium-poly(ethylene glycol)-UW solution [Institut Georges Lopez 1 solution (IGL-1)], and Dulbecco's modified Eagle's medium (DMEM) culture medium (control) were tested at 2°C or 37°C for lentiviral vector transduction efficiencies to the hepatoma cell line Huh-7 and primary human or mouse hepatocytes. Lentiviral vectors expressing short hairpin RNA were used to target HCV replication. With a potent short hairpin RNA vector, transductions were directly correlated to the therapeutic effect, with low transduction yielding low knockdown and vice versa. Green fluorescent protein (GFP) reporter gene expression was observed with vector incubation times as short as 10 minutes. The highest transductions were seen, after 2-hour 37°C incubation, in UW (62% ± 6 SEM); they were significantly higher than those in HTK (21% ± 7 SEM). Neither adenosine nor glutathione, present in UW, provided any increase in transduction when supplemented to HTK, although the addition of hydroxyethyl starch (HES) significantly improved transductions. To rule out size exclusion as a mechanism of HES, IGL-1 was tested but did not result in better transductions than HTK or DMEM. When supplemented to UW, anionic compounds reduced transduction, and this indicated a charge interaction mechanism of HES. In conclusion, this study demonstrates that effective vector delivery can be achieved under conditions of hypothermic liver perfusion. UW provides superior transduction to hepatocytes over nonstarch solutions. ***Liver Transpl 14:1708-1717, 2008.*** © **2008 AASLD.**

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To date, various gene therapies have been successful in vitro and in small clinical studies. Several studies have shown that gene therapies can be applied to protect a graft against acute rejection[1-3] or recurrent diseases such as hepatitis C virus (HCV).[4] However, the current inability to narrow the tropism of viral vectors as well as the safety concerns regarding the infection of patients with viral vectors has hampered the progress of new studies. Genetic therapy treatments for hematopoietic disorders are now being performed ex vivo, with the target cells being mobilized, treated in culture, and reimplanted.[5] This represents a vast improvement for specific targeting with vectors that have high multiple tropisms. The liver or specific cell populations can be removed and treated without worry of nontarget transductions to areas deemed sensitive or dangerous to vector deliveries.

During the process of liver transplantation, the donor undergoes multiple perfusions to clear the liver of

Abbreviations: 7AAD, 7-amino-actinomycin D; Aden, adenosine; cDMEM, complete Dulbecco's modified Eagle's medium; DMEM, Dulbecco's modified Eagle's medium; EH, EloHaes; GFP, green fluorescent protein; Glut, glutathione; HCV, hepatitis C virus; HES, hydroxyethyl starch; HTK, histidine tryptophan ketoglutarate; IGL-1, Institut Georges Lopez 1 solution; LV-GFP, lentiviral vector/green fluorescent protein; MFI, mean fluorescence intensity; MOI, multiplicity of infection; PBS, phosphate-buffered saline; RNAi, RNA interference; UW, University of Wisconsin solution; VSV-G, vesicular stomatitis virus glycoprotein.

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blood, preserve it, and prepare it for transplant. Hypothermic liver perfusion is most commonly performed with University of Wisconsin solution (UW) or histidine tryptophan ketoglutarate (HTK) preservation solution.[6] After the liver is recovered from the donor within the minimum warm ischemic time (<30 minutes), a cold perfusion is performed to clear the vasculature and cool the liver for storage. The graft is then placed on ice and is potentially ready for up to 12 to 18 hours for allocation to the receiving hospital. Generally, a second perfusion is performed with cold human albumin at the receiving hospital, in part to remove transportation outflow and also to dilute the high potassium levels in some perfusion solutions. This time between donor and patient opens a window of opportunity in which pretransplant gene therapies can be delivered to the donor graft safely and efficiently with minimal perturbance in the existing protocols for liver transplantation.

Within experimental settings, third-generation lentiviral vectors can achieve high transductions with wide tropisms because of the incorporation of the vesicular stomatitis virus glycoproteins (VSV-Gs) in their envelope. These VSV-G–pseudotyped lentivectors enter the cell through receptor-mediated endocytosis followed by low pH–induced membrane fusion in the endosome.[7,8] Although the molecules involved in VSV-G binding are largely unknown, previous work has suggested that a specific receptor as well as an interaction between phosphatidylserine residues on the membrane surface is essential for VSV-G–membrane interaction.[9,10] We have previously shown the development of a VSV-G–pseudotyped lentivirus capable of down-regulating multiple HCV or HCV-related targets simultaneously.[4] Lentiviral-directed transduction of RNA interference (RNAi) has been proven an effective method to silence HCV gene expression.[4,11-13] Through the delivery of therapy during perfusion, before transplantation, one can impart RNAi-based protection to the new graft before it enters an HCV-infected patient, thus reducing or even eliminating the chance of recurrence. It is currently unknown whether these vectors are capable of transduction within the hypothermic conditions of liver perfusion and storage, so the transduction potentials of the pretransplant environment will need to be determined.

The aim of this study was to determine the effect of organ preservation solutions on the transduction efficiency of lentiviral vectors at temperatures used for liver preservation and perfusion. We show that lentiviral binding and subsequent transduction to hepatocytes can occur under normothermic and hypothermic conditions in vitro. It has been found that the type of perfusion solution used makes a distinct impact on the levels of transduction.

MATERIALS AND METHODS

Cell Culture

Cell monolayers of the human embryonic kidney epithelial cell line HEK 293T and human hepatoma cell line Huh-7[14] and Huh-7 endothelial cells containing a subgenomic HCV replicon (I_{389}/NS3-3′/LucUbiNeo-ET)[15] were maintained in Dulbecco's modified Eagle's medium (Invitrogen-Gibco, Breda, The Netherlands) and complemented with 10% vol/vol fetal calf serum (PerBio Science), 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2mM L-glutamine [complete Dulbecco's modified Eagle's medium (cDMEM); Invitrogen-Gibco]. Huh-7 cells were maintained under 250 μg/mL G418 (Sigma, Zwijndrecht, The Netherlands) selection. Cryofrozen primary neonatal mouse hepatocytes (a kind gift from Suzanne van de Nobelen, Erasmus MC, Rotterdam, The Netherlands) were plated at near confluence and maintained in Williams' E medium with Glutamax (Gibco) supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin, 10 nM dexamethasone (Sigma), 1 mM insulin (Sigma), and 10% vol/vol fetal calf serum (PerBio Science). Fresh primary human hepatocytes (Lonza, Verviers, Belgium) were maintained in a hepatocyte maintenance medium with Ultra-Glutamax (Clontetics, Verviers, Belgium) and supplemented with 10 μM insulin, 10 μM dexamethasone, 50 mg/mL gentamicin, and 50 ng/mL amphotericin supplied as mini-quot additives (Lonza).

Reagents

Cells were transduced with green fluorescent protein (GFP) expressing lentiviral vectors (LV-GFP) in various solutions to determine the optimal solution for hypothermic transduction: UW (ViaSpan, Barr Laboratories, Pomona, NY), HTK (Pharmapal, Athens, Greece), Elo-Haes (EH; Fresenius Kabi, Bad Homburg, Germany), and sodium-poly(ethylene glycol)-UW solution [Institut Georges Lopez 1 solution (IGL-1); Laboratoire Cair LGL, Madrid, Spain; a kind gift from Professor R. Ploeg, University of Groningen, Groningen, The Netherlands]. Various components contained within UW were added to HTK to investigate their individual influences on lentiviral transduction. HTK was supplemented with various concentrations of Viastarch [250-kDa hydroxyethyl starch (HES); a kind gift from Professor R. Ploeg, University of Groningen, Groningen, The Netherlands], 3 mM glutathione (Sigma), or 5 mM adenosine (Sigma), and transduction efficiencies were determined. UW or HTK was also supplemented with anionic polymers, 100 μg/mL dextran sulfate (Sigma, or 20 mM heparin (LeoPharma, Breda, Netherlands), to determine the dependence of charge interactions in lentiviral transduction.

Lentiviral Production and Transduction

A third-generation lentiviral packaging system, originally described by Dull et al.,[14] in combination with viral backbone plasmid containing the chicken albumin promoter, GFP expression cassette and the woodchuck hepatitis virus post-transcriptional regulatory element (pND-CAG/GFP/WPRE) was used to produce a high-titer VSV-G–pseudotyped lentivirus expression GFP reporter construct (LV-GFP) or both GFP and short hair-

pin RNA targeting the HCV NS5b (LV-shNS5b), as previously described.[4,15]

To test the effect of LV-shNS5b vectors on HCV replication, Huh-7 replicon cells were plated in cDMEM at 70% to 80% confluence. After a period of 3 to 4 hours, LV-shNS5b, at increasing multiplicity of infection (MOI), was added and incubated under normothermic conditions for 120 minutes. The cells were washed with warm phosphate-buffered saline (PBS), fresh complementary DNA was added, and the cells were returned to 37°C and cultured for 48 hours. Luciferin substrate was added, and counts per minute were measured with a scintillation counter.

To determine the effectiveness of perfusions solutions to act as transduction agents, Huh-7 cells were plated in cDMEM at 70% to 80% confluence. After a period of 3 to 4 hours, the medium was removed, and cells were incubated in a variety of solutions: UW, HTK, EH, and cDMEM. Cells, in all solutions, were incubated under either hypothermic (2°C) or normothermic (37°C) conditions and transduced with LV-GFP at matched MOI for 10, 30, 60, or 120 minutes. After incubation with LV-GFP, the solutions were removed, and the cells were washed with warm PBS and replaced by cDMEM. The cells were then returned to 37°C and cultured for 48 hours. Cells were harvested via trypsin/ethylene diamine tetraacetic acid digestion.

All transduction efficiencies (percentage of GFP-positive cells and geometric mean fluorescence intensity) were determined by flow cytometry (FACSCalibur, BD BioSciences, Mountain View, CA).

Luciferase Assay

Luciferase activity in Huh-7 cells was quantified with a Top Count scintillation/luminescence counter (Top Count-NXT, Packard BioSciences, Milan, Italy). Cells were lysed, and luciferin substrate was added with the Steady Glo luciferase system (Promega) according to the manufacturer's protocols.

Cell Viability and Proliferation

Cell viability was determined by 7-amino-actinomycin D (7AAD; BD BioSciences) staining followed by flow cytometry analysis (data not shown). 7AAD-negative cells were considered viable, whereas late-apoptotic and necrotic cells were positive for 7AAD.[16] Cell quantification was assessed with Giemsa (Merck, Darmstadt, Germany) total protein staining.[17] Adherent cells were fixed with methanol, and this was followed by 20 minutes of staining with a 40% Giemsa solution. After 5 washes with PBS, the cell-bound Giemsa was resolved in methanol and quantified with a spectrophotometer at 655 nm with a model 680 microplater reader (Bio-Rad, Hercules, CA). The cell count per well is directly related to the intensity of the optical density; as such, the expansion of cultures can be measured by the comparison of optical densities of treated cells and cells fixed at time zero (before transduction).

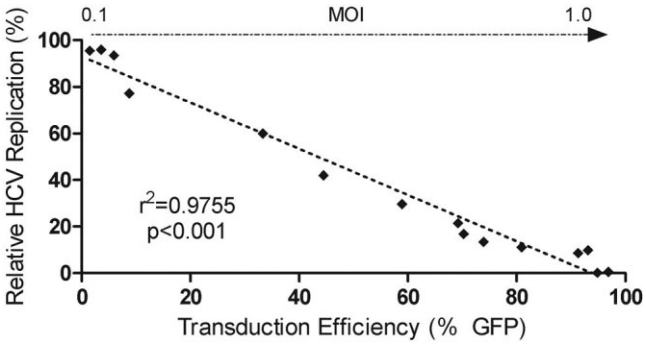

Figure 1. Increasing MOI increases transduction efficiency and HCV inhibition. Huh-7 replicon cells were treated with shNS5b-containing lentivectors through a range of MOIs for 2 hours. Cells were washed and incubated for 48 hours to allow for integration and expression. Luciferase (HCV replication) and GFP (transduction efficiency) contents were measured, and a correlation was drawn. Increasing vector input increased the transduction of hepatocytes and correlated inversely to the ability of the shNS5b to knockdown HCV (r^2 = 0.9755, $P < 0.001$). Abbreviations: GFP, green fluorescent protein; HCV, hepatitis C virus; MOI, multiplicity of infection.

Statistical Analysis

Statistical analysis was performed with a nonparametric matched pair test (Wilcoxon) or unpaired *t* test for comparing individual conditions or 1-way analysis of variance with the Tukey multiple comparison post test for comparing across time points with GraphPad Prism 4.0 software.

