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DEVELOPING USE OF RECOMBINANT LENTIVIRAL VECTORS FOR DELIVERY OF ANTI-HEPATITIS B VIRUS MICRO RNA MIMICS

Dejana Ivacik

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree

of

Doctor of Philosophy

Johannesburg, 2014

DECLARATION

I, Dejana Ivacik declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……. (Signature)

…….....day of ….....……, 2014

DEDICATION

Lord, help make my intelligence amenable to all truths, give me the wisdom to know the meaning and purpose of my learning, so that my knowledge, understanding, and skills may help in some little ways to make this world a better place, for Your greater glory.
Amen.

"We can do no great things; only small things with great love."
Blessed Mother Teresa of Calcutta

To my husband Nicholas

A tribute for providing the inspiration to follow my dreams and being my everlasting source of hope, love and strength.

PUBLICATIONS AND PRESENTATIONS RELATED TO THESIS

Journal Publications

1. Ivacik, D, Ely, A, and Arbuthnot, P (2011). Countering hepatitis B virus infection using RNAi: how far are we from the clinic? *Rev Med Virol* **21**: 383-396.

2. Pichard, V, *et al.* (2012). Specific Micro RNA-Regulated TetR-KRAB Transcriptional Control of Transgene Expression in Viral Vector-Transduced Cells. *PLoS One* **7**: e51952.

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Book Chapters

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Conference Proceedings

1. South African Society of Biochemistry and Molecular Biology Congress. University of the Free State, Bloemfontein, South Africa 18th -20th January 2010.

2. Viral Vectors in Gene Therapy: Applications and Novel Production Methods. A.I. Virtanen Institute, University of Eastern Finland, Kuopio, Finland 26th August - 4th September 2010.

3. European Society for Gene and Cell therapy and the British Gene Therapy Society Congress 2011. The Brighton Centre, Brighton UK, 27th - 31st October 2011.

4. University of the Witwatersrand / Faculty of Health Sciences Research Day and Postgraduate Expo, 19th September 2012.

5. The 20th anniversary collaborative congress of the European Society for Gene and Cell Therapy and French Society of cell and gene therapy. Versailles, Paris, France, 25th-29th October 2012.

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ABSTRACT

Persistent infection with hepatitis B virus (HBV) is associated with an increased risk for development of serious life-threatening complications such as cirrhosis and hepatocellular carcinoma (HCC). This common malignancy is aggressive and has a very poor prognosis. Currently available HBV therapies have variable efficacy and do not diminish the risk for development of HCC associated with infection with the virus. Consequently the development of novel therapies aimed at providing effective treatment for chronic HBV infection remains a medical priority.

The therapeutic potential of harnessing the RNA interference (RNAi) pathway to achieve specific and potent silencing of pathology-causing genes has been explored for the development of novel and improved therapy to counter chronic HBV infection. Highly effective expressed anti-HBV RNAi-based sequences have been demonstrated to be capable of mediating impressive silencing of viral gene expression *in vitro* and *in vivo*. Nevertheless, accomplishing stable delivery to hepatocytes and sustained, long-term expression of therapeutic effecters remains the greatest challenge impeding the clinical translation of RNAi-based anti-HBV gene therapy.

Stable integration of transgenes that may be achieved with recombinant lentiviruses, derived from the human immunodeficiency virus type 1 (HIV-1), makes these vectors particularly useful for attaining sustained expression of RNAi activators. This property is suited to countering HBV persistence, making these delivery vehicles advantageous for gene transfer of expressed RNAi anti-HBV sequences to infected hepatocytes. To advance potential clinical application of RNAi-based anti-HBV gene therapy, second generation self-inactivating (SIN) lentiviral vectors were engineered to include liver specific, RNA polymerase II (Pol II) cassettes that generate HBV-silencing primary micro RNA (pri-miR) mimics. The antiviral pri-miR sequences, placed under the

control of the liver-specific mTTR promoter, are derived from natural pri-miR-31 and engineered to generate anti-HBV RNAi guide sequences targeting single (monocistronic pri-miR sequences) or multiple (polycistronic pri-miR sequences) sites within the highly conserved multifunctional HBV X protein (HBx) open reading frame (ORF) of the viral genome. The HBV silencing lentiviruses (LVs) stably transduced liver-derived Huh7 and HepG2.2.15 cells. Northern blot analysis of RNA extracted from stably transduced Huh7 cells verified that the integrated anti-HBV pri-miR sequences were processed to form RNAi-activating guide strands according to the intended design. When stably transduced Huh7 cells were transfected with a HBV replication-competent plasmid, potent inhibition of markers of viral replication was achieved. Sustained, hepatotropic HBV silencing was effected with limited disruption of endogenous miR function. Silencing of a mutated HBV sequence in stably transduced cells was caused by HBV-silencing LV expressing a polycistronic pri-miR effecter. Rigorous evaluation of the anti-HBV efficacy of the polycistronic silencing LV in the HepG2.2.15 HBV replication cell line demonstrated potent antiviral activity.

Preclinical analysis of the efficacy of the RNAi-activating lentiviral vector system was assessed using the HBV transgenic mouse model. HBV silencing LVs encoding a liver-specific polycistronic pri-miR 31/5-8-9 sequence was delivered to young adult animals via a tail-vein injection as well as to neonatal mice via a superficial temporal vein injection. The HBV-silencing LV was capable of mediating stable hepatic delivery and sustained expression of anti-HBV pri-miR sequences *in vivo*. Furthermore, the therapeutic sequences were processed *in vivo* according to the intended design and mediated powerful knockdown of HBsAg, circulating viral particle equivalents and intrahepatic viral RNA without inducing hepatotoxicity. To our knowledge this is the first study to evaluate the efficacy of RNAi-activating anti-HBV lentiviral vectors in the HBV transgenic mouse

model. Although the investigation constitutes a preliminary step towards clinical application of HBV silencing RNAi-based lentiviral vectors, this study has provided proof of principle for a therapeutic strategy that can be potentially useful for the treatment of chronic HBV infection.

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LIST OF SYMBOLS

α	- alpha
β	-beta
γ	-gamma
μ	- micro
Ψ	- psi

LIST OF ABBREVIATIONS

5'-OAS	5'-oligoadenylate synthetase
AAV	adeno associated virus
Ad	adenovirus
ADA	adenosine deaminase
Ago2	Argonaute 2
ALT	alanine transaminase
APCs	antigen presenting cells
ATP	adenosine triphosphate
C/EBP	CCAAT/enhancer binding protein
cccDNA	covalently closed circular DNA
CMV	cytomegalovirus
cPPT	central polypurine tract
CREB	cyclic AMP response element binding protein
DGCR8	DiGeorge critical region 8
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulphoxide
DPBS	Dulbecco's Phosphate-Buffered Saline
ds	double stranded
E. coli	Escherichia coli
EF-1 alpha	the human elongation factor- 1 alpha
eGFP	enhanced GFP
ELISA	enzyme linked immunosorbent assay
endo-siRNAs	endogenous siRNAs

FCS	foetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
hBA	human β Actin
HBcAg	hepatitis B virus core antigen
HBeAg	hepatitis B virus E antigen
HBsAg	hepatitis B virus surface antigen
HBV	hepatitis B virus
HBx	hepatitis B virus X protein
HCC	hepatocellular carcinoma
HDAd	helper dependent Ad
HEK	human embryonic kidney cells
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus type 1
HNF	hepatic nuclear factor
IFN	interferon
ITR	inverted terminal repeats
kb	kilobases
lhRNA	long hairpin RNA
lhRNA	long hairpin RNA
LTR	long terminal repeat
LV	lentivurus
miR	micro RNA
MOI	multiplicity of infection
mTTR	murine transthyretin promoter