RESULTS

Vector Concentration Is Proportional to Transduction and to Inhibition of HCV Replication

The success of any viral vector–based gene therapy is limited by the effectiveness of the treatment itself and perhaps more importantly by the number of cells that can be reached during administration. Thus, even with a very effective RNAi vector, such as LV-shNS5b, therapeutic success is limited to the level of transduction that can be achieved to targeted cells. To determine a correlation between the level of transduction and the effectiveness of LV-shNS5b, Huh-7 replicon cells were treated with increasing MOI of LV-shNS5b, and both GFP and HCV contents were determined.

The correlation is shown in Fig. 1: increasing the vector MOI increases transduction, which, in turn decreases the level of HCV content of the replicon cells (r^2 = 0.9755, $P < 0.001$) by delivering more RNAi to more cells.

Lentiviral Transduction Is Achieved in Hypothermic or Normothermic Perfusion Solutions

In order to determine if lentiviral vectors could be delivered to hepatocytes under the conditions of cold liver

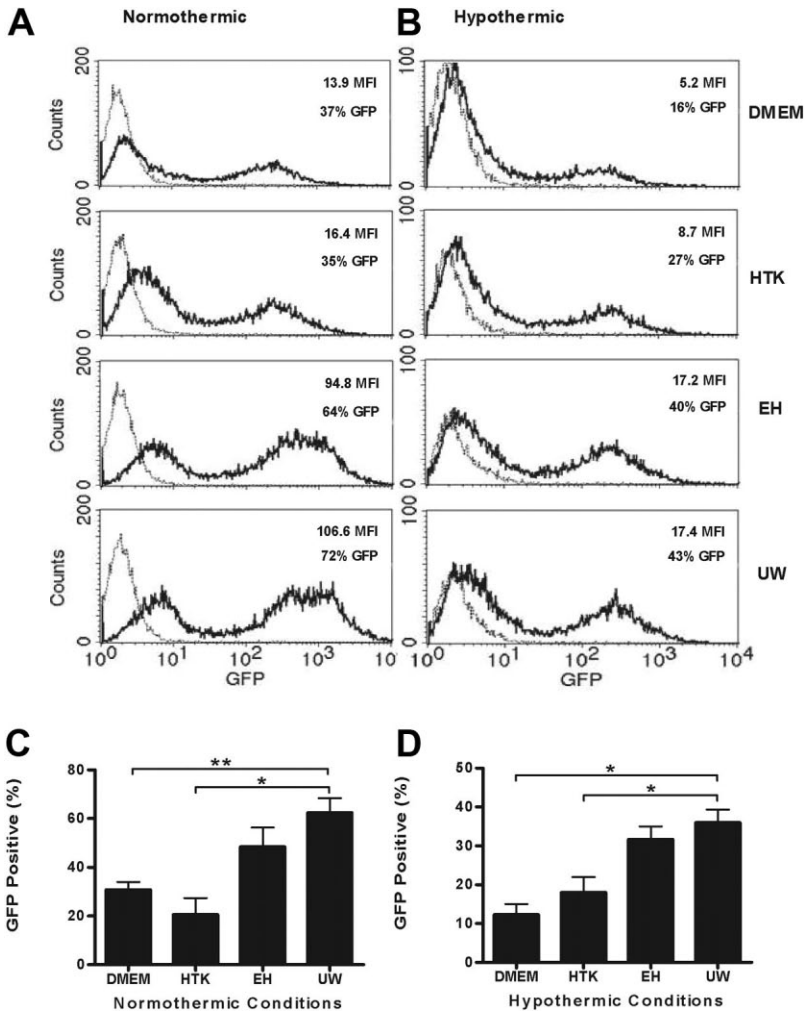

Figure 2. UW promotes lentiviral transduction under normothermic and hypothermic conditions. GFP-positive Huh-7 cell percentages and geometric MFIs were measured 24 hours after 2-hour lentiviral incubation in either (A) normothermic (37°C) or (B) hypothermic (2°C) solutions (cDMEM, HTK, UW, and EH). UW gave higher transduction than other solutions (72% GFP, normothermic, and 43% GFP, hypothermic). One representative fluorescence-activated cell sorting plot is shown of 5 to 8 independent experiments. The number of GFP-positive cells was significantly greater than the number in cDMEM or HTK under both (C) normothermic (n = 5) and (D) hypothermic (n = 5) conditions (*$P < 0.05$, **$P < 0.01$). Abbreviations: cDMEM, complete Dulbecco's modified Eagle's medium; DMEM, Dulbecco's modified Eagle's medium; EH, EloHaes; GFP, green fluorescent protein; HTK, histidine tryptophan ketoglutarate; MFI, mean florescence intensity; UW, University of Wisconsin.

perfusion and storage, Huh-7 hepatoma cells were incubated in different preservation solutions containing submaximal MOI of LV-GFP under either hypothermic (2°C) or normothermic (37°C) conditions.

Figure 2 shows a representative fluorescence-activated cell sorting profile of the 4 conditions under normothermic conditions (Fig. 2A) and hypothermic (Fig. 2B) conditions. The use of normothermic UW (62% ± 6 SEM) showed a significantly increased transduction percentage over that of both HTK (21% ± 7 SEM; $P = 0.03$) and cDMEM (31% ± 3 SEM; $P = 0.007$; Fig. 2C). Under hypothermic conditions (Fig. 2D), the best transductions were seen with UW (36% ± 3 SEM), which showed significantly improved transduction over that of HTK (18% ± 3.9 SEM; $P = 0.03$) or cDMEM (12% ± 3 SEM; $P = 0.03$).

Kinetics of Hypothermic and Normothermic Transduction

To determine the time dependence of transduction in perfusion solutions, subconfluent Huh-7 cells were incubated for different times with LV-GFP under normothermic (Fig. 3A) or hypothermic (Fig. 3B) conditions.

Under hypothermic conditions, GFP expression could be observed 48 hours afterwards with LV-GFP incubation times as short as 10 minutes and, as expected, increased with longer (2-hour) time periods. The transduction levels appeared to reach a maximum at 1-hour vector exposure and dropped, though not significantly, at 2 hours with HTK. Similar trends were found under normothermic conditions. UW provided a significantly better LV-GFP transduction environment than cDMEM or HTK solutions in both hypothermic ($P < 0.01$ and $P < 0.05$, respectively) and normothermic ($P < 0.05$ and $P < 0.05$, respectively) environments across all time points.

Cell Viability and Growth

High tissue survival is one of the main aims of pretransplant graft perfusion, and as such, the protocols used are optimized to ensure maximum tissue viability postreperfusion. We determined whether cells would remain viable under hypothermic and normothermic conditions during the longest lentiviral incubation time point (120 minutes). Overall, cells remained vital (data not shown), and as shown in Fig. 4, cells continued to proliferate after both normothermic (Fig. 4A) and hypothermic (Fig. 4B) transduction incubations.

Interestingly, after hypothermic conditions, cells in-

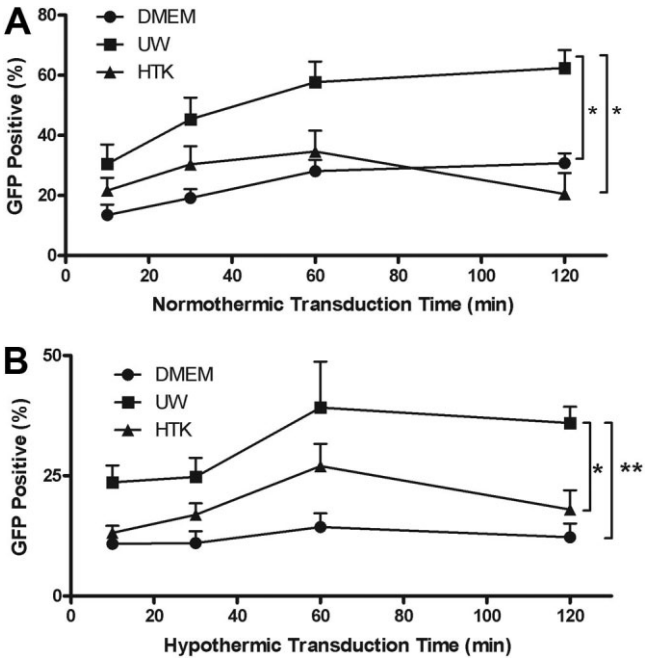

Figure 3. Transduction increases with incubation vector exposure time. Huh-7 cells were incubated with matched-input MOI of LV-GFP and incubated in normothermic (37°C) or hypothermic (2°C) solutions for a range of times (10 minutes to 2 hours). GFP-positive cells were measured via flow cytometry 48 hours post–vector incubation. GFP-positive cells increased with the vector incubation time. (A) Under normothermic conditions (n = 6), UW gave significantly higher transductions than HTK or cDMEM at all time points. (B) Under hypothermic conditions (n = 6), UW gave significantly higher transductions than HTK or cDMEM. A transduction plateau was observed at 1 hour with all solutions, although HTK transductions were reduced with incubation longer than 1 hour (*P = 0.05, **P = 0.01). Abbreviations: cDMEM, complete Dulbecco's modified Eagle's medium; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; HTK, histidine tryptophan ketoglutarate; LV-GFP, lentiviral vector/green fluorescent protein; MOI, multiplicity of infection; UW, University of Wisconsin.

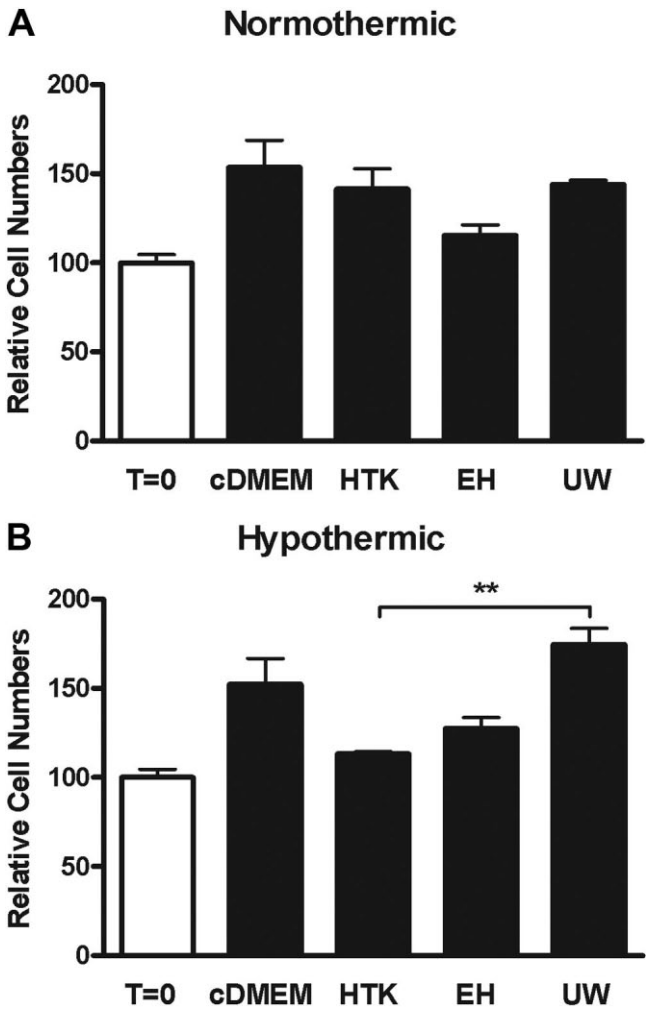

Figure 4. UW does not affect cell proliferation after normothermic or hypothermic incubations. Giemsa total protein stain was used to determine the total protein content, an indicator of cellular growth. Giemsa staining was determined at 0 and 24 hours post-incubation. (A) Under normothermic conditions, cell growth was not significantly different across all solutions (n = 3), but (B) after hypothermic incubation, cells in UW had increased in numbers significantly more than those that were incubated in HTK (n = 3; **P = 0.01). Abbreviations: cDMEM, complete Dulbecco's modified Eagle's medium; EH, EloHaes; HTK, histidine tryptophan ketoglutarate; UW, University of Wisconsin.

cubated in UW (75% ± 9 SEM increase over Time = 0) showed more proliferation than those in HTK (13% ± 1 SEM increase over Time = 0; P = 0.016), possibly in part because of the colloid (HES) and superior impermeant (lactone) components of UW.

HES Is Responsible for Superior Transduction Efficiency by UW

Although UW and EH gave similar transductions, these solutions differ in their component makeup as detailed in Table 1. As UW is used in the clinic as a perfusion and a preservation solution, the solution is much more complex than EH, which is used mainly as a vascular volume expander during cardiovascular surgery. Though used for different purposes, these 2 solutions contain HES, with UW containing 5% Viastarch (250-kDa HES pentafraction) and EH containing 6% Viastarch (200-kDa HES pentafraction; Table 1).