MW	molecular weight
nt	nucleotide
NTCP	sodium taurocholate cotransporting polypeptide
ORF	open reading frame
PEG	polyethylene glycol
PEI	polyethylenimine
pgRNA	pregenomic RNA
PIC	pre integration complex
piRNAs	PIWI-interacting RNAs
PKR	protein kinase R
Pol II	RNA polymerase II
pre-miR	precursor miR
pri-miR	primary miR
qPCR	quantitative PCR
rcDNA	relaxed circular DNA
RIG- 1	retinoic-acid-inducible protein I
RISC	RNA induced silencing complex
RNAi	RNA interference
RRE	Rev-responsive element
RSV	Rous sarcoma virus
SCID	severe combined immunodeficiency disorder
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
shRNA	short hairpin RNA
SIN	self-inactivating

siRNA	small interfering RNA
snRNA	small nucleolar RNA
TAR	trans-activation-responsive region
TBE	Tris-Borate EDTA
TLR	toll-like receptor
TRBP	TAR RNA binding protein
TU	transducing units
UTR	untranslated region
VSV-G	vesicular stomatitis virus G glycoprotein
VPEs	viral particle equivalents
WPRE	woodchuck hepatitis virus posttranscriptional regulatory

1 INTRODUCTION

1.1 Hepatitis B virus biology, pathogenesis, epidemiology and current treatment

1.1.1 Hepatitis B virus biology and life-cycle

The hepatitis B virus (HBV) is a member of the hepadnavirus family, a family of hepatotropic viruses with partly double stranded DNA genomes (1). The virus has a relaxed circular (rc), partly double stranded DNA genome consisting of a negative strand of 3.2 kilobases (kb) and a positive strand DNA that is variable in length (2) (Figure 1.1). The infectious virion, also known as a Dane particle (3), consists of an inner nucleocapsid core enclosing the viral genome and an outer lipid envelope in which small, middle and large surface glycoproteins (HBV surface antigens (HBsAgs)) are embedded. In addition to infectious Dane particles, an excess of spherical and filamentous subviral particles, which exclusively consist of a lipid envelope and surface glycoproteins, are found in the blood of individuals infected with the virus. These particles lack the nucleocapsid core, are non-infectious and play an important role in immune evasion as antibody decoys (reviewed in (4)).

The Australia antigen, now known as HBsAg, was the first HBV-specific infection marker identified (5). The discovery of the HBsAg heralded the development of sensitive HBV detection assays commonly used today, such as the HBsAg enzyme linked immunosorbent assay (ELISA) which has become an indispensable tool in the screening and diagnosis of viral infection as well as in monitoring the efficacy of anti-viral therapy (reviewed in (6)). The HBV surface glycoproteins have been the subject of numerous investigations aiming to identify the receptor used by the virus to gain access to

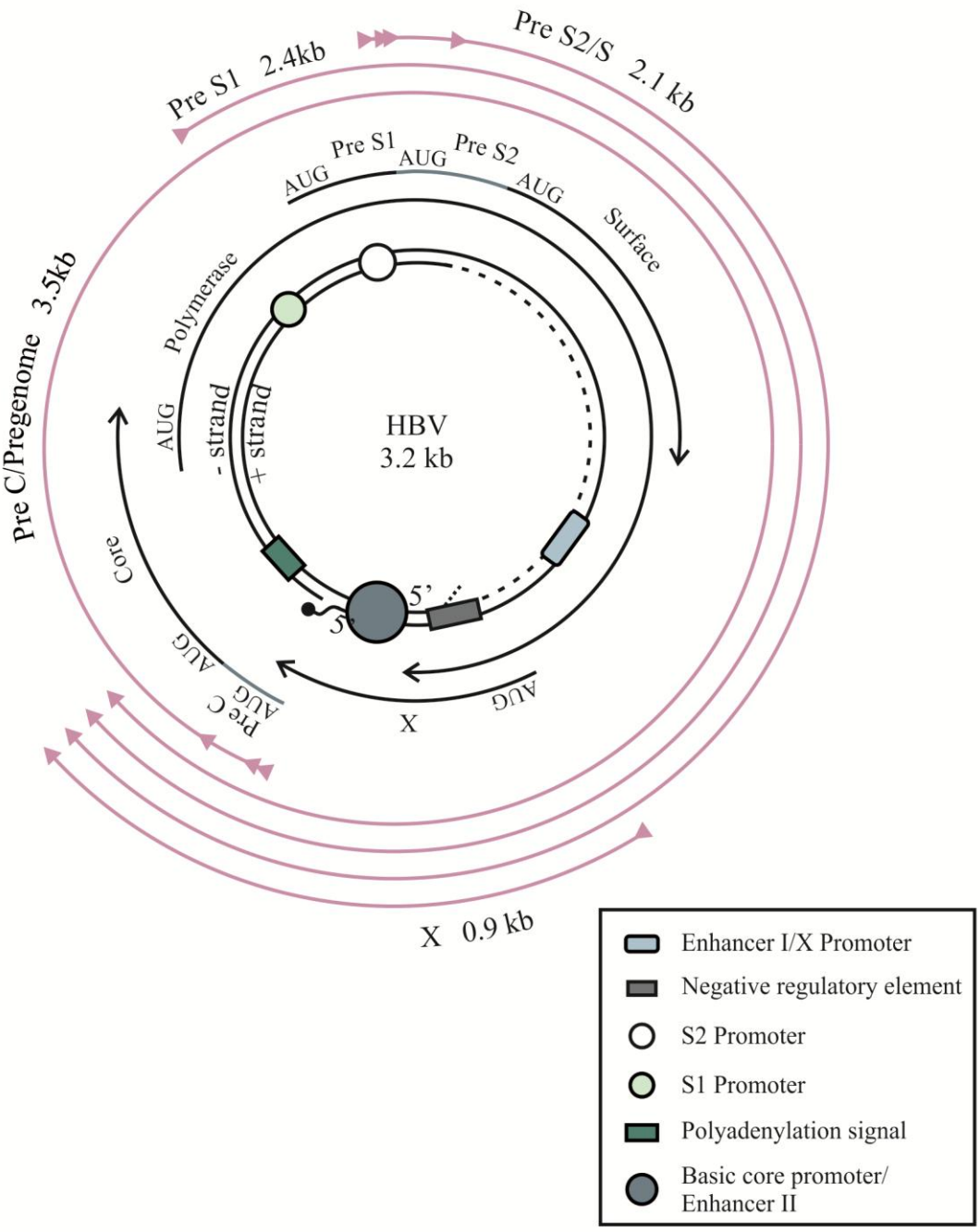


Figure 1.1: <u>Hepatitis B virus genome organisation.</u>

The partially double stranded genome is indicated with transcription regulatory elements. Viral open reading frames are shown as black arrows immediately surrounding the genome. The outermost, pink arrows represent the four major viral transcripts that terminate at a single polyadenylation site. The figure has been adapted from reference (7).

hepatocytes. Recently, the sodium taurocholate cotransporting polypeptide (NTCP) receptor was identified as the receptor used by the virus to enter hepatocytes. This hepatic transmembrane transporter is expressed on the surface of liver cells and interacts with the large surface protein of HBV (8). Following entry via the NTCP receptor, HBV nucleocapsids translocate to the nucleus where the rcDNA is offloaded and subsequently converted to covalently closed circular DNA (cccDNA) (9).

The cccDNA encodes four overlapping open reading frames (ORFs) from which seven HBV viral proteins are synthesised. Four different viral promoters and two enhancers drive unidirectional transcription of four viral RNAs from the cccDNA template (10) (Figure 1.1). A single polyadenylation signal terminates the transcription of all viral mRNAs at a universal 3' end. Viral mRNAs include: the 0.9, 2.1 and 2.4 kb subgenomic mRNAs and the 3.5 kb greater-than-genome length pregenomic RNA (pgRNA). Translation of the 0.9 kb mRNA yields the HBV X protein (HBx), while translation of the 2.1 and 2.4 kb mRNA sequences produces the small, middle and large surface envelope glycoproteins. The 3.5 kb pregenomic RNA serves as the template for reverse transcription as well as translation of polymerase and core proteins (11). An additional viral mRNA exists, which is slightly longer than the pgRNA at its 5' end. This mRNA is translated to the precore/core protein, which is the precursor of secreted HBV e antigen (HBeAg) (11).