HTK and UW are similar in that the solutions have comparable concentrations of cations and anions; however, HTK contains no starch but instead contains mannitol to reduce hypothermic swelling. In addition, UW contains numerous active agents to replenish energy and scavenge oxygen radicals (Table 1), which are not found in HTK or EH. To determine whether any or all of these component differences could account for the increase in transduction efficiency, HTK was supplemented with UW-comparable concentrations of adenosine, glutathione, or HES. After a 2-hour normothermic LV-GFP incubation and a nominal period of 48 hours post-transduction, flow cytometry analysis for GFP-positive cells was performed. As shown in Fig. 5, no increased transduction was observed after HTK was

TABLE 1. Annotated Components

UW	
Poly(2-0 hydroxethyl) starch (250 kDa)	Colloid: reduction of interstitial edema and endothelial cell swelling
Lactobionic acid (as lactone)	Impermeant: suppression of hypothermic cell swelling
Potassium phosphate monobasic	pH buffer: maintenance of intracellular sodium and potassium concentrations, restoration of high-energy phosphate
Magnesium sulfate heptahydrate	Preservation of intracellular magnesium concentration
Raffinose pentahydrate	Impermeant suppression of hypothermic cell swelling
Adenosine	Restoration of high-energy phosphate
Allopurinol	Inhibition of xanthine oxidase activity and purine metabolism/reduction of oxygen free radicals
Total glutathione	Antioxidant
Potassium hydroxide	Preservation of intracellular potassium concentration
H_2O	
pH 7.4 (37°C)	
Osmolaity:320 mosmol/kg	
HTK	
Sodium chloride	Isotonic balance
Potassium chloride	Preservation of intracellular potassium concentration
Potassium-hydrogen-2-oxoglutarate	Substrate for aerobic energy production
Magnesium chloride–$6H_2O$	Preservation of intracellular magnesium concentration
Calcium chloride–$2H_2O$	Isotonic balance
Histidine HCL–H_2O	pH buffer: extends capacity
Histidine	pH buffer: extends capacity
Tryptophan	Membrane integrity
Mannitol	Sugar alchohol: impermeant: suppression of hypothermic cell swelling, maintainence of intracellular glucose levels
H_2O	
pH 7.4 (37°C)	
Osmolality: 310 mosmol/kg	
EH	
Poly(2-0 hydroxethyl) starch (200 kDa)	Colloid: reduction of interstitial edema and endothelial cell swelling
Sodium chloride	Isotonic balance
Sodium hydroxide	pH control
Hydrochloric acid	pH control
H_2O	
pH 3.5–6.0 (37°C)	
Osmolality: 308 mosmol/kg	

Abbreviations: EH, EloHaes; HTK, histidine tryptophan ketoglutarate; UW, University of Wisconsin.

supplemented with glutathione or adenosine, and this indicated that neither of these components was responsible for the additional transduction potential of UW.

However, when HTK was supplemented with increasing concentrations of Viastarch, significant improvement in transductions, nearing that of UW alone (Fig. 5), was observed. This indicated that HES is the most important component responsible for the increased transduction potential of UW.

Enhanced Transduction by HES Is Due to Charge Interaction and Not Size Exclusion

The presence of Viastarch in UW helps to minimize the free liquid while maintaining osmotic pressure, allowing a more concentrated ion flow during perfusion. It could be postulated that the greater transduction potential of UW could be in part due to the nature of HES, which excludes total free liquid, thereby concentrating the initial input of the vector. To test this, UW was compared to machine perfusion solution IGL-1, a solution very similar to UW, with the main exception that HES is replaced by poly(ethylene glycol) (PEG 35) and sodium replaces potassium (see Maathuis et al.[6] for detailed differences). As shown in Fig. 6A, transductions in IGL-1 (25% ± 3 SEM) were not significantly different than those in HTK (19% ± 4 SEM) but were significantly lower than the transduction achieved with UW solution (48% ± 4 SEM, $P < 0.009$).

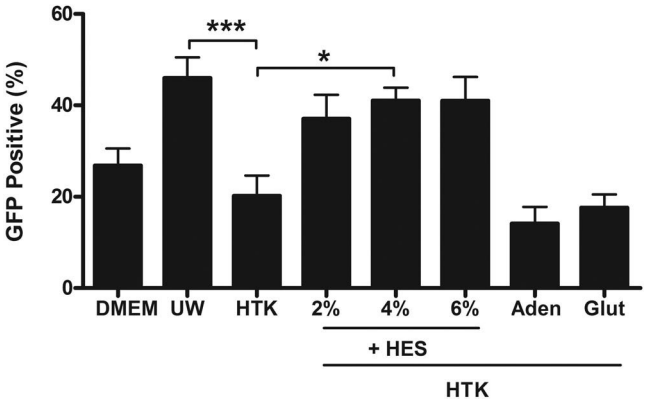

Figure 5. Increased transduction was dependant on the presence of HES but not Aden or Glut. Huh-7 cells were incubated for 2 hours in normothermic perfusion solutions with LV-GFP. Transduction was determined in HTK supplemented with additional components found in UW (Aden, Glut, and HES). The addition of Aden (n = 5) or Glut (n = 3) did not significantly increase transduction of LV-GFP over that of HTK alone (n = 12). When HTK was supplemented with HES (n = 3-8) at percentages lower and higher than that of UW (5% HES), a general increase was observed, with the 4% HES/HTK solution showing a significant increase in transduction over that of HTK alone (*P = 0.05). Abbreviations: Aden, adenosine; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; Glut, glutathione; HES, hydroxyethyl starch; HTK, histidine tryptophan ketoglutarate; LV-GFP, lentiviral vector/green fluorescent protein; UW, University of Wisconsin.

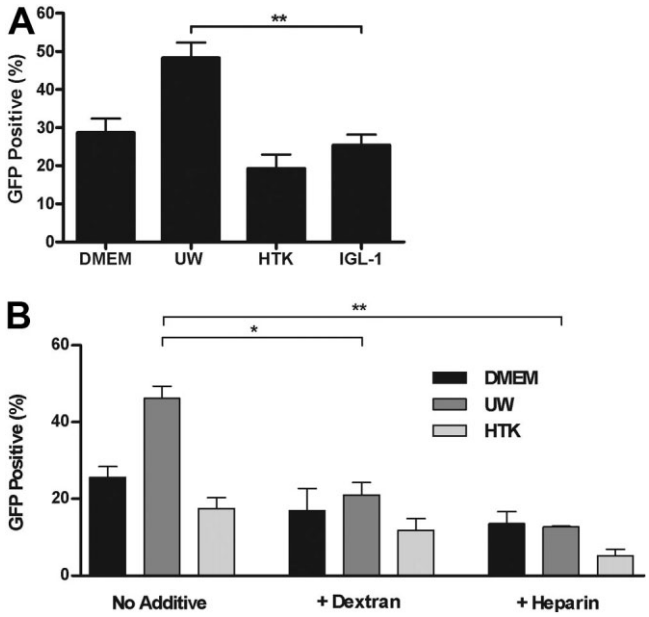

Figure 6. Increased transduction in UW is due to a charge interaction and not size exclusion concentration of the vector. Huh-7 cells were incubated for 2 hours in normothermic perfusion solutions with LV-GFP; transductions were measured by flow cytometry 48 hours post-incubation and then compared to transductions in the machine perfusion solution IGL-1. (A) Transduction percentages were nonsignificantly increased in IGL-1 (n = 5) over that of HTK but remained significantly lower than the transductions observed in UW. (B) Huh-7 cells were incubated in normothermic solutions (complete DMEM, HTK, or UW) supplemented with anionic polymers (dextran sulfate or heparin sulfate) and LV-GFP at matched MOI for 2 hours. Transduction was significantly reduced with both dextran sulfate (n = 6) and heparin (n = 3) supplementation to UW in comparison with UW alone (*P = 0.05, **P = 0.01). Abbreviations: DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; HTK, histidine tryptophan ketoglutarate; IGL-1, Institut Georges Lopez 1 solution; LV-GFP, lentiviral vector/green fluorescent protein; MOI, multiplicity of infection; UW, University of Wisconsin.

Charged polymers alter the kinetics of viral adsorption and thereby modulate the transduction efficiency independent of their tropism. To determine if an HES/vector charge-dependent interaction was present in our setting, cDMEM, HTK, or UW was supplemented with anionic polymers before the addition of matched MOI LV-GFP, and then the level of transduction was determined. Figure 6B shows the effects of the addition of heparin or dextran sulfate on the transduction potentials. Both heparin (13% ± 0.1 SEM) and dextran sulfate (21% ± 3 SEM, $P < 0.009$) supplementation to UW significantly ($P = 0.07$ and $P = 0.01$, respectively) reduced the percentage of GFP-positive cells in comparison with UW alone (46% ± 3 SEM), whereas their addition to cDMEM or HTK resulted in nonsignificant reductions.

UW Enhances Transduction in Primary Hepatocytes

Although high transductions were achieved in a hepatoma cell line, these cells may not be representative of primary hepatocytes. To show the effectiveness of UW in delivering lentiviral vectors to primary hepatocytes, mouse and human hepatocytes were subjected to the same perfusion conditions as the hepatoma cell lines and transduced with LV-GFP for 2 hours. In primary mouse hepatocytes, significantly higher relative transductions were achieved in UW (26.81% ± 1.5 SEM) than in HTK (15.4% ± 0.7 SEM; $P = 0.0002$, n = 5) with a relatively low dose of 3 MOI (Fig. 7A). Primary human hepatocytes were transduced for 2 hours under conditions similar to those used for the primary mouse hepatocytes, although we increased the MOI range from 30 to 50 and supplemented HTK with 5% HES. HTK alone gave similar transductions (24.6% ± 6 SEM at an MOI of 50), as seen in the primary mouse hepatocytes, although when it was supplemented with 5% HES, transductions improved (37% ± 11 SEM at an MOI of 50). UW again gave the highest transductions (46% ± 21 SEM), although the significance between solutions was not tested because of low experimental numbers (n = 2). Although transductions to primary hepatocytes were lower than the results obtained with hepatoma cell lines, it was clear that transductions were possible and were notably increased with the use of UW or the addition of 5% HES.

DISCUSSION

In the current study, we have demonstrated that transduction is inversely correlated to the effectiveness of an

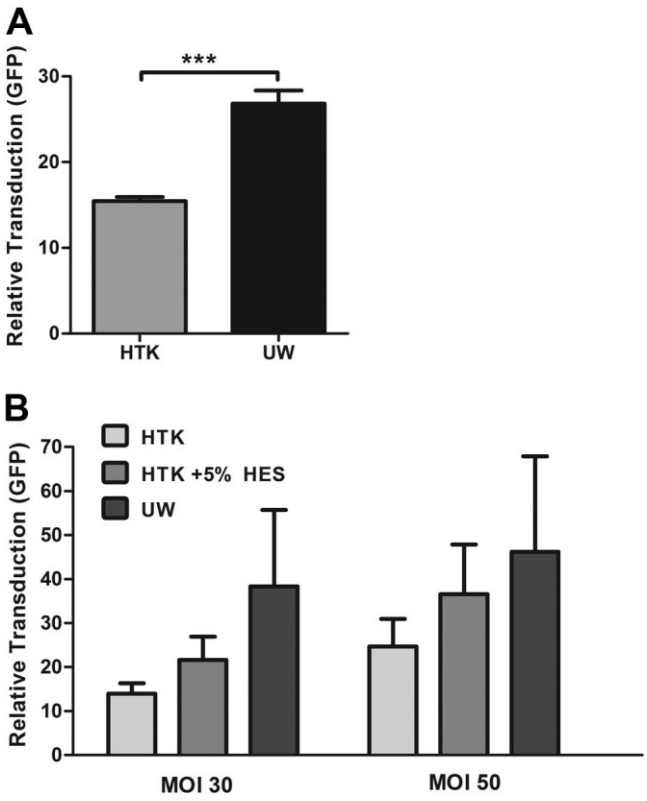

Figure 7. UW enhances transduction in primary hepatocytes. Primary hepatocytes were incubated for 2 hours in normothermic perfusion solutions with LV-GFP. (A) UW significantly increased the transduction of primary mouse hepatocytes over that of HTK (26.81% ± 1.5 SEM and 15.4% ± 0.7 SEM, respectively; *P* = 0.0002, n = 5). (B) In human primary hepatocytes, a similar trend of results was obtained when transductions were performed in normothermic solutions for 2 hours. With the addition of 5% HES to HTK, transductions increased to levels near those obtained with UW alone (n = 2). Abbreviations: GFP, green fluorescent protein; HES, hydroxyethyl starch; HTK, histidine tryptophan ketoglutarate; LV-GFP, lentiviral vector/green fluorescent protein; MOI, multiplicity of infection; UW, University of Wisconsin.

RNAi therapy (Fig. 1) and that UW perfusion solution creates a superior transduction environment, improving the transduction of hepatocytes in vitro, by lentiviral vectors. UW provided a much better environment that facilitated a greater number of transduced cells under both normothermic and hypothermic conditions (Fig. 2). Therefore, liver preservation before transplantation may provide a unique opportunity to safely apply viral vector–based gene therapies that target, for instance, recurrent liver diseases after liver transplantation, such as hepatitis C.

Recurrent HCV rapidly infects a new liver graft after transplantation and will thrive under the immunosuppression needed to prevent graft rejection. In order to prevent HCV from overwhelming the new graft, a substantial proportion of hepatocytes need to express the given genetic therapy, imparting a selective advantage over nontreated cells. With only a small window of opportunity to deliver therapeutics to a liver graft, it is important that high transductions be achieved in not only the shortest period of time but also under the conditions of cold perfusion. We determined in vitro that transductions were possible with incubation times ranging from 10 minutes to 2 hours, with the 1-hour time point showing the highest transductions. In the clinical setting, this could be achieved during the second cold perfusion of the graft, with longer incubation times producing higher transductions. UW's superior transduction potential was observed across the range of times under hypothermic and normothermic conditions. Surprisingly, we were able to achieve reasonable transductions with an incubation time as short as 10 minutes; this indicated a rapid interaction between vector particles and the cell monolayer (Fig. 3).

Although lentiviral vectors are capable of transducing mitotically quiescent cells,[18,19] the levels attainable in vivo are generally lower than those in vitro,[20] possibly because of lentiviral vectors needing some level of cell cycling in vivo to effectively transduce hepatocytes.[21] Given this, it is not surprising that we saw lower transductions in both primary mouse hepatocytes (Fig. 7A) and primary human hepatocytes (Fig. 7B). The trends were similar, in that UW gave better transductions than other solutions used, indicating that UW could be a viable transduction agent for transfer of a genetic therapy to liver grafts during perfusion, although in order to increase transductions to therapeutic levels, additives may be required such as hyper-interleukin-6[20] or growth factors such as hepatocyte growth factor or epidermal growth factor.[22]

UW has become the standard solution for the preservation of most organs in transplantation since its introduction by Southard and Belzer[23] in the late 1980s. Preservation solutions have been designed to help reduce the physiological and biochemical effects of ischemia due to cold storage. One of the main purposes of these solutions is to prevent cell swelling and interstitial edema formation by including substances that are osmotically active and impermeable to the cell.[24] Compounds such as colloids are used in perfusion solutions to counteract the hydrostatic force during the initial washout, thereby creating a homeostatic environment that precludes the need for osmotic regulation. A major component of UW is HES (Viastarch), a colloid used to prevent hypothermic-induced cell damage during major surgery. HES has been shown to decrease transvascular fluid flux and edema formation, maintain colloid osmotic pressure, and preserve the microvascular barrier.[25] As EH, a 6% HES solution, showed transduction similar to that of UW, it was logical that this shared component was, at least in part, responsible for the increase in transduction that was observed in vitro. Indeed, the addition of HES to HTK solution increased transductions to levels nearing those of UW alone (Fig. 5).

Traditionally, charged polymers have been used to increase the transduction potentials of viral vectors.[26-29] Charged polymers alter the kinetics of viral adsorption, thereby modulating transduction efficiency, independently of their tropism.[30] Cationic polymers such as diethylaminoethyl-dextran and polybrene

can enhance negatively charged adenovirus binding and transduction by reducing electrostatic repulsion between negatively charged viruses and cells,[27,31] although they can almost always be inhibited by the addition of anionic polymers through the increase in electrostatic repulsion.[30,32] Most retroviruses, including lentiviruses, contain positively charged surface domains, which probably create a repulsion force that prevents natural aggregation and is similar to that which that prevents the natural aggregation of erythrocytes.[33] However, in the presence of HES, human[24] and rat[33] red blood cells aggregate into large stacks known as rouleaux formations. The exact mechanism for this red blood cell aggregation is not known, although Morariu et al.[24] showed a strong correlation between colloids with high molecular weights and rouleaux formation. They suggested that the effect can be explained by the theory of macromolecular bridging,[34] in which polymers and plasma proteins with a large molecular mass insert between adjacent erythrocytes, decreasing the electrostatic repulsive forces of erythrocytes. HES may influence vector electrostatic charges in this way as well. This reduction in the normal repulsive state may force vector aggregation,[28] increasing the number of vector to individual cell contacts, or increase the speed at which vectors are able to sediment and bind to cell surfaces by neutralizing repulsive forces between vectors and cells.[35] Most likely, both situations occur.

Although HES is a nonionic compound, at pH 7.4 (such as in UW), it contains weak negative charges in flux. This flux possibly creates temporary surface dipoles altering charge distribution across the large irregular surface of the starch. Lentiviruses with VSV-G pseudotypes are positively charged and as such could be attracted to the negative regions and form temporary aggregation complexes with the starch. The vector/HES complex sediments to the cell surface, where it shields against the positively charged residues, exposing more negatively charged residues, and the vector shifts from vector/HES to vector/cell, attracted by the more negatively charged phospholipids. These 2 effects produce much greater transductions in solutions with HES compared to solutions without HES.

This study details the possibility of using UW to transfer lentiviral-based gene therapy vectors to hepatocytes. In the context of liver transplantation, this opens a distinct window of opportunity to increase the ex vivo transfer of vectors, even under cold conditions. As the use of vector-based RNAi therapies approaches clinics, the need for ex vivo delivery becomes increasingly obvious. The ex vivo approach can target specific cells or whole organs while avoiding off-target integrations common with systemic administration. The use of UW could allow for ex vivo transduction under both hypothermic and normothermic conditions, allowing for a customized approach to gene delivery. Although it is effective in this in vitro setting, a more robust in vivo perfusion model is needed to fully explore the potential of UW for vector delivery to organ grafts.

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Supplementary data (unpublished)

Isolated Liver Perfusion
Protocols for the isolated liver perfusion (IHP) in rat were approved by the local Erasmus MC ethics committee and performed under strict biological containment. The lentiviral vector used is based on pRRL.PPT.SF.EGFP.WPRE4*.SIN (van Til et al., Appendix i). To generate pRRL.PPT.SF.LUC.WPRE4*.SIN (LV-SF-LUC), the eGFP cDNA was replaced by luciferase cDNA excised from vector pGL3-Basic (Promega). Luciferase is expressed by a ubiquitous, non-liver specific transcription unit consisting of the Spleen Focus Forming Virus (SSFV) LTR promoter/Murine Embryonic Stem cell Virus (MESV) enhancer.

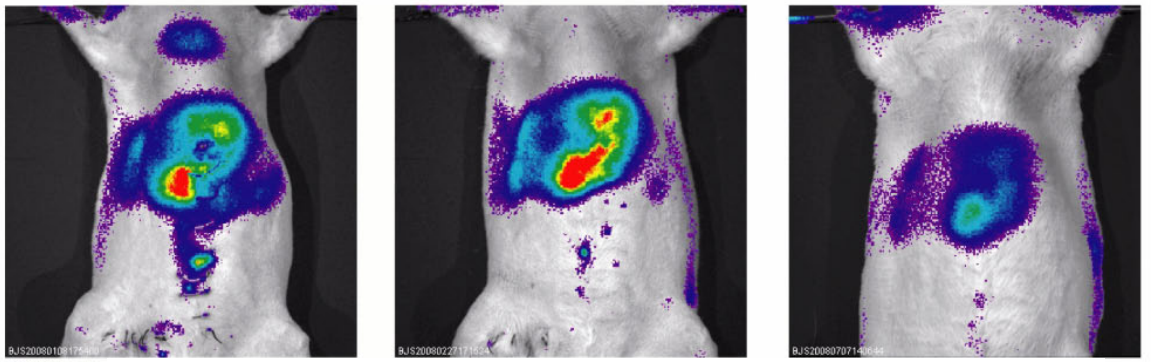
The IHP procedure was performed as described earlier with minor modifications. In brief, male Wistar rats, >280 g body weight and ~25 weeks of age were subjected to oxygenated IHP by cannulation of the inferior vena cava (via the femoral vein) and only the gastroduodenal side branch of the common hepatic artery. To isolate the liver vascular bed the portal vein, hepatic artery, suprahepatic caval vein and mesenteric artery were temporarily clamped. At an orthograde perfusion rate of 5 ml/min, the perfusate containing 2.5 $x10^8$ transducing units (TU) of LV-SF-LUC diluted in cold (4°C) heparinized UW (Viaspan, DuPont Pharma) was circulated for 10 minutes, followed by a 15 ml wash out with warm (38°C) heparinized blood plasma substitute (Gelofusin, B. Braun) to wash out non-adhered vector particles and to prevent hyperkalemic cardiac arrest due to the high potassium concentration in UW (125 mmol/L, Rat reference value is ~5 mmol/L). At the end of the procedure, clamps and cannulas were removed to restore the blood flow to the liver and the operation wound was sutured. The complete procedure takes less than 1 hr.

For real time *in vivo* bioluminescence imaging, rats were anaesthetized with isoflurane and intraperitoneally injected with 25 µl per 10 gram body weight D-Luciferin potassium salt (Caliper Life Sciences) in PBS (30mg/ml). During a 10 minute incubation time the abdominal fur was trimmed and subsequently luciferase expression was quantified for 5 minutes from the ventral side of the animal using an IVIS Imaging System 200 Series (Caliper Life Sciences).

Transduction of lentiviral vectors to hypothermic isolated hepatic perfusion is possible with UW solution in rats
Isolated hepatic perfusion was used to determine if the effect we had observed with the *in vitro* perfusion settings would be applicable to deliver lentiviral vectors directly to the liver *ex vivo*. The liver was isolated from the body by cannulation and a full perfusion performed with cold UW so a predictive model could be assessed. After perfusion with lentiviral vectors expressing the luciferase gene, rats were surveyed for luciferase expression 19, 69 and 200 days (Fig. S1). Clear liver-restricted expression is detected at all time points. Non-liver specific signals are assumed to be due to thoracic cavity and hair-related scatter. A portion of the signal overlaps with the former operation wounds, and presumably originates from scatter or reduced thickness of the skin at these sites. The signal was reduced after 200 days, possibly due to loss of episomal vector copies or partial silencing of the vector.

Supplementary figures

Fig. S1. Hypothermic isolated rat liver perfusion deposits lentiviral vectors specifically to the liver. VSV-G pseudotyped lentiviral vectors carrying a SSFV promoter driven luciferase expression cassette were delivered directly to the liver during a 10 min hypothermic perfusion with UW solution. Luciferase expression was measured by using equal detector and software settings after 19 days (A), 69days (B) and 200 days (C) post perfusion.



day 19 **day 69** **day 200**

Chapter VI

Summary and Discussion

Summary and Discussion

The focus of this thesis is the use of HIV-1 derived lentiviral vectors to treat liver disease. We have studied the application of lentiviral vectors in two clinically relevant experimental models, congenital hyperbilirubenemia (Crigler-Najjar disease) and Hepatitis C virus infection (HCV). In this chapter we summarize our conclusions, and discuss the prospects for further studies and future clinical applications.

6.1 Intravenous administration of lentiviral vector in a rat model of Crigler-Najjar disease results in complete remission

Crigler-Najjar disease is a heritable form of bilirubin conjugation deficiency in the liver. It is caused by a defect in the UGT1A1 gene that leads to the accumulation of bilirubin in the blood to toxic levels. We have shown that the hyperbilirubinemic phenotype observed in the Gunn rat, an animal model for Crigler-Najjar disease, can be restored to normal after a single intravenous injection of 5×10^8 TU of a liver-specific UGT1A1 expressing lentiviral vector (LV-ALB-UGT) in juvenile animals (Chapter 2). Plasma bilirubin levels were reduced by 88% on average when compared to non-treated mutant animals and bile analysis indicated total restoration of bilirubin conjugation and excretion. An average of two integrated LV-ALB-UGT vector copies per cell was required to obtain this beneficial effect. Vector administration resulted in stable expression of UGT1A1 mRNA in approximately 34% of the hepatocytes as determined by *in situ* hybridization. Southern- and qPCR analysis indicated significant off-target vector deposition in the spleen, however UGT1A1 antigen was detected only in the liver. Immunohistochemical analysis of liver sections did not show increased lymphocyte infiltration, and, in combination with the sustained reduction in plasma bilirubin levels, this indicates the absence of a severe immune response directed against the UGT1A1 antigen in this experiment.

None of the treated animals showed adverse effects in terms of carcinogenesis after macroscopic and microscopic analysis of liver tissue. However, considering the limited number of analyzed animals (n=11) and the relatively short duration of the experiment (range 200-500 days), this is not sufficient to draw conclusions about such adverse events after treatment of humans. Therefore, an analysis of the safety of lentiviral vector deposition to the liver is not within the scope of this experiment and this should be determined separately in dedicated toxicity studies in a relevant model.