The viral polymerase binds covalently to an RNA stem-loop structure at the 5' end of the pgRNA and triggers packaging of the viral pgRNA into core particles (12). Viral replication occurs within nucleocapsids in the cytoplasm and proceeds from reverse transcription of the pgRNA. The viral polymerase has reverse transcriptase activity and acts as a primer for DNA synthesis (13). Encapsidation initiates reverse transcription of pgRNA to synthesize the negative DNA strand. The minus strand serves as template for the synthesis of the plus strand to form a complete rcDNA genome. Nucleocapsids bud

through the endoplasmic reticulum where they are enveloped and soon after secreted from host cells, to continue infection or traffic to the nucleus of the same cell where additional cccDNA is formed and a stable pool of cccDNA is established (14-16). HBV DNA persists in the nucleus of infected hepatocytes as an episomal cccDNA minichromosome (17). In addition, the virus has a propensity to randomly integrate portions of its DNA into the host genome via recombination (18-22).

Since the sophisticated replication strategy employed by HBV hinges on production of the pgRNA, the development of therapeutic strategies that target and/or degrade this RNA replication intermediate is important, as this class of therapeutic agents may potentially deplete intrahepatic cccDNA and counter persistent viral replication.

1.1.2 Epidemiology, immunopathogenesis and HBV-associated hepatocarcinogenesis

HBV is a non-cytopathic human virus that causes both acute and chronic infection of the liver. It is estimated that over 2 billion people have been infected with the virus and more than 350 million individuals have progressed to chronicity (23). Geographically, sub-Saharan Africa, east and south east Asia and the western Pacific islands are the regions most affected by chronic HBV infection, followed by regions in the Amazon and some southern parts of central and eastern Europe (23). Ten diverse HBV genotypes (A-J) have been described (24). These have varied geographical prevalence and have been reported to influence the clinical course of the infection as well as responsiveness to antiviral therapy.

In highly endemic regions, the virus is generally transmitted perinatally from mother to child or horizontally among young children. Parenteral transmission via sexual contact in adults or the use of contaminated needles is more common in areas of low endemicity. While 90% of infants that have acquired the infection at birth and up to 50%

of children that have acquired the infection before the age of 6 will develop chronic hepatitis, clinical symptoms are resolved and protective immunity is developed by the majority of acutely infected adults (reviewed in (25)). Viral infection is usually undetected by the innate immune system and consequently does not result in the induction of a vigorous innate immune response (26). Viral spread following infection is typically slow, however HBV is capable of infecting the entire hepatocyte population in the liver (26-28). Despite the absence of a vigorous innate immune response, an effective adaptive immune response involving the clearance of cells expressing viral antigens by cytotoxic CD8+ T cells is considered responsible for the viral clearance typically observed in immunologically competent adults (28). Nevertheless, failure of the adaptive immune response to mount an efficient response and to clear HBV-infected cells leads to the development of chronic infection. The above has been attributed to evasion and/or inhibition of adaptive immune responses including T-cell anergy, ignorance and exhaustion (reviewed in (29)). High levels of HBsAg in chronically infected individuals contribute to exhaustion of the functional HBV-specific cytotoxic T cell reservoir and a blunted T-cell response which results in viral immunotolerance instead of viral eradication (30, 31). In addition, specific HBV proteins have been proposed to function in inducing tolerance to adaptive anti-HBV immune responses. The X protein has been reported to be capable of mediating inhibition of the cellular proteosome which can result in reduced antigen processing and presentation (32). The HBeAg has been proposed to play a role in viral persistence by suppressing HBV core antigen (HBcAg)-specific T cell responses and inhibiting the clearance of HBcAg-expressing infected cells (33). Finally, viral persistence induces an immune-mediated, chronic necroinflammatory disease which has been identified as one of the triggers of HBV-induced hepatocarcinogenesis (34). Together hepatitis B and C virus infection are the two major risk factors for the development of liver

cancer (35). Individuals who are chronically infected with the virus are at an increased risk of developing hepatocellular carcinoma (HCC) and cirrhosis (36). Together hepatitis B and C virus infection are the two major risk factors for the development of liver cancer (35). Worldwide, HCC is the second leading cause of cancer-related death in men and the sixth leading cause of cancer-related death in women (37). Chronic HBV infection is commonly observed in the rural, black South African population (38, 39). Approximately 10 % of individuals in the South African black population have been chronically infected with HBV and infection with the virus is typically the cause of majority of cases of HCC that occur in the respective population (38, 39). Unfortunately, HCC is one of the most aggressive malignancies and it has a very poor prognosis (40). Viral mutations (41), age, gender, HBeAg status, serum HBsAg, HBV DNA levels (42, 43) as well as the viral genotype are among some of the factors that influence the development and severity of chronic inflammation and HCC (reviewed in (44)). For instance, infection with subgenotype A1 (45), which is endemic in South Africa, and genotype C (46) has been reported to be associated with high risk for developing liver cancer.

Several mechanisms have been proposed to play a role in the pathogenesis of HBV-associated HCC. As mentioned previously, HCC may develop as a result of virus-induced chronic necroinflammatory disease (34). In addition, oncogenic gene transactivation by the carcinogenic HBx protein as well as insertional mutagenesis mediated by HBV DNA integration have been implicated in the onset and progression of HCC (19, 47-49). HBV integration promotes tumorigenesis via three mechanisms. These include virus-mediated *cis* transactivation of oncogenes or altered activity of tumour suppressor genes (49-51), enhanced genomic instability (19) and lastly the generation of mutated viral proteins with carcinogenic potential such as truncated HBx and large surface proteins (52, 53). The critical function of the HBx protein in the induction of HBV-associated HCC has been

highlighted in a plethora of reports (reviewed in (47)). HBx has been proposed to promote carcinogenesis by several mechanisms. This oncogenic protein is well known for its ability to induce transactivation of host and viral genes involved in cell survival and proliferation by enhancing transcription directly or modulating the activity of various transcription factors, such as the Yes-associated protein (54), cyclic AMP response element binding protein (CREB) (55) and p53 (56, 57). The interaction of HBx with the UV-damage DNA binding factor UV-DDB1, a protein that plays a key role in cellular DNA repair, has been well documented and reported to contribute to malignant cell transformation (58, 59). Other mechanisms of HBx-mediated induction of tumorigenic transformation include: the activation of signalling pathways involved in cell survival (60, 61) as well as the stimulation of epigenetic changes (reviewed in (62)).

Globally, chronic HBV infection and associated life-threatening sequelae such as the development of cirrhosis, liver-failure and HCC remain a significant cause of public health problems. A safe and effective vaccine against HBV has been available for over three decades and implementation of mass immunisation on the recommendation of the World Health Organization has indeed contributed to a significant reduction in the rate of chronic infection (23, 63, 64). However, new cases of HBV infection continue to be reported as a result of poor immunisation coverage, particularly in some of the countries with the highest incidence of new infections (65, 66). Moreover, the vaccine is prophylactic and although it has reduced the number of new infections, it does not benefit individuals that have already developed chronic HBV infection.

1.1.3 Current treatment

Currently seven therapies are licensed for use in the management of chronic HBV, these include: immunomodulators (interferon-α (IFN-α) 2b and IFN-α 2a modified with

polyethylene glycol (PEG) (PEG-IFN-α 2a)), nucleoside analogues (lamivudine, telbivudine and entecavir) and nucleotide analogues (adefovir dipivoxil and tenofovir) (67). As a natural immunomodulator, the use of interferon in the treatment of HBV has a long-standing history (68). Standard interferon has been replaced with PEG-modified interferon which has an improved pharmacokinetic profile and hence improved efficacy (69, 70). IFN exerts its antiviral effect by two distinct mechanisms (71). Firstly, interferons stimulate the expression of numerous genes leading to the initiation of multiple antiviral pathways and inhibition of viral replication. Secondly, interferon amplifies the cellular immune response against HBV-infected hepatocytes by stimulating the activity of B cells and cytotoxic T cells. However, interferon treatment causes sustained antiviral effects in only a third of patients who have chronic HBV and its use is associated with numerous side effects thus limiting the tolerability of the treatment (68, 69).