6.2 Lentiviral vector administration via Isolated Hepatic Perfusion

The introduction of a large quantity of lentiviral vector directly into the bloodstream of live subjects has several major drawbacks that impede clinical application of this vector system. Systemic vector delivery is associated with: vector inactivation by human serum, the potential of a lethal acute immune response, the risk of germ line vector transmission and the induction of cancer by off-target genomic vector integration in actively dividing stem cells or progenitor cells. In an attempt to develop a more clinically relevant and controlled delivery method for lentiviral vectors to the liver, we have explored the effectiveness of local vector administration during isolated hepatic perfusion in the Gunn rat. Vector delivery via this route was feasible and after application of a dose of 1.5×10^9 TU LV-ALB-UGT, we show a significant reduction in plasma bilirubin levels of 36 % with 0.27 vector copies per cell in the liver (Chapter 3). Kupffer cell depletion followed by IHP with a three times lower vector dose of 5×10^8 TU LV-ALB-UGT resulted in a similar effect of 34% reduction in plasma bilirubin levels and 0.23 copies per cell in the liver. Morbidity and mortality in the IHP experiments was mainly due to the invasive and complicated surgical procedure, which proved to be particularly challenging in Gunn rats. Vector delivery, using relatively crude preparations was well tolerated in rats using dosages comparable to those previously used in intravenous injections.

Despite the relatively short ten-minute perfusion time, vector deposition during IHP appeared at least as efficient (TU/gram target tissue) as intravenous injection in juvenile Gunn rats or adult mice. By extrapolation of our current results we can expect that complete correction of hyperbilirubinemia in the Gunn rat by IHP can be achieved with a high vector dose of 2×10^9 TU LV-ALB-UGT in combination with Kupffer cell depletion. Experiments with these high vector dosages were performed and to be able to generate sufficient amounts of vector, tangential flow filtration pre-concentration of vector supernatants was used. However, these experiments were unsuccessful due to frequent clogging of the perfusion set-up by impurities present in the vector preparations. These impurities were mainly due to co-concentration of high molecular weight contaminants in the production medium. This resulted in bad vector re-suspension after ultracentrifugation and the formation of aggregates after thawing of frozen vector preparations. In future experiments, the amount of contaminants in vector preparations produced by our current protocol based of TFF pre-concentration in combination with ultracentrifugation could be easily reduced by using protein-free production media [1]. Another option that will presumably result in enhanced purity is to implement one of the various chromatography-based purification methods described in the literature, which combine vector concentration and purification in a single procedure [2-4]. Of these methods, the use of anion-exchange chromatography by using mustang Q membranes deserves special interest.

We conclude that IHP is an efficient option for lentiviral vector delivery in rats, with potential benefits when applied in humans. Kupffer cell depletion was shown to improve the transduction efficiency, presumably by preventing vector sequestration by this cell type, and can potentially help to prevent macrophage induced acute and cellular immune responses. Improvement is required in the quality of vector preparations and expression level of the integrated vector. Analysis of acute vector induced hepatotoxicity is still a genuine concern, but should be performed with purified clinical grade vector preparations.

Large-scale vector production is a limiting factor

A level equivalent to five percent of the average normal UGT1A1 activity is considered sufficient to avoid pathology in Gunn rats and humans. To achieve this by lentiviral vector transduction, the number of transcriptionally active integrations, the expression per integration and the stability of transcription activity are the decisive parameters. The human liver is obviously a very large target compared to a rodent liver. Therefore, not only vector quality but also vector production volume is an important issue in future studies. A major obstacle in the large-scale application of lentiviral vectors is the low efficiency of the production system. From our experiments, it appears that the number of production cells needed is similar to the number of target hepatocytes *in situ*.

To allow effective vector deposition in adult Gunn rats, we needed to develop a more efficient, and larger-scale vector production protocol than the standard laboratory procedure. Replacement of the calcium phosphate transfection protocol for a method based on polyethylenimine (PEI) resulted in better reproducibility and enabled up scaling of vector production to 6500 cm^2 tissue culture vessels. Furthermore, optimization of the PEI transfection protocol, decreasing the amount of production medium, more frequent vector harvesting, and direct filtration of harvested media, aided to generate larger and more concentrated vector batches. Ultracentrifugation time was minimized by pre-concentration of vector supernatant up to fifteen times by hollow fiber tangential flow filtration. These modifications in the production procedure enable the production of twenty times more vector (TU) by a single person in an equal amount of time as compared to the generic procedure.

An alternative to improve the therapeutic effect in the Gunn rat is to increase the UGT1A1 expression rate per vector copy. This could be achieved by performing transgene sequence optimization and by changing the internal enhancer-promoter. The latter option calls for caution, since the use of strong (retroviral) enhancer-promoter combinations has been associated with an increased risk of insertional mutagenesis [5]. Probably the best current option is to make use of an efficient, non-viral, liver specific promoter in combination with a microRNA target sequence that prevents off-target transgene expression in leukocytes [6].

Minimally invasive surgery for IHP in humans
The invasive open surgical isolated perfusion as performed here in rats may be problematic in a clinical setting, especially in diseased patients. However, advances in the field of isolated perfusion have led to techniques based on balloon catheters that can be performed with minimally invasive surgery and may result in a more feasible method of vascular isolation of the liver [7]. As opposed to the traditional IHP procedure, these balloon catheter based strategies are associated with minor leakage, making this approach less suitable for the administration of high dose cytostatics [8, 9]. However, for gene therapy purposes, these minimally invasive strategies may be an acceptable compromise between the risk of surgery and the reduction of off-target vector deposition. Alternatively, the classical open surgical IHP procedure is also subject of constant improvement to reduce costs, operation time, blood loss and patient survival [10].

6.3 shRNA expression for treatment of hepatitis C virus infection

The latest advances in inhibitory RNA technology may allow treatment of hepatitis C by interfering with one or more genes required for viral replication or infection. In order to create a liver-directed gene therapy strategy for the treatment of HCV infection, we developed and analyzed a lentiviral vector that directs expression of multiple anti-HCV shRNAs. Here, a single lentiviral vector was demonstrated to simultaneously express up to three different shRNAs designed to inhibit both HCV replication and infection of the virus *in vitro*. In an HCV replicon system, lentiviral shRNAs directed against the viral genome prevented replication of viral sequences, and efficient down regulation of cellular CD81, a putative HCV co-receptor, reduced binding of its viral ligand (Chapter 4).
Since HCV exhibits an exceptionally high mutation rate, this multi-targeted approach is required to prevent mutational escape of the virus. Only a single nucleotide mismatch between an RNAi molecule and its target sequence can diminish the inhibitory effect. Therefore, the most conserved regions of the HCV genome were chosen as a target: the IRES that is required for translation initiation and the NS5b region that encodes the viral RNA polymerase. Because of the high mutation rate of HCV, RNAi based approaches should not be limited to the vector genome, and the incorporation of additional cellular targets such as the CD81 receptor is expected to add to the overall HCV inhibiting effect and further prevent possible viral escape.

Incorporating multiple shRNA's in a single vector assures that the target cell receives a full range of therapeutic sequences in a single integration event. This is especially important, since in a clinical context, transduction rates reaching multiple copies per cell may not be feasible. Such high-dose transduction would also increase the risk of vector-induced toxicity upon administration and insertional mutagenesis. Furthermore, uncontrolled, high-level gene transfer could lead to saturation of the RNAi pathway in the liver, which can have severe detrimental side effects [11].

The current vector design can be improved by avoiding repetitive promoter sequences, which cause vector recombination during reverse transcription. This can be realized by using different polymerase III promoter sequences or by modifying endogenous polycistronic microRNAs by exchanging their active sequences for custom sequences of interest [12, 13]. A benefit of the latter approach is that miRNAs can be expressed by conventional polymerase II promoters so that their expression can be better regulated.

The requirement for drug-induced selection to retain the HCV replicon in the system used here, did not permit long-term analysis of mutational escape rates after vector administration [14]. Therefore, the long-term efficiency of shRNA based lentiviral gene therapy on inhibiting HCV, and assessment of the prevention of mutational escape should be evaluated by a different approach. Possibly, recently developed more genuine *in vitro* infection systems will be more suitable for this purpose [15]. Animal models for HCV infection based on a chimeric murine/human liver have been described, but these models are extremely complicated in their use and the development of more practical solutions is highly anticipated [16].

6.4 Lentiviral transduction during liver transplantation

Recurrence of HCV infection after liver transplantation is considered a serious clinical problem with few therapeutic options and is associated with high morbidity and mortality. In principle, HCV recurrence in liver grafts could be prevented by generating a pool of HCV-resistant hepatocytes prior to transplantation. A lentiviral vector expressing multiple anti-HCV shRNAs as described above may be suitable to realize such an effect. Liver transplantation protocols involve flushing of the donor liver with cold preservation solution to clear it from blood and to prevent organ degeneration during transport. Before transplantation, the liver is then usually flushed with a warm human albumin solution to reheat the liver and wash out the preservation solution. The time between resection and transplantation of the liver graft, may provide a unique opportunity for gene therapeutic intervention.

It was determined *in vitro* that it is feasible to realize efficient lentiviral vector deposition under cold perfusion conditions (Chapter 5). In fact, the generally used starch-containing preservation solution (UW), was shown to favor lentiviral vector transduction over another frequently used non-starch based preservation solution (HTK) and tissue culture medium. Finally, we provided preliminary evidence that a lentiviral vector expressing the luciferase gene could be successfully delivered during a method that resembles liver transplantation *in vivo*, i.e. isolated perfusion with cold UW in combination with a warm wash-out of unbound vector.

Future research will be necessary to determine the transduction rates needed to prevent HCV recurrence in animal liver transplantation models and perfused human liver tissue, and whether this shRNA based approach can prevent or decrease HCV replication to prevent cirrhosis of the liver graft.

6.5 Clinical application of lentiviral vectors

While our studies provide further proof of the versatility and efficiency of lentiviral vectors, many issues have to be dealt with before clinical applications in chronic liver disease can be considered.

Insertional mutagenesis

The major concern associated with clinical application of lentiviral vector is genotoxicity caused by genomic vector integration. HIV-1 integration on itself is not known to be carcinogenic during natural infection and carcinogenesis caused by HIV-1 derived vectors has not yet been reported in animal studies *in vivo*. However, clear evidence for preferential integration in active genes has been presented [17]. This suggests a scenario that involves activation of proto-oncogenes by vector sequences. So far, published data suggest that lentiviral vectors have a lower potential to induce insertional mutagenesis than their gammaretroviral counterparts that were shown to induce leukemia in 5 out of twenty three patients treated in two clinical X-SCID trials [18-20]

Considering the low transformation rates observed so far, risk assessment for insertional mutagenesis by lentiviral vectors will require analysis of thousands of small experimental animals to approach the number of transduction events required for the treatment of human liver disease [21]. Even then, the significance of this approach remains questionable considering the longevity of human subjects compared to rodents, and differences in genetic makeup that affect carcinogenesis. On the other hand, safety evaluation in sufficient numbers of large animals models is very expensive and time consuming. Considerable investments in large-scale, clinical grade vector production will be required first. An *in vitro* system to assess the risk of insertional mutagenesis of different vector designs in primary HSC's has been recently developed and can be used to verify various concepts of improvement of vector safety [5, 22]. How these results will relate to liver carcinogenesis is difficult to predict, and only a system based on liver cells will enable safety analysis of lentiviral vectors tailored to the liver.

One of the main determinants of integrational mutagenesis is the upregulation of a proto-oncogene by the enhancer present in the integrated vector. Therefore, the incorporation of stronger enhancer-promoters to reduce the total number of vector copies needed to obtain a gene therapeutic effect may not result in a safer gene therapy approach [5]. The incorporation of insulators, boundary elements or locus control regions into lentiviral vectors has been proposed to limit the interaction of vector derived enhancers with promoters in the host genome and simultaneously prevent transgene silencing and position effects [23] . However, the availability of these elements and the knowledge about their mechanism of action is limited to several well-studied textbook cases [24, 25]. Random integration of these elements may result in non-specific host-DNA interactions with an unpredictable outcome. Also, the use of these elements has been shown to interfere with lentiviral vector function, which may make them unsuited for incorporation in lentiviral vectors [26].

Availability of clinical grade vector preparations

GMP production of clinical grade lentiviral vector batches has been established for current clinical trials that involve transduction and transplantation of relatively small amounts of CD4+ T-cells and HSCs [2] . However, with current production techniques that are based on transient transfection, it will be difficult to generate sufficient amounts of lentiviral vector for *in vivo* transduction of large organs, such as the liver. The development of stable lentiviral vector packaging or producer cell lines is expected to overcome these problems. Despite various efforts (mentioned in the papers referred to at the end of this section), such cell lines are not available to date and their development is hampered by the cytotoxicity associated with the constitutive expression of HIV-1 protease and the VSV-G envelope protein. Possibly recently described strategies using cell lines that contain leakage-free inducible packaging or producer systems can provide a solution to this problem [27, 28].

Adverse effects

A detrimental and underestimated factor in the field of gene therapy is that the introduction of a foreign therapeutic protein can induce immune responses. These adverse events can not only diminish the gene therapeutic effect but may also be dangerous. For example, an unforeseen CTL response against the introduced antigen in large numbers of transduced hepatocytes may induce considerable liver damage. The generation of an antibody-directed immune response, however, is considered less dangerous and tends to affect the functionality of gene therapy applications that involve the expression of excreted proteins (e.g. F.IX or GAA), rather than that of proteins that remain intracellular (e.g. UGT1A1). On the other hand, the liver is an immunologically distinct organ that has evolved to tolerate various antigens supplied via the intestines [29]. High-level expression of the introduced transgene has been associated with the induction of liver tolerance [30]. MiRNA directed de-targeting of expression from lentiviral vectors in inadvertently transduced leukocytes in combination with hepatic gene transfer was shown to induce immune tolerance [6].

An advantage of the application of shRNA expressing vectors is the absence of the generation of potentially immunogenic foreign antigens. However, other problems are associated with RNAi based strategies [31]. Cytoplasmic dsRNA and specific sequences around the transcription start of polymerase III driven shRNA expression systems have been reported to lead to the induction of the IFN cascade. However, this seems to be partially overcome by expressing the RNAi target sequences of choice in modified forms of naturally occurring pri-miRNAs. Since the miRNA pathway is an important biological regulatory mechanism, overexpression of shRNA sequences has been associated with lethal liver toxicity in mice [11]. Here, shRNAs and modified pri-miRNAs may be more potent in inhibiting the endogenous RNAi pathway than synthetic siRNAs, since competition is thought to be at the nuclear export level of small RNAs.

Clinical trials and future applications

The first clinical trial using lentiviral vectors has been carried out for HIV-1 infection and involved the transplantation of autologous CD4+ T cells transduced with a lentiviral vector

expressing an antisense RNA directed against the HIV-1 *env* gene [32]. This trial paved the way of lentiviral vectors to the clinic in terms of GMP vector production and regulatory matters [33]. The gene therapeutic approach used here is considered relatively safe, since it does not involve the transduction of long living or actively dividing cells and does not require the expression of non-self antigens. A second clinical trial takes lentiviral gene therapy a step further and involves autologous transplantation of lentivirally corrected HSC's for the treatment of X-linked adrenoleukodystrophy. Preliminary results were successful and no adverse effects were reported [34].

As of September 2008, other clinical trials based on HSCT in combination with lentiviral gene transfer have been approved and aim for treatment of: beta-thalassemia major, sickle cell anemia, Wiskott-Aldrich Syndrome, mucopolysaccharidosis VII and Fanconi anemia (http://www.wiley.co.uk/ genetherapy/clinical/). These trials are expected to shed further light on the general safety of lentiviral vectors in terms of genotoxicity. If insertional mutagenesis is not apparent, this may render the use of gammaretroviral vectors redundant and other applications based on this strategy are likely to follow. Furthermore, a successful and safe gene transfer protocol for HSCs is expected to result in investments to increase the production capacity for clinical grade lentiviral vector batches.

Recently, significant advances in the field of AAV-directed vector administration to the liver have been made and the first partially successful clinical trial with this vector type has been performed [35]. Here, a CTL response directed against the AAV serotype 2 vector capsid in transduced hepatocytes presented a drawback. However, in future trials, this problem is expected to be overcome by using different vector serotypes or transient immuno-suppression at the time of vector administration. Nevertheless, in the long run, the true solidity of AAV directed gene transfer to the liver remains to be awaited. The transient nature of the episomal transgenes delivered by AAV vectors may require vector re-administration several years after the first treatment. Furthermore, these mitosis-induced dilution effects may be enhanced by the fact that AAV forms concatamers and its therapeutic response depends on high-level expression in a limited amount of transduced cells. Common infections with e.g. Epstein-Barr virus (EBV) or cytomegalovirus (CMV) enhance the turnover rate of hepatocytes and may result in a quick loss of the transgene, causing a threat when metabolic disease returns.

The future of systemic administration of lentiviral vectors for liver-directed gene therapy of inherited metabolic deficiencies will largely depend on the outcome of the clinical trials mentioned above. For the prevention of HCV recurrence in liver grafts, however, lentiviral vectors remain a viable choice and more studies are required to further explore this appealing treatment option. Possibly, the application of gene therapy in liver grafts can be expanded to prevent graft rejection or improve graft survival. This might lead to reconsideration of current immunosupression regimes or improvement of transplantation outcomes with lower quality donor livers derived from e.g. non-heart beating donors.

Lentiviral vectors are also expected to find their way into liver cell transplantation. They could be used to improve the expression rate of a deficient protein in differentiated donor hepatocytes to reduce the amount of cells required for transplantation. As for Crigler-Najjar disease, it has been shown in the Gunn rat that a successful transplantation rate of 1% with UGT1A1 over-expressing hepatocytes in the liver can have a significant beneficial effect on the plasma bilirubin level [36]. Rapid developments in stem cell technology may allow a transplantation procedure with engineered autologous stem cells in the future [37]. This approach can have major advantages, since cells can be transduced with a low amount of vector and subsequently expanded. Rejection of transplanted cells is prevented and the risks of acute toxicity upon vector delivery and gonadal transduction are excluded. However, an efficient and safe clinical liver cell transplantation protocol remains to be developed [38]. Moreover, expansion of a stem cell population transduced with an integrating lentiviral vector is associated with risks that have to be carefully balanced against the morbidity and lethality of the disease treated.

6.6 General conclusion

Here we explored the use of lentiviral vectors for gene therapy applications that cover treatment of a broad spectrum of both hereditary and acquired liver disease. Our studies confirm and extend the conclusion that lentiviral vectors are promising therapeutic tools for liver directed gene transfer. Translation of these applications to the clinic seems feasible, but there are still major issues to be addressed. More study is required to optimize vector design to ensure safe, efficient and long-term transgene expression. Furthermore, the production methods for clinical grade lentiviral vector preparations for pre-clinical and clinical studies need to be improved. Most importantly, the risk of insertional mutagenesis by lentiviral vectors needs to be further assessed and possible strategies to reduce this risk need to be evaluated. Finally, the most effective and safe method of vector delivery needs to be established, depending on the specific application.

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Chapter VII

Samenvatting en Discussie

Samenvatting en Discussie

Dit proefschrift richt zich op het gebruik van HIV-1 afgeleide lentivirale vectoren voor de behandeling van leverziekte. We onderzochten de toepassing van lentivirale vectoren in twee klinisch relevante experimentele modellen voor humane leverziekte: aangeboren hyperbilirubinemie (de ziekte van Crigler-Najjar) en infectie met hepatitis C virus (HCV). In dit hoofdstuk vatten wij onze conclusies samen en bespreken we de vooruitzichten betreffende verdere studies en toekomstige klinische toepassingen.

6.1 Intraveneuze toediening van lentivirale vector in een rat model van de ziekte van Crigler-Najjar resulteert in volledige remissie

De ziekte van Crigler-Najjar is een erfelijke vorm van bilirubine conjugatie deficiëntie in de lever. Het wordt veroorzaakt door een defect in het UGT1A1 gen, hetgeen leidt tot de stapeling van bilirubine in het bloed tot een toxisch niveau. We ontwikkelden een een lever-specifieke, UGT1A1 expresserende lentivirale vector (LV-ALB-UGT) en toonden aan dat met een enkele intraveneuze injectie van vijfhonderd miljoen actieve virus deeltjes (5×10^8 TU) van deze vector het hyperbilirubinemische fenotype van jonge Gunn ratten, een diermodel voor de ziekte van Crigler-Najjar, kan worden gecorrigeerd (Hoofdstuk 2). Het plasma bilirubine niveau daalde met gemiddeld 88% in vergelijking met onbehandelde mutante dieren en analyse van de gal liet een totaal herstel van bilirubine conjugatie en excretie zien. Om dit effect te bewerkstelligen was een gemiddelde van twee vector kopieën LV-ALB-UGT per levercel vereist. *In situ* hybridisatie toonde aan dat toediening van de vector resulteerde in stabiele expressie van UGT1A1 mRNA in ongeveer 34% van de hepatocyten. Southern- en qPCR analyse duidde op significante vector depositie in de milt, maar expressie van UGT1A1 werd alleen aangetoond in de lever. Immunohistochemische analyse liet geen verhoogde lymfocyt infiltratie in de lever zien, en dit, in combinatie met de langdurige daling van het plasma bilirubine niveau, wijst niet op de aanwezigheid van een ernstige immuunrespons tegen het UGT1A1 antigen in dit experiment.

Door macro- en microscopische analyse van de lever kon in geen van de behandelde dieren carcinogenese worden aangetoond. Echter, gebaseerd op het beperkte aantal geanalyseerde dieren (n=11) en de relatief korte duur van het experiment (200-500 dagen) kunnen geen harde conclusies worden getrokken omtrent de veiligheid van deze behandeling bij de mens. Een veiligheidsanalyse van lentivirale vectordepositie in de lever maak dan ook geen deel uit van dit experiment, en deze dient afzonderlijk te worden uitgevoerd in speciale toxiciteitsstudies in een relevant model.

6.2 Toediening van lentivirale vector via geïsoleerde leverperfusie

De introductie van een grote hoeveelheid lentivirale vector rechtstreeks in de bloedbaan van levende proefpersonen heeft een aantal serieuze nadelen die de klinische toepassing van dit vectorsysteem in de weg staan. Systemische vectortoediening is mogelijk onderhevig aan: vector inactivatie door humaan serum, het mogelijk optreden een letale acute immuunrespons, het risico op vector-transmissie door geslachtscellen en oncogenese door off-target vector integratie in het genoom van actief delende stam- of voorlopercellen. Met het oog op de ontwikkeling van een klinisch relevante methode van gecontroleerde lentivirale vectortoediening aan de lever onderzochten we de effectiviteit van lokale vector depositie tijdens geïsoleerde leverperfusie (IHP) in de Gunn rat. Toediening van vector via deze route bleek haalbaar en bij een dosis van $1.5\text{x } 10^9$ TU LV-ALB-UGT, zagen we een significante daling in het plasma bilirubine niveau van 36% met 0,27 vector kopieën per cel in de lever (hoofdstuk 3). Kupffer cel depletie, gevolgd door IHP met een drie keer lagere vector dosis van $5\text{x}10^8$ TU LV-ALB-UGT resulteerde in een vergelijkbaar effect van 34% reductie in het plasma bilirubine niveau en 0,23 kopieën per levercel. Morbiditeit en mortaliteit in de IHP-experimenten was voornamelijk te wijten aan de zware, gecompliceerde chirurgische

procedure, welke in Gunn ratten extra lastig uitvoerbaar bleek. Toediening van relatief onzuivere vector preparaten tijdens IHP in hoeveelheden vergelijkbaar met doses eerder gebruikt bij intraveneuze injectie werd door ratten goed verdragen.

Ondanks de relatief korte perfusietijd van tien minuten, bleek dat de vectordepositie tijdens IHP minstens zo efficiënt (TU / gram target-weefsel) verloopt als bij intraveneuze injectie in juveniele Gunn ratten of volwassen muizen. Als we deze resultaten extrapoleren, kan worden verwacht dat volledige correctie van hyperbilirubinemie in de Gunn rat tijdens IHP kan worden bewerkstelligd door een hoge vectordosis van 2×10^9 TU LV-ALB-UGT in combinatie met Kupffer cel depletie. Experimenten met deze hoge vectordoseringen zijn uitgevoerd en om voldoende hoeveelheden vector te produceren werd pre-concentratie van vector supernatant door tangential flow filtratie gebruikt. Echter, deze experimenten waren niet succesvol, vermoedelijk omdat verontreinigingen in de vector preparaten coagulatie van de perfusie vloeistof en verstopping van de bloedvaten veroorzaakten. Deze verontreinigingen zijn hoofdzakelijk het gevolg van de co-concentratie van hoog moleculaire bijproducten in het productie medium. Dit resulteerde in slechte vector resuspensie na ultracentrifugatie en de vorming van aggregaten na het ontdooien van de vector preparaten. In toekomstige experimenten zou de hoeveelheid verontreinigingen in vector-preparaten gegenereerd met ons huidige protocol op basis van TFF pre-concentratie in combinatie met ultracentrifugatie kunnen worden verminderd door het gebruik van eiwitvrije productie media [1]. Een ander alternatief wat vermoedelijk resulteert in een verbeterde zuiverheid is het gebruik van een van de verschillende in de literatuur beschreven zuiveringsmethoden gebaseerd op chromatografie, welke vector concentratie en zuivering combineren in een enkele procedure [2-4]. Van deze methoden verdient anion-exchange chromatografie met behulp van Mustang Q membranen bijzondere belangstelling.