In contrast to interferon, nucleoside/nucleotide analogues have an improved safety profile and can be taken orally. The nucleoside/nucleotide analogues resemble endogenous nucleotides and are phosphorylated following cellular uptake. Phosphorylated nucleoside/nucleotide analogues are subsequently incorporated into the growing viral DNA chain and function as chain terminators or by direct inhibition of HBV polymerase. This class of therapeutic agents has been shown to be capable of mediating potent reduction of viraemia in chronically infected patients. Nevertheless, long-term treatment with nucleoside/nucleotide inhibitors, especially lamivudine and adefovir, has been shown to be associated with the emergence of viral escape mutants (72-74). Although some of the recently approved therapeutic agents from this class seem to have a somewhat improved barrier to resistance (75, 76), preclinical studies have shown that long-term administration of nucleoside/nucleotide analogues does not lead to complete clearance of cccDNA from infected cells (77, 78). As a result, treatment interruption results in recurrence of viraemia,

limiting the curative efficacy of these agents (reviewed in (79)). Currently available HBV treatments are costly, have limited therapeutic efficacy and their use is associated with the emergence of viral escape mutants. Consequently the development of novel therapies aimed at providing effective treatment for chronic HBV infection remains a medical priority. Novel and improved therapies should ideally be safe, provide a high barrier to emergence of escape mutants and exhibit potent and durable silencing of persistent viral replication to avert the progression of cirrhosis and the development HCC. Harnessing the RNA interference (RNAi) pathway to accomplish powerful silencing of pathology-causing genes represents a new avenue that could be exploited for the development of improved therapy to counter chronic HBV infection.

1.2 Harnessing RNA interference to counter chronic HBV

1.2.1 The endogenous RNAi pathway

The RNAi pathway was originally discovered by Fire and colleagues, following their observations of potent gene silencing activity consequent to the introduction of double-stranded (ds) RNA molecules into the nematode *Caenorhabditis elegans* (80). Subsequent studies identified RNAi as a highly-conserved posttranscriptional gene silencing mechanism which functions in diverse multicellular eukaryotic organisms (81-83). In addition, RNAi was associated with previously documented gene silencing events in plants ('co-suppression') (84) and fungi ('quelling') (85). The molecular mechanism of RNAi was initially elucidated using *Drosophila in vitro* assay systems (86, 87). Introduction of exogenous long dsRNA typically resulted in the generation of small (21 to 23 nucleotide (nt) long) RNA duplexes with characteristic 5' terminal phosphates and 2 nt

3' overhangs. These small dsRNAs or small interfering RNAs (siRNAs) were identified to be the activators of the RNAi pathway.

The applicability and functionality of the RNAi pathway in mammals remained in doubt following the discovery of the novel pathway, as long dsRNA molecules had been shown to stimulate a non-specific IFN response in mammalian cells. Yet, it was not long after the discovery of siRNAs that artificially synthesised siRNA molecules were demonstrated to be capable of activating the endogenous RNAi pathway (Figure 1.2) in mammalian cells without triggering the innate immune system (88). This discovery was set to herald a revolution in the development of novel therapies as the knockdown of any gene of interest in mammalian cells could be achieved with artificially engineered siRNAs.

A new class of ~22 nt long non-coding RNAs, known as micro RNA (miR), was recognised as the principal group of activators of the mammalian RNAi pathway (89). Additional non-coding small RNA sequences capable of activating the RNAi pathway in mammals include endogenous siRNAs (endo-siRNAs) (90) as well as small RNAs that interact with the PIWI subfamily of Argonaute proteins, PIWI-interacting RNAs (piRNAs) (91). Genes encoding miRs have been identified in introns, exons, intergenic regions as well as in antisense orientation to previously described genes (89, 92-94). Since their discovery, miRs have been associated with the regulation of a wide spectrum of physiological and pathological cellular processes such as differentiation, proliferation, apoptosis, development and tumorigenesis (95, 96). Canonical miR biogenesis typically entails two-step processing of primary miR (pri-miR) transcripts to precursor miR (pre-miR) and finally mature miR sequences (Figure 1.2). Pri-miRs are transcribed by RNA polymerase (Pol) II, and have characteristic hairpin motifs. These molecules may contain a single stem-loop (monocistronic) or multiple stem-loops (polycistronic). Initially, pri-miRs are processed in the nucleus to form pre-miR intermediates of 60-80 nt in length.

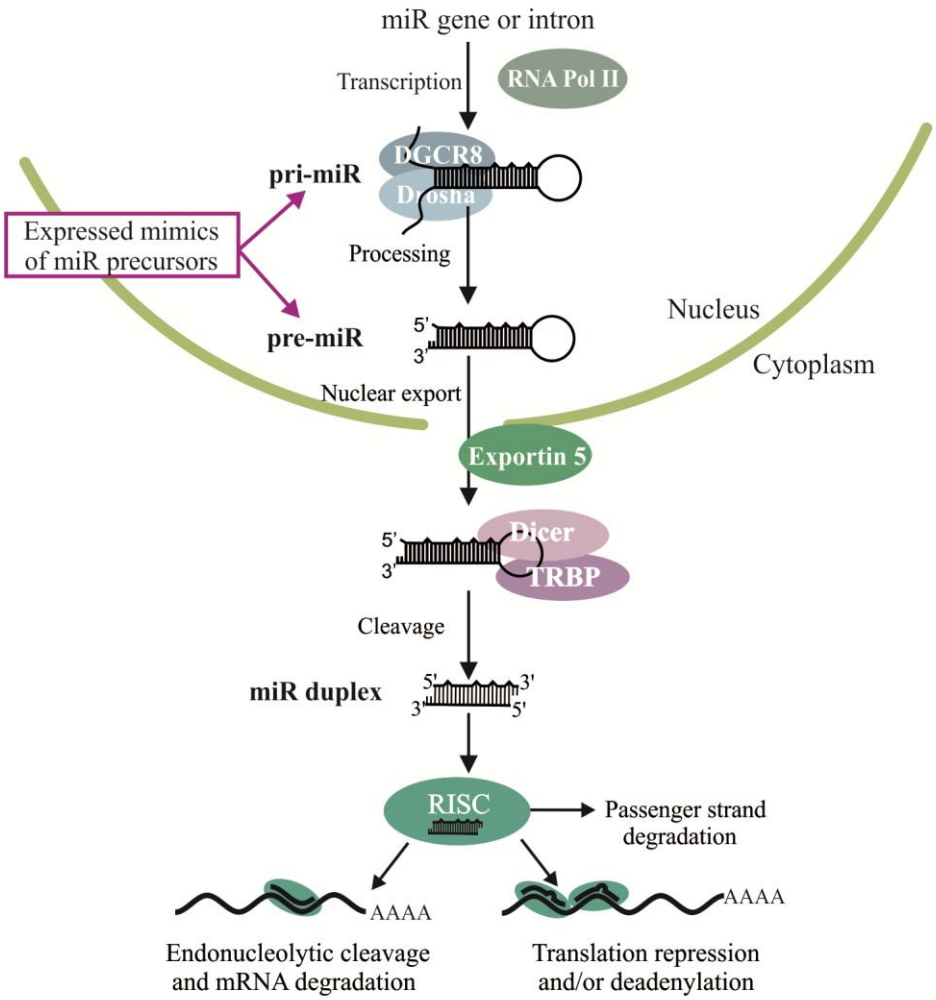


Figure 1.2: <u>The canonical miR biogenesis pathway.</u>

Canonical miR biogenesis involves the production of mature miRs from RNA Pol II-derived pri-miR transcripts. pri-miRs are cleaved in the nucleus by the microprocessor complex (Drosha-DGCR8) to generate pre-miR molecules. Nuclear export of the miR precursors is mediated by the karyopherin Exportin 5. In the cytoplasm, pre-miRs are further processed by the RNase Dicer and its dsRNA binding protein TRBP to mature miR duplexes and loaded into RISC. Once the passenger strand is released, activated RISC is guided by the mature miR guide strand to the target mRNA and effects silencing. The endogenous RNAi pathway can be activated to effect therapeutic silencing following the delivery of exogenous pri-miR and/or pre-miR mimics.

This step is catalysed by the nuclear microprocessor complex, which includes Drosha (an RNase III enzyme) and its dsRNA binding partner, DiGeorge critical region 8 (DGCR8) (94, 97, 98). Drosha-mediated processing of pri-miRs transcribed from Pol II promoters, as well as intron-encoded pri-miRs, occurs co-transcriptionaly (99, 100). Recent studies have revealed the existence of alternative pathways for miR biogenesis, involving generation of miRs from pre-miRs encoded within debranching introns (mirtrons) (101), small nucleolar RNAs as well as tRNA transcripts (reviewed in (102)).

Pre-miRs are exported to the cytoplasm by the Ran-GTP dependent nucleocytoplasmic transport factor Exportin 5 (103) where they are processed by the RNase III endonuclease- Dicer (104). *In vitro* studies have demonstrated that human Dicer cleaves pre-miRs ~22 nt away from the 3' (105) and the 5' end (106), which removes the loop to generate a miR duplex with characteristic dinucleotide 3' overhangs and monophosphorylated 5' ends. Recently published data has also highlighted the importance of the loop position in pre-miRs and shRNAs for accurate Dicer processing *in vivo* (107). This study showed that a distance of 2 nt between the cleavage site and upstream bulge/loop structures is necessary for precise Dicer-mediated cleavage and preventing the generation of heterogeneous miR duplexes. Processed miR duplexes are loaded into the RNA-induced silencing complex (RISC) by Dicer and the trans-activation-responsive region (TAR) RNA binding protein (TRBP) (108). One of the strands in the duplex is selected as the mature miR guide, based on differential thermodynamic stabilities of the RNA duplex ends, while the passenger strand is released and degraded (109). Typically, the strand with lower 5' thermodynamic stability is selected as the guide strand and retained within RISC. The miR guide sequence directs activated RISC to the 3' untranslated (UTR) region of target mRNA to effect silencing by sequence-specific cleavage or translational repression (110). Typically, miRs are partly complementary to

their targets and hybridisation between the seed region of the miR guide sequence (nt 2 to 8 from the 5' end) and target mRNA results in the decrease of mRNA translational efficiency or mRNA deadenylation. Localisation of mRNA associated with activated RISCs within cytoplasmic processing bodies is likely to play an important role for translational repression (111) (reviewed in (110)). Initially, the predominant mechanism of miR-mediated suppression of protein synthesis was thought to be translational suppression of mRNA transcripts (reviewed in (110)). miR-mediated translational suppression can occur at different stages of translation. For example, blocking the recognition of the mRNA 5′-terminal 7-methylguanosine cap (112, 113) or the joining of the 60S ribosomal subunit (114) inhibits translation at the initiation stage. Additionally, translational repression can be caused by slowed elongation or ribosome 'drop-off' resulting in termination of elongation (115, 116). Nevertheless, recent data has revealed that miR-mediated mRNA deadenylation which in turn promotes destabilisation and degradation of targeted mRNA transcripts is accountable for the observed reduction of translation and decrease in protein production (117).

Less frequently, endogenous miR sequences can be completely complementary to their cognate mRNA targets and mediate sequence-specific mRNA degradation. This mechanism is caused by the slicing effecter of RISC, the Argonaute 2 (Ago2) protein (118). The potency and specificity of gene silencing is amplified when the miR guide is perfectly complementary to its cognate mRNA target, a silencing mechanism that has been exploited in the rational design of potentially therapeutic exogenous RNAi activators.

1.2.2 Exogenous RNAi effecters

The natural RNAi pathway can be activated by exogenous RNAi effecters to accomplish specific and potent knockdown of gene expression. These molecules are

delivered as synthetic or expressed RNAi effecters capable of entering and thereby activating the RNAi pathway (119). Typically, activators of the RNAi pathway are designed to be completely complementary to genes of interest to effect sequence-specific degradation of target mRNA. Synthetic siRNA sequences are generally in the form of 21-25 nt duplexes with characteristic 2 nt 3' overhangs and thus resemble Dicer cleavage products that can enter and activate RISC. In contrast, expressed RNAi effecters generally mimic pri-miR or pre-miR sequences and are encoded in DNA cassettes downstream of a Pol III or Pol II promoter. Expressed pri- or pre-miR mimics enter the RNAi pathway as substrates for the microprocessor complex or Dicer/TRBP (Figure 1.2).

The most important considerations for designing potentially therapeutic antiviral RNAi activators concern safety and efficacy. RNAi activators have been shown to cause cytotoxicity by mediating unintended silencing of endogenous genes as well as triggering the innate immune system (120-122).

Cytotoxicity as a result of unintended silencing of endogenous genes may occur as the requirement for miR-mediated silencing is not rigorous and it is limited to hybridisation of the guide seed region (nucleotides 2-8) to mRNA (122, 123). In addition, the passenger strand of the RNAi effecter sequence could potentially be incorporated into RISC and cause unintended silencing. Various chemical modifications are being employed to overcome unintended silencing of cellular transcripts by synthetic siRNAs (reviewed in (124)), some of these have included the incorporation of DNA as well as 2'-*O*-Me modifications of nucleotides in the seed region. Unlike synthetic siRNAs, expressed RNAi effecters cannot be chemically modified to reduce unwanted off-target effects. Design algorithms may be used to facilitate the generation of RNAi effecters with improved safety and efficacy (125). Importantly, the efficacy and safety of expressed RNAi effecters can be improved by optimizing their design to allow for accurate processing and biased

incorporation of the intended miR guide sequence, the sequence designed to have lower thermodynamic stability at its 5' end, into RISC (107, 126-128). Recent data elucidating the mechanism of Dicer-mediated cleavage of shRNAs and pre-miRs (107) has highlighted some important features that should be incorporated in the design of therapeutic shRNAs/pre-miRs, such as the inclusion of an optimal distance between the cleavage site and upstream loop or bulge structures. Inaccurate Dicer processing can result in the generation of mature miR guides with altered seed region as well as the duplex ends (107, 129), which are likely to mediate non-specific silencing of untargeted genes.

In addition to non-specific gene silencing, therapeutic RNAi effecters can induce innate immune responses. The factors responsible for recognising foreign RNA or DNA generated during viral infection (reviewed in (130, 131)) have been shown to be triggered by exogenous RNAi activators particularly synthetic siRNAs. Non-viral vector-delivered synthetic siRNAs enter target cells via endosomal compartments and as a consequence activate Toll-like receptors (TLRs) and subsequent secretion of inflammatory cytokines (type I interferon, interleukin-1/6 and tumour necrosis factor α). siRNA activation of TLR 3, TLR 7 and TLR 8, which are activated by dsRNA sequences or RNA sequences with guanosine and/or uridine rich sequence motifs, has been reported to trigger innate immune responses (120, 121). A number of different chemical modifications of siRNAs can be utilised to attenuate unwanted immunostimulatory effects (reviewed in (132)) .

In contrast to non-viral vector delivered siRNAs, RNAi activators encoded in various expression systems circumvent TLR activation as they originate in the nucleus (133). Nevertheless, CpG motifs, frequently present in DNA expression cassettes, can trigger an immune response via TLR 9 signalling (134). A recent study has demonstrated that shRNA expressed from an integrated lentiviral vector exhibits little or no unintended immunostimulatory effects compared to equivalent synthetic siRNA sequence (135).

In addition to TLR receptor activation, expressed RNAi effecters may also activate other intracellular receptors responsible for detecting foreign RNA, including the RNA helicase retinoic-acid-inducible protein I (RIG-1), the protein kinase RNA-activated receptor (PKR), 5'-oligoadenylate synthetase (5'-OAS) and the melanoma differentiation-associated protein 5 (MDA5) (136-138). Nevertheless, traditional siRNA sequences and Dicer-generated miR mimics possess 2 nt 3' overhangs and resemble self RNA, thus these sequences are non immunostimulatory (139) and avoid detection by intracellular receptors (140).

Several groups have developed synthetic anti-HBV siRNA effecters and these have been demonstrated to mediate potent inhibition of viral replication (141-143). Efficient delivery of therapeutic siRNA effecters can be accomplished with the aid of synthetic conjugates or non-viral vectors (reviewed in (144)). Nevertheless, siRNA-mediated knockdown is transient, necessitating repeated administration to maintain a durable therapeutic effect. Accomplishing durable silencing is critical for successful treatment of persistent viral infections, such as chronic HBV. As a result, the use of synthetic siRNA technology for anti-HBV RNAi-based gene therapy is limited. Unlike siRNAs, expressed anti-HBV RNAi effecters can be incorporated into viral vectors to enable long-term expression of therapeutic sequences as well as durable silencing following a single administration. These properties make expressed RNAi effecters particularly useful to counter chronic viral infections such as HBV.

1.2.3 Developing RNAi-based anti-HBV gene therapy

A plethora of reports has provided overwhelming evidence that RNAi effecters can be exploited to knockdown virtually any gene of therapeutic interest (reviewed in (145-147)). Indeed, just a decade after its discovery, RNAi-based therapeutic agents were being

evaluated for their efficacy in a number of clinical trials, highlighting the potential of this exciting novel technology to transform conventional therapeutic approaches (119, 148, 149). Harnessing the RNAi pathway can be a potentially effective strategy to develop improved therapeutics against chronic viral diseases that cannot be effectively treated with conventional therapies (150-154). Antiviral RNAi strategies have typically involved targeting the genome of RNA viruses, RNA intermediates of DNA viruses, transcripts encoding essential viral genes or host factors necessary for viral replication (155).

The replication cycle of HBV, involves the use of an RNA intermediate which makes the virus amenable to RNAi-mediated silencing. The compact arrangement of the HBV genome, embodied by its small size and partially overlapping ORFs limits the ability of the virus to alter its genetic sequence. In addition, HBV has a single polyadenylation signal and thus all major viral transcripts terminate at a common 3' end. The aforementioned points allow for the development of RNAi-based effecters capable of targeting highly conserved viral sequences with a shared 3' end thus enabling simultaneous silencing of all HBV mRNAs while limiting the potential of escape mutants.

Importantly, successful application of this novel technology can be potentially effective in overcoming some of the major limitations of currently licensed treatments for chronic HBV infection. This includes the inability of licensed therapy to eradicate or cause sustained suppression of chronic viral replication or provide a high barrier to emergence of escape mutants. The enormous potential of RNAi to silence pathology-causing genes as well as the amenability of HBV to therapies based on nucleic-acid hybridisation prompted the development of RNAi-based approaches for the treatment of chronic HBV infection (153, 156-158).

1.2.4 Engineering expressed effecters for RNAi-based anti-HBV gene therapy

To date a number of different strategies employing expressed RNAi effecters has been utilised to accomplish knockdown of HBV gene expression (153, 156, 157, 159, 160). The expression of shRNA sequences from Pol III promoters, such as the U6 and H1 transcriptional elements, was the initial method employed to activate the RNAi pathway with expressed RNAi effecters (161, 162) (Figure 1.3). This approach has been applied extensively to induce RNAi and several groups have demonstrated potent knockdown *in vitro* and *in vivo,* using anti-HBV shRNA sequences expressed from Pol III promoters (153, 156-158). Since shRNAs are structurally very similar to pre-miRs, these sequences enter the RNAi pathway as substrates of Dicer, are subsequently processed to siRNAs, incorporated into RISC and mediate silencing of target mRNA. shRNA technology was further adapted for the expression of anti-HBV long hairpin RNA (lhRNA) sequences, characterised by long (62 bp) stem regions (163) (Figure 1.3). lhRNAs are processed by Dicer into multiple siRNA sequences, allowing for simultaneous targeting of several genes, which is potentially beneficial if long-term silencing of a mutable virus is required (163, 164). However, processing of individual siRNAs across the span of the lhRNA duplex is not equally effective resulting in varying concentrations of mature miR guides and consequently variable silencing efficacy.

U6-driven expression of these types of sequences, especially of shRNAs, has however been shown to result in severe toxicity *in vivo* as a consequence of saturation of components of the RNAi pathway (165, 166). Overexpression of shRNA sequences *in vivo* was demonstrated to result in saturation of Exportin 5 leading to perturbation of endogenous miR function and ultimately fatality in the mice (165). A subsequent study revealed that saturation of the Argonaute protein family also plays a role in

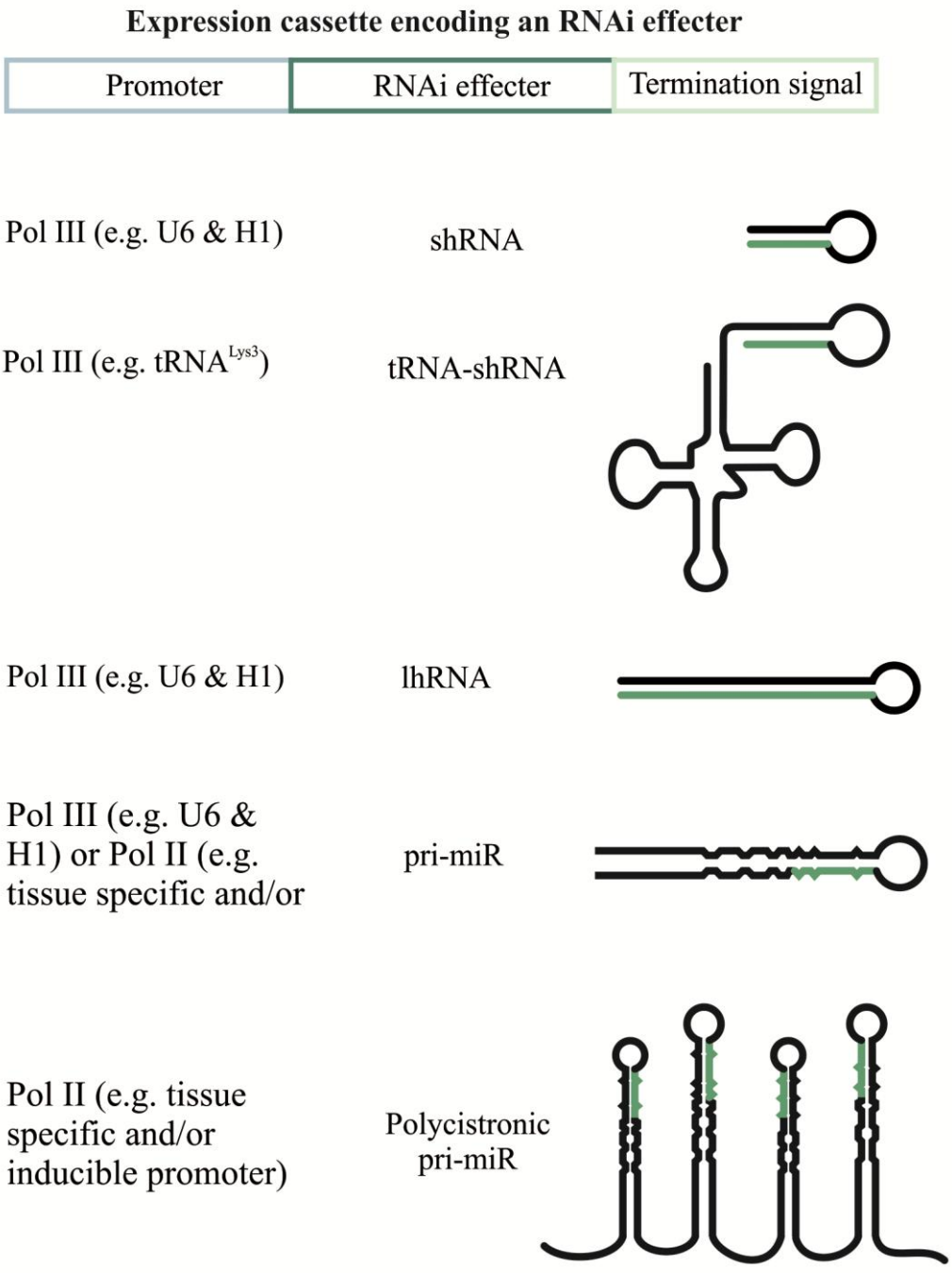


Figure 1.3: <u>The evolution and design of expression systems encoding RNAi effecters.</u>

Different RNAi effecters are shown in order of increasing safety and efficacy beginning with Pol III compatible shRNA effecters. Schematics of the transcript structure are shown with encoded guide sequences highlighted in green. Promoters compatible with the individual effecters are indicated.

shRNA-induced toxicity *in vivo* (166). These studies highlighted the need to improve the existing RNAi expression systems. Optimising RNAi expression systems has involved altering the promoter sequence as well as designing potent RNAi activators which exert a therapeutic effect at a low concentration. Alternative promoters, such as the inherently weaker H1 Pol III promoter (167), as well as tRNA promoters which generate a tRNA/shRNA hybrid that circumvents Exportin5-mediated nuclear export (168-170), have been used as a safer alternative to Pol III promoters (Figure 1.3). Moreover, Pol II promoters have been incorporated into RNAi-based expression cassettes in an attempt to overcome overexpression of RNAi effecters and saturation of cellular RNAi components (171) (Figure 1.3). Pol II promoters allow for exogenous control over the onset and level of transgene expression as well as tissue-specific and inducible expression, enabling spatial and temporal control of gene expression (172-174).

The functionality of a number of different Pol II transcriptional sequences in hepatocytes has been explored. The widely used, promiscuous Pol II cytomegalovirus (CMV) immediate-early enhancer promoter mediates effective transgene expression in hepatocytes (175) and its utility and functionality in driving efficient RNAi effecter expression in the liver has been established (160). Nevertheless shutdown of CMV promoter activity in the murine liver has been reported (176, 177), making this promoter unsuitable for applications requiring long-term transgene expression. Mammalian Pol II promoters, such as the ubiquitously expressing human elongation factor-1 alpha (EF-1 alpha) promoter, can be used to drive robust transgene expression in hepatocytes (178). Ideally, the use of hepatospecific promoters (179, 180) would enable efficient and controllable expression of therapeutic anti-HBV RNAi effecters in hepatocytes while limiting unintended off-target effects in non-targeted tissues. Investigations aimed at improving the safety profile of RNAi expression systems ultimately led to the development

of the novel Pol II expression systems encoding siRNA guide sequences embedded within naturally occurring pri- or pre-miR scaffolds (159, 160, 181, 182). Utilisation of Pol II-expressed artificial pri-miR mimics allows for low level expression and efficient processing of desired RNAi effecters, limiting the risk of potential saturation of the endogenous miR biogenesis pathway (159, 160, 181). In addition, pri-miRs often occur as polycistrons, a property that has been exploited to generate polycistronic pri-miR mimics capable of targeting multiple targets (159, 182, 183). The efficacy of novel, artificial miR-based expression systems encoding multiple miRs has to be determined empirically. Silencing may be influenced by the selection of the natural miR scaffold (159), the number and sequential order of individual miR effecters encoded in the scaffold (159, 184, 185) as well as potential recombination between equivalent sequences in multimeric scaffolds when used in the context of lentiviral vectors (183, 185, 186).

The use of a multitargeted RNAi approach is a potentially beneficial strategy for long-term gene silencing of chronic HBV infection. HBV replication is error prone as it involves the use of reverse transcriptase, which lacks proofreading activity (187), and emergence of viral escape mutants has been reported when RNAi effecters targeted against a single HBV site are utilised (188). Nevertheless, HBV has a limited sequence flexibility as a result of its overlapping ORFs and a considerably lower rate of emergence of escape mutants compared to other viruses, such as the human immunodeficiency virus (HIV), that replicate using reverse transcription of their genomes (189). It has been reported that effective inhibition of HIV using a multi-targeted RNAi-based approach required simultaneous expression of four potent effecters (182). Accordingly, it is likely that an effective anti-HBV RNAi-based multi-targeted approach would require simultaneous expression of less than four effecters. The use of highly efficacious Pol II-expressed pri-miR mimics derived from natural pri-miR-31 targeting a single (monocistronic) (160) and

multiple (polycistronic) sites (159) in the conserved *HBx* ORF was explored in the present set of research endeavours to develop an effective RNAi-based therapy for HBV. The *HBx* sequence had been selected as it is common to all viral mRNAs enabling simultaneous targeting of all transcripts. In addition, HBx plays a key role in the establishment of viral infection (190), viral replication (191) as well as the development of HBV-associated hepatocarcinogenesis (7).

Despite the availability of highly efficacious RNAi-based anti-HBV expression systems (159, 160), the development of a clinically relevant RNAi-based therapeutic strategy for the treatment of chronic HBV is dependent on the availability of an efficient delivery system capable of mediating stable hepatic delivery and durable expression of the therapeutic molecules.

1.3 Viral vector delivery of expressed anti-HBV RNAi effecters

Recombinant viral vectors mediate effective transduction of target tissues and could be exploited to accomplish efficient hepatic transfer of expressed anti-HBV pri-miR effecters. Adenoviruses (Ads), adeno-associated viruses (AAVs) and retroviruses (gammaretroviruses and lentiviruses (LVs)) have been engineered to be effective vehicles for the delivery of therapeutic genes. These vectors are increasingly being used for various preclinical and clinical gene therapy applications on account of their unique features (192-194). These include the ability to integrate in the host genome and enable sustained expression of therapeutic genes, ease of preparation, broad and/or cell-specific tissue tropism, safety and immunogenicity. Viral vector selection for any application will depend on the nature of the disease as well as the desired therapeutic outcome. Accomplishing effective silencing of a chronic viral infection, such as HBV, would require efficient

transduction of the majority of hepatocytes coupled with durable expression of antiviral RNAi activators to achieve sustained silencing of HBV gene expression.

1.3.1 Adenoviral vectors

Recombinant adenoviral vectors are naturally hepatotropic and are capable of transducing hepatocytes *in vivo* with high efficiency, making them attractive candidates for delivery of expressed anti-HBV RNAi sequences (157, 158, 195, 196). In addition, adenoviral vectors are capable of infecting dividing as well as non dividing cells, do not integrate in the host genome, have very high transgene capacity and are amenable to large-scale, high titre production (197). Consequently, recombinant adenoviral vectors have become a frequently utilised delivery tool for various gene therapy applications. First-generation adenovirus vectors, commonly derived from Ad 2 or Ad 5 serotypes, have been generated by substituting the immediate early gene E1 with therapeutic DNA. Deletion of E1 results in a replication defective genome. Production of viral particles requires supplementation of E1 *in trans*, usually provided by human embryonic kidney 293 (HEK293) packaging cells (198). Unfortunately, the use of these vectors has been shown to cause direct cytotoxicity and transient gene expression as a result of cytotoxic T-cell mediated destruction of transduced hepatocytes (199). Subsequent optimisation of Ad vectors included deletion of other early adenoviral genes (E2, E3 and E4) to generate second generation Ad vectors (200, 201).

Parks and colleagues further modified recombinant adenoviral vectors by engineering third generation helper dependent Ad (HDAd) vectors that are devoid of all viral sequences except those necessary for encapsidation and initiation of viral replication (202). The propagation of third generation Ads necessitates expression of all essential viral structural proteins *in trans* by a helper virus (202). Deletion of viral genetic material

significantly increased the transgene capacity of the HD or 'gutless' Ad vectors (~36 kb). However, efficient encapsidation of recombinant adenoviral particles is dependent on the size of the vector genome. As a result the incorporation of DNA stuffer sequences is typically required to restore the natural Ad genome size and enable efficient packaging (203). The choice of stuffer sequence can affect vector stability, transgene expression and immunogenicity (204).

Nevertheless, systemic administration of first and second generation recombinant adenoviruses induces innate and adaptive immune responses involving liver residing antigen presenting cells, known as Kupffer cells (205-207). Kupffer cell-mediated uptake of Ad particles results in a vigorous immune response against the viral capsid proteins. Activation of the PI3K and ERK/MAPK pathways and NFκB induced expression of chemokines leads to leukocyte recruitment, immune-mediated clearance of vector particles, inflammation and transient liver toxicity (208, 209). Sadly, the highly immunogenic nature of adenoviruses led to the first reported death of a patient in a gene therapy clinical trial. After intravenous, high-dose administration of the recombinant adenovirus the patient developed systemic inflammatory response syndrome and died soon thereafter (210). This incident highlighted the risks associated with high-dose, systemic administration of adenoviruses.

A number of strategies has been employed since to improve the safety and efficacy of Ad vectors such as: the development of HDAds, immunosuppression, use of different serotypes, chemical modifications and genetic engineering of the Ad capsid (211, 212). Importantly, ablation of viral genes has considerably improved the immunogenic profile of HDAd vectors, allowing for a more sustained, episomal transgene expression following a single administration. Gutless Ad vectors have been successfully used for hepatotropic delivery of therapeutic genes and recently an RNAi expression cassette *in vivo* (213-216).

Pre-administration of the glucocorticoid drug dexamethasone, has been shown to be useful in attenuating Ad vector stimulated immune responses (217). Furthermore polymer modification of adenoviral capsids with PEG or poly-N-(2-hydroxypropyl) methacrylamide has been demonstrated to be an effective tool in diminishing immunostimulation (218). Importantly, PEGylation of recombinant Ads attenuates vector immunogenicity and depending on the protocol used, does not interfere with the native, hepatotropism of the virus (195, 219). Despite these improvements the utility of these delivery vehicles is limited to applications where life-long expression of the transgene is not required for the desired therapeutic outcome. In addition, technical considerations limit the large-scale propagation of pegylated Ad for clinical use.

1.3.2 Adeno-associated viral vectors

Recombinant AAV vectors are characterised by episomal persistence and low-frequency integration with a preference for actively transcribing genes, making them well suited for stable delivery and sustained expression of therapeutic genes (220, 221). These recombinant viruses are stable, have low pathogenicity, infect dividing and non dividing cells and are less immunogenic and safer than recombinant adenoviruses (222, 223). However pre-existing immunity to AAVs has been shown to reduce the transduction efficiency of recombinant vectors in non human primates, and this has important implications for applying this technology to a clinical setting (224). Immunosuppression has been explored as a means of overcoming this hurdle and thereby improving the *in vivo* transduction with AAV vectors (225). The AAV genome comprises a ~4.7 kb long, linear single stranded DNA (ssDNA) flanked by inverted terminal repeats (ITRs) which include *cis* regulatory elements required for viral replication and packaging (226). Two intervening ORFs, *rep* and *cap*, encode replication and capsid proteins, respectively (227).

Recombinant AAV vectors are devoid of the two ORFs, which are replaced by transgene cassettes. Propagation of recombinant AAVs involves a transient transfection of HEK293 cells with an AAV helper plasmid, encoding rep and cap proteins, the AAV transfer vector encoding the two ITRs and transgene of interest as well as an Ad helper plasmid (228). Alternatively, recombinant AAV can be produced in insect cells following infection with baculoviruses expressing *cis*-acting elements required for viral replication and packaging (229) or by co-infection with wild type Ads or herpes viruses (230).

A factor contributing to low gene transfer by AAVs has been the requirement for nuclear import and capsid uncoating as well as conversion of the ssDNA genome to dsDNA before initiation of gene expression (231, 232). The use of double stranded or self complementary AAV (scAAV) vectors circumvents the need for dsDNA conversion enabling robust transgene expression and efficient transduction using a lower dose of recombinant virus (233). However, as a consequence of creating the double stranded scAAVs the transgene capacity of these vectors is halved.

The diverse tissue tropism of AAVs, with over 100 different serotypes described, has provided the basis for the development of recombinant AAVs for a broad range of gene therapy applications (234). Typically, transgenes flanked by AAV-2 ITRs can be cross packaged into virions comprising capsids of different serotypes. This is possible by simultaneous expression of AAV-2 rep proteins with capsid proteins of a different serotype (235). The AAV-8 vector is of particular interest for liver gene therapy since it has been demonstrated to confer efficient hepatocyte transduction (236). AAV-8 vectors have been used to deliver expressed anti-HBV RNAi sequences and accomplish potent inhibition of HBV replication (165, 237). Sequential use of different hepatotropic AAVs can be exploited to overcome pre-existing immunity to a particular serotype and enable long-term HBV silencing (167). A recent paper has also demonstrated enhanced *in vivo* AAV

transduction of HBV infected hepatocytes (238). Improved transduction was shown to be mediated by HBx. The novel finding could be exploited to develop improved AAV-based anti-HBV gene therapy.

Importantly, unlike their Ad counterparts, recombinant AAV administration in preclinical studies has not been associated with significant toxicity (239, 240). Preclinical studies in animal models of haemophilia demonstrated durable transgene expression and expression at therapeutic levels in the absence of immune responses. However, T cell-mediated clearance of transduced cells attributed to short-term transgene expression was observed in patients during a clinical trial (241-243). The trial involved administration of recombinant AAV-2 carrying the human *factor IX* gene to haemophilia B patients via the hepatic artery. Administration of the recombinant vector was not associated with severe toxicity and a therapeutic effect, albeit limited to 8 weeks, was observed. The eventual loss of the therapeutic effect correlated with a transient increase in liver transaminases and it was concluded that this was as a result of immune-mediated clearance of infected cells. Subsequently it was demonstrated that memory CD8+ T-cells against AAV-2 were reactivated upon administration of the recombinant vector (244). In addition to mediating immune clearance, pre-existing AAV neutralising antibodies can interfere with efficient viral vector-mediated gene transfer precluding long-term expression of therapeutic genes. Serotype switching or transient immunosuppression can be potentially effective strategies to overcome the above limitations (245, 246). Nevertheless, further improvement of these vectors is necessary if stable transduction of the liver and sustained expression of therapeutic genes is to be realised while administering a minimal virus dose.

1.3.3 Lentiviral vectors

Gamma (γ)- retroviral vectors, derived from the murine leukaemia virus (MLV), were the first generation of retroviral vectors used in a clinical setting to demonstrate stable gene transfer and successful long-term gene correction in patients with severe combined immunodeficiency (SCID) (247-249). However, some of the successfully treated patients in the more recent X-linked SCID clinical trial, developed leukaemia as a result of retroviral mediated proto-oncogene activation (250). In addition to having a prominent genotoxic profile, recombinant retroviruses can only transduce dividing cells which complicates and limits their use in treatments requiring gene transfer to non-dividing cells. The need for a recombinant vector with an improved