We concluderen dat IHP een efficiënte optie is voor lentivirale vector toediening in ratten met mogelijke voordelen voor toepassing in de mens. De Kupffer cel depletie verbeterde de transductie-efficiëntie, vermoedelijk door het voorkomen van vector inactivering door dit celtype, en kan mogelijk macrofaag geïnduceerde acute en cellulaire immuunresponsen helpen voorkomen. In vervolgexperimenten dient de kwaliteit van de vector preparaten en het expressieniveau van de geïntegreerde vector te worden verbeterd. Analyse van de mate van acute hepatotoxiciteit geïnduceerd door de vector dient te worden uitgevoerd met opgezuiverde vectorpreparaten.

Grootschalige vectorproductie is een beperkende factor

Een niveau van vijf procent van de normale UGT1A1 activiteit in de lever wordt voldoende geacht om ziekteverschijnselen in de Gunn rat en de mens te voorkomen. Om dit expressieniveau te evenaren door middel van lentivirale gentherapie zijn het aantal transcriptioneel actieve vector integraties, het expressieniveau per vector integratie en de stabiliteit van genexpressie de doorslaggevende parameters. Vergeleken met een knaagdierlever is de menselijke lever een buitengewoon groot doelwit. Hierdoor is niet alleen de vectorkwaliteit, maar ook de productiecapaciteit voor vectoren een belangrijk gegeven in toekomstige studies. Een groot obstakel voor de grootschalige toepassing van lentivirale vectoren is de lage efficiëntie van het productiesysteem. Uit onze experimenten blijkt dat met de huidige methode het aantal benodigde productiecellen vergelijkbaar is met het aantal te raken hepatocyten *in situ*.

Voor het bewerkstelligen van effectieve vector depositie in volwassen Gunn ratten was het nodig een vector productie protocol te ontwikkelen dat efficiënter en grootschaliger is dan de standaard laboratoriumprocedure. Vervanging van het op calciumfosfaat gebaseerde transfectie protocol door een methode gebaseerd op polyethylenimine (PEI) resulteerde in een verbeterde reproduceerbaarheid en maakte opschaling van de vectorproductie in celkweek units van 6500 cm^2 mogelijk. Verdere hebben de optimalisatie van het PEI transfectie protocol, het verminderen van de hoeveelheid productie medium, het meer frequent oogsten van vector en het toepassen van directe filtratie van geoogst vectormedium zonder vooraf te centrifugeren, geresulteerd in het genereren van grotere en meer geconcentreerde vector batches. De tijd benodigd voor ultracentrifugatie werd geminimaliseerd door pre-concentratie van het vector supernatant tot vijftien keer door middel

van “hollow fiber tangential flow filtratie”. Deze wijzigingen in de productieprocedure maken de productie van twintig keer meer vector (TU) in een gelijke hoeveelheid tijd mogelijk.

Een alternatief voor het verbeteren van het therapeutisch effect in de Gunn rat is het verhogen van de mate van UGT1A1 expressie per vector kopie. Dit kan worden bereikt door optimalisatie van de sequentie van het transgen en door het vervangen van de interne enhancer-promotor voor een sterkere variant. De laatste optie vergt enige voorzichtigheid, omdat het gebruik van sterke (retrovirale) enhancer-promotor combinaties in verband wordt gebracht met een verhoogd risico op integratie-gerelateerde mutagenese [5]. Waarschijnlijk is de beste optie het gebruik van een efficiënte, niet-virale, leverspecifieke promotor in combinatie met een microRNA doel-sequentie die expressie van het transgen in leukocyten voorkomt [6].

Minimaal invasieve chirurgie voor IHP bij de mens
Het open invasieve karakter van de chirurgische geïsoleerde leverperfusie procedure zoals uitgevoerd in ratten kan problematisch zijn in een klinische setting, met name bij zieke patiënten. Echter, recente ontwikkelingen op het gebied van geïsoleerde perfusie hebben geleid tot technieken gebaseerd op ballonkatheters die kunnen worden uitgevoerd met minimaal invasieve chirurgie, hetgeen kan leiden tot een meer klinisch relevante methode van vasculaire isolatie van de lever [7]. In tegenstelling tot de traditionele IHP procedure gaan deze op ballonkatheter gebaseerde technieken gepaard met enige lekkage, waardoor deze aanpak minder geschikt is voor de toediening een van hoge doses cytostatica aan de lever [8, 9]. Echter, voor gentherapie doeleinden kunnen deze minimaal invasieve strategieën een aanvaardbaar compromis vormen tussen het risico van de operatie en de vermindering van off-target vector depositie. Daarnaast wordt de klassieke open chirurgische IHP eveneens voortdurend verbeterd om de operatiekosten te verlagen, de operatietijd te verkorten, bloedverlies te beperken en de overleving van de patiënt verder te verhogen [10].

6.3 shRNA expressie voor de behandeling van hepatitis C-virus infectie

Recente ontwikkelingen op het gebied van inhibitory RNA technologie bieden perspectief voor de behandeling van hepatitis C door remming van een of meerdere genen benodigd voor HCV replicatie of infectie. Voor de ontwikkeling van een levergerichte gentherapie voor de behandeling van HCV infectie hebben we een lentivirale vector ontworpen en getest welke gelijktijdig drie verschillende anti-HCV shRNAs tot expressie brengt om zowel infectie als replicatie van HCV te remmen. In een *in vitro* HCV replicon systeem bleek deze vector in staat de replicatie van het virale genoom te voorkomen en de expressie van cellulair CD81, een vermeende HCV co-receptor, te verlagen om zo de binding van virale liganden te verminderen (hoofdstuk 4).

Aangezien HCV een uitzonderlijk hoge mutatiesnelheid heeft is deze multi-target aanpak essentieel om te voorkomen dat het virus door mutatie ontsnapt. Een mismatch van slechts een enkel nucleotide tussen een RNAi-molecuul en zijn target sequentie leidt al tot een significante vermindering van de remmende werking. Daarom zijn in deze aanpak de meest geconserveerde regio's van het HCV genoom als target gekozen: de IRES die nodig is voor de initiatie van virale translatie en het NS5b gen dat codeert voor het virale RNA-polymerase. Vanwege de hoge mutatiegraad van HCV, moeten behandelingen gebaseerd op RNAi niet worden beperkt tot het virusgenoom. De opname van cellulaire targets zoals de CD81 receptor zal waarschijnlijk helpen het algemene remmende effect op HCV verhogen en de kans op mogelijke escape van het virus verminderen.

Het samenvoegen van meerdere shRNA sequenties in een enkele vector verzekert dat de cel door een enkele vectorintegratie het volledige scala aan therapeutische sequenties verkrijgt. Dit is vooral van belang omdat, in een klinische context, de realisatie van meerdere vectorkopieën per cel waarschijnlijk niet haalbaar is. Daarnaast verhoogt de toediening van de een hoge dosis vector die benodigd is om meerdere kopieën per cel te realiseren het risico van vector-geïnduceerde toxiciteit en integratie-gerelateerde mutagenese. Bovendien zou

ongecontroleerde genoverdracht op grote schaal kunnen leiden tot verzadiging van de RNAi pathway in de lever, hetgeen ernstige bijwerkingen kan hebben [11].

Het huidige vectorontwerp kan worden verbeterd door het vermijden van identieke promotor sequenties welke recombinatie van de vector kunnen veroorzaken tijdens reverse transcriptie. Dit kan worden gerealiseerd door gebruik te maken van verschillende polymerase III promotor sequenties of door aanpassing van endogene polycistronische microRNAs door vervanging van hun natuurlijke sequenties door synthetische versies [12, 13]. Een voordeel van deze laatste benadering is dat miRNAs tot expressie worden gebracht door conventionele polymerase II promoters zodat hun expressie beter kan worden gereguleerd.

Door de vereiste toevoeging van selectie aan het celkweekmedium van het hier gebruikte HCV replicon systeem was het niet mogelijk om na vectortoediening eventuele mutatie-gerelateerde escape op lange termijn te analyseren [14]. Daarom moet de lange termijn efficiëntie van op shRNA gebaseerde lentivirale gentherapie voor de remming van HCV, en de evaluatie van mutatie gerelateerde escape op een andere manier worden bepaald. Mogelijk is een recent ontwikkeld *in vitro* HCV infectiesysteem meer geschikt voor dit doel [15]. Diermodellen voor HCV-infectie op basis van een chimere humane lever in muizen zijn beschreven, maar deze modellen zijn zeer gecompliceerd in hun gebruik en de ontwikkeling van meer praktische oplossingen is noodzakelijk [16].

6.4 Lentivirale transductie tijdens lever transplantatie

Herinfectie van de lever met HCV na levertransplantatie is een ernstig klinisch probleem met weinig therapeutische opties en een hoge morbiditeit en mortaliteit. In principe zou de herinfectie van levertransplantaten met HCV kunnen worden voorkomen door het genereren van een bepaald percentage HCV-resistente hepatocyten voorafgaand aan transplantatie. Een lentivirale vector die, zoals hierboven beschreven, meerdere anti-HCV shRNAs tot expressie brengt zou een dergelijk effect kunnen bewerkstelligen. Levertransplantatie protocollen gaan al gepaard met het spoelen van de donor lever met koude preservatieoplossing om het transplantaat van bloed te ontdoen en om degeneratie van het orgaan tijdens transport te voorkomen. Voorafgaand aan transplantatie wordt het levertransplantaat meestal geperfuseerd met een warme albumineoplossing voor opwarming van het orgaan en het uitspoelen van de preservatieoplossing. Het tijdvlak tussen resectie en transplantatie van de donorlever biedt in principe een unieke gelegenheid voor gentherapeutische interventie.

We hebben *in vitro* aangetoond dat het mogelijk is om efficiënte lentivirale vectordepositie te bewerkstelligen onder de condities van koude perfusie (hoofdstuk 5). Tevens toonden we aan dat de transductie-efficientie door lentivirale vectoren onder deze condities in een algemeen gebruikte preservatieoplossing op basis van zetmeel (UW) superieur is aan een andere veelgebruikte niet op zetmeel gebaseerde preservatieoplossing (HTK) en normaal celkweekmedium. Ten slotte hebben we *in vivo* aangetoond dat depositie en expressie van een lentivirale vector met het luciferase gen kan worden bewerkstelligd tijdens een procedure die veel gelijkenissen vertoond met een lever transplantatie, d.w.z. geïsoleerde leverperfusie met koude UW in combinatie met een warme wash-out van ongebonden vector.

Toekomstig onderzoek in dierlijke levertransplantatie modellen en geperfuseerd humaan leverweefsel zal nodig zijn om de mate van transductie te bepalen die benodigd is om herinfectie met HCV te voorkomen, en of deze op shRNA gebaseerde aanpak HCV replicatie en de daarmee gepaard gaande levercirrose in donororganen kan verminderen of voorkomen.

6.5 Klinische toepassing van Lentivirale vectoren

Terwijl onze studies de veelzijdigheid en efficiëntie van lentivirale vectoren bevestigen, dienen een aantal zaken verder te worden uitgezocht voordat klinische toepassingen voor chronische leverziekte kunnen worden overwogen.

Mutagenese door genomische integratie
De grootste zorg omtrent de klinische toepassing van lentivirale vectoren is de mogelijke genotoxiciteit die gepaard kan gaan met genomische integratie van de vector. Het is bij een normale HIV-1 infectie nooit aangetoond dat genomische integratie op zich oncogeen is en carcinogenese veroorzaakt door HIV-1 afgeleide vectoren *in vivo* is eveneens nooit gerapporteerd. Echter, het is evident dat lentivirale vectoren bij voorkeur integreren in actieve genen [17]. Dit schept een mogelijk scenario voor de activatie van proto-oncogenen door sequenties die zich in de vector bevinden. Uit de literatuur blijkt dat lentivirale vectoren een lager potentieel hebben voor het induceren van integratie-gerelateerde mutagenese dan hun gamma-retrovirale tegenhangers, welke verantwoordelijk worden geacht voor het opwekken van leukemie in vijf van de drieëntwintig patiënten behandeld in twee klinische trials voor X-SCID [18-20].

Gezien de lage tot dusver waargenomen genotoxiciteit zal een risicoanalyse voor integratie-gerelateerde mutagenese door lentivirale vectoren de analyse van duizenden kleine proefdieren vergen om zo het aantal integraties dat nodig is voor de behandeling van menselijke leverziekte te benaderen [21]. Zelfs dan blijft de betekenis van de uitkomst van deze aanpak twijfelachtig gezien de lange levensduur van de mens vergeleken met knaagdieren, en genomische verschillen met betrekking tot oncogenese. Ook is een risicoanalyse in een voldoende aantal grote diermodellen zeer duur en tijdrovend. Hiervoor zullen tevens aanzienlijke investeringen op het gebied van grootschalige vectorproductie voor klinisch gebruik nodig zijn. Een *in vitro* systeem voor het beoordelen van de risico's van integratie-gerelateerde mutagenese van verschillende vectorontwerpen in primaire HSC's is recentelijk ontwikkeld en kan worden gebruikt om verschillende concepten voor de verbetering van de veiligheid te evalueren [5, 22]. In hoeverre deze resultaten betrekking zullen hebben op de lever is moeilijk te voorspellen en alleen een systeem gebaseerd op levercellen zal in staat zijn de veiligheid van lentivirale vectoren die zijn afgestemd op de lever te evalueren.

Een van de belangrijkste oorzaken van integratie-gerelateerde mutagenese is de opregulatie van proto-oncogenen door de in de geïntegreerde vector aanwezige enhancer. Daarom zal het gebruik van sterke enhancer-promoter combinaties om het aantal vector kopieën benodigd voor het verkrijgen van een bepaald gentherapeutisch effect niet altijd resulteren in een veiliger gentherapieprotocol [5]. Het is voorgesteld om insulators, boundary elementen en locus control regions in lentivirale vectoren op te nemen om zo de interactie van de in de vector aanwezige enhancers met promotoren in het genoom tegen te gaan en tegelijkertijd silencing van expressie en het optreden van positie effecten tegen te gaan [23]. Echter, de beschikbaarheid van deze elementen en de kennis over hun werkingsmechanisme is beperkt tot enkele goed bestudeerde gevallen [24, 25]. Willekeurige integratie van deze elementen in het genoom kan leiden tot a-specifieke DNA interacties in de targetcel waarvan het effect moeilijk voorspelbaar is. Ook blijken deze elementen te interfereren met het goed functioneren van lentivirale vectoren, waardoor hun gebruikt verder wordt beperkt [26].

Beschikbaarheid van clinical-grade vector preparaten
De productie van lentivirale vector batches volgens GMP richtlijnen is gerealiseerd voor de huidige klinische trials die zich richten op de transductie en transplantatie van relatief kleine aantallen CD4+ T-cellen en HSCs [2]. Echter, de productie van voldoende hoeveelheden lentivirale vector voor de transductie van grote organen als de lever zal met de met de huidige op transfectie gebaseerde productietechnieken moeilijk haalbaar zijn. De verwachting is dat de ontwikkeling van stabiele packaging of producer cellijnen voor lentivirale vectoren deze problemen op kan lossen. Echter, ondanks diverse inspanningen zijn deze cellijnen tot op heden niet beschikbaar en hun ontwikkeling wordt belemmerd door de cytotoxiciteit die gepaard gaat met de stabiele expressie van HIV-1 protease en het VSV-G envelopeiwit. Mogelijk bieden de recent beschreven strategieën gebaseerd op cellijnen met lekkagevrije, induceerbare expressiesystemen een oplossing voor dit probleem [27, 28].

Schadelijke neveneffecten
Een nadelige en vaak onderschatte factor op het gebied van gentherapie is dat de introductie van een vreemd therapeutisch eiwit een immuunrespons kan veroorzaken. Deze bijwerkingen kunnen niet alleen het gentherapeutisch effect tenietdoen, maar kunnen ook gevaar met zich meebrengen. Een onvoorziene CTL respons tegen een geïntroduceerd antigeen dat in grote aantallen getransduceerde hepatocyten aanwezig is zou tot aanzienlijke leverschade kunnen leiden. Het opwekken van antilichamen tegen het geïntroduceerde antigen wordt als minder gevaarlijk beschouwd en is vaak alleen van invloed op de functionaliteit van het door gentherapie geïntroduceerde eiwit. Hier kan een onderscheid worden gemaakt tussen eiwitten die worden uitgescheiden in de bloedbaan (bijv. F.IX of GAA) welke door antilichamen in hun functie worden beperkt, en eiwitten die intracellulair aanwezig blijven (bijv. UGT1A1) en geen nadelig effect van antilichamen ondervinden. Aan de andere kant is de lever, immunologisch gezien, een bijzonder orgaan dat heeft geleerd verschillende antigenen die het lichaam via de darmen binnenkomen te tolereren [29]. Een hoog epressieniveau van een geïntroduceerd transgen in lever is eveneens in verband gebracht met de inductie van tolerantie [30]. Het voorkomen van de expressie door lentivirale vectoren in off-target getransduceerde leukocyten door middel van een endogene miRNA sequentie in combinatie met levergerichte genoverdracht bleek eveneens immuuntolerantie te induceren [6].

Een voordeel van de toepassing van shRNA expressie vectoren is dat deze niet gepaard gaan met de expressie van potentieel immunogene eiwitten. Er kleven echter een aantal andere mogelijke nadelen aan gentherpie strategieën gebaseerd op RNAi [31]. Cytoplasmatisch dsRNA en specifieke sequenties rond de transcriptie start van op polymerase III gebaseerde shRNA expressiesystemen kunnen leiden tot inductie van de interferon (IFN) cascade. Echter, dit lijkt gedeeltelijk verholpen te kunnen worden door de expressie van RNAi sequenties te realiseren door ze te incorporeren in natuurlijk voorkomende pri-miRNAs. Tevens is de miRNA pathway een belangrijk biologisch regulatiemechanisme en de overexpressie van shRNA sequenties is in verband gebracht met letale levertoxiciteit bij muizen [11]. Hier zijn shRNAs en gemodificeerde pri-miRNAs wellicht meer geneigd de endogene RNAi pathway te blokkeren dan synthetische siRNAs, omdat de blokkade plaatsvindt op het niveau van nucleair export van kleine RNAs.

Klinische trials en toekomstige toepassingen
De eerste klinische trial met lentivirale vectoren is uitgevoerd voor de bestrijding van HIV-1 infectie. Hierbij werd gebruikt gemaakt van transplantatie van autologe CD4+ T cellen getransduceerd met een lentivirale vector welke de expressie van een antisense RNA gericht tegen het HIV-1 *env* gen [32]. Deze trial heeft de weg voor lentivirale vectoren naar de kliniek vrijgemaakt op het gebied van GMP vector productie en regelgeving [33]. De hier gebruikte gentherapeutische aanpak wordt relatief veilig geacht, aangezien er geen transductie van langdurig levende of actief delende cellen plaatsvindt en de expressie van neo-antigenen is niet aan de orde. Een tweede klinische trial gaat een stap verder en richt zich op transplantatie van lentiviraal gecorrigeerde HSC's voor de behandeling van X-gebonden adrenoleukodystrofie. Voorlopige resultaten zijn succesvol en zonder negatieve effecten [34].

Met ingang van september 2008 zijn een aantal andere klinische trials gebaseerd op HSCT in combinatie met lentivirale genenoverdracht goedgekeurd voor de behandeling van: bèta-thalassemie major, sikkelcelanemie, Wiskott-Aldrich syndroom, mucopolysaccharidose VII en Fanconi anemie (http://www.wiley.co.uk/genetherapy/clinical/). Deze studies zullen naar verwachting een verder licht werpen op de algemene veiligheid van lentivirale vectoren in termen van genotoxiciteit. Als integratie-gerelateerde mutagenese niet aantoonbaar is, kan dit het gebruik van gamma-retrovirale vectoren overbodig maken en andere toepassingen op basis van deze strategie zullen dan waarschijnlijk snel volgen. Bovendien zal een succesvol en veilig protocol voor genoverdracht naar HSC’s naar verwachting resulteren in nieuwe investeringen om de productiecapaciteit voor GMP lentivirale vector batches te vergroten.

Onlangs is er aanzienlijke vooruitgang geboekt op het gebied van toediening van AAV-vectoren aan de lever en de eerste gedeeltelijk succesvolle klinische trial met dit vectortype is

reeds uitgevoerd [35]. Deze trial ging gepaard met de inductie van een CTL respons gericht tegen het vector capside van AAV serotype 2 in getransduceerde hepatocyten. In toekomstige trials zou dit probleem kunnen worden omzeild door gebruik te maken van een ander vector serotype of tijdelijke immuunsuppressie op het moment van vectortoediening. De stabiliteit van op AAV-gebaseerde genoverdracht in de lever op de lange termijn dient nog te worden afgewacht. De instabiliteit die inherent is aan het episomale karakter van op AAV-gebaseerde genoverdracht kan herhaalde vectortoediening enkele jaren na de eerste behandeling noodzakelijk maken. Het verdunningseffect wordt hier veroorzaakt door mitose en kan worden versterkt door het feit dat AAV-afgeleide transgenen concatameren vormen en de therapeutische respons afhangt van een hoog expressieniveau in een beperkt aantal getransduceerde cellen. Algemene infecties met bijvoorbeeld Epstein-Barr virus (EBV) of cytomegalovirus (CMV) verhogen de mitotische index van hepatocyten en dit kan resulteren in een progressief verlies van het transgen waardoor een stofwisselingsziekte, inclusief alle daarmee gepaard gaande gevaren, acuut terug kan keren.

De toekomst van systemische toediening van lentivirale vectoren voor levergerichte gentherapie van erfelijke stofwisselingsziekten zal grotendeels afhangen van de hierboven genoemde klinische trials. Echter, voor de preventie van HCV herinfectie in transplantatielevers bieden lentivirale vectoren nog steeds het beste alternatief en verdere studies zijn nodig om deze potentievolle behandelingsoptie verder uit te werken. Mogelijk kan de toepassing van gentherapie in transplantatielevers in de toekomst worden ingezet om de levensduur van het transplantaat te verlengen en afstoting te voorkomen. Dit kan leiden tot een heroverweging van de huidige immunosupressie regimes of verbetering van de resultaten van transplantatie met donorlevers van een lagere kwaliteit afkomstig van zogenaamde “non-heart beating” donoren.

Lentivirale vectoren zullen naar verwachting ook toepasbaar zijn op het gebied van levercel transplantatie. Ze zouden kunnen worden ingezet om het expressieniveau van een bepaald deficiënt eiwit in gedifferentieerde donor hepatocyten te verhogen om zo het aantal cellen benodigd voor transplantatie te verminderen. Voor de ziekte van Crigler-Najjar is aangetoond dat in de Gunn rat een transplantatieniveau van 1% met UGT1A1 over-expresserende hepatocieten in de lever een significant positief effect op het plasma bilirubineniveau kan bewerkstelligen [36]. De snelle ontwikkelingen op het gebied van stamceltechnologie zouden in de toekomst transplantatie met genetisch gemodificeerde autologe stamcellen mogelijk kunnen maken [37]. Deze aanpak heeft als voordeel dat stamcellen kunnen worden transduceerd met een kleine hoeveelheid vector en vervolgens kunnen worden geëxpandeerd. Afstoting van de getransplanteerde cellen is niet aan de orde en het risico van acute toxiciteit na vectortoediening en transductie van geslachtscellen wordt uitgesloten. Echter, de ontwikkeling van een efficiënt en veilig klinisch-relevant protocol voor leverceltransplantatie laat nog op zich wachten [38]. Bovendien kleven er een aantal mogelijke risico’s aan de expansie van stamcellen en de transductie van deze cellen met een integrerende lentivirale vector. Deze risico’s dienen zorgvuldig te worden afgewogen tegen de morbiditeit en letaliteit van de te behandelen ziekte.

6.6 Algemene conclusie

In deze studie hebben we het gebruik van lentivirale vectoren voor de behandeling van zowel erfelijke als niet-erfelijke leverziekte voor gentherapie doeleinden onderzocht. We onderbouwen en bevestigen het feit dat lentivirale vectoren veelbelovende therapeutische hulpmiddelen voor levergerichte genoverdracht zijn. De vertaling van deze behandelmethoden naar de kliniek lijkt haalbaar, maar daartoe dient eerst een aantal problemen te worden opgelost. Zo is er allereerst verder onderzoek nodig naar het meest optimale vectorontwerp om zo een veilige, efficiënte en langdurige expressie van het transgen te bewerkstelligen. Daarnaast dient de productiemethode voor lentivirale vector preparaten van voldoende kwaliteit voor het uitvoren van (pre-)klinische studies te worden verbeterd. Nog belangrijker is verder onderzoek naar het risico van mutagenese door genomische integratie van lentivirale vectoren en naar mogelijke strategieën om dit risico te beperken. Ten slotte dient, afhankelijk van de specifieke gentherapietoepassing, de meest effectieve en veilige methode van vector toediening te worden bepaald.

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Curriculum Vitae

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van der Wegen P, aan de Wiel-Ambagtsheer G, van der Laan LJ, ten Hagen TL, Scholte BJ, Liver-restricted administration of lentiviral vector results in significant reduction of plasma bilirubin levels in the adult Gunn rat. In preparation

Grants

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International conferences	
- 2nd World Congress of Pediatric Gastroenterology, Hepatology and Nutrition, Paris, France, July 3-7, 2004 (presentation)	2004
- Tenth Annual Meeting of the American Society of Gene Therapy, Seattle, USA, May 30-June 3, 2007 (poster)	2007
- XVth Annual Congress of the European Society of Gene and Cell Therapy, Rotterdam, The Netherlands, October 27-30, 2007 (presentation and poster)	2007

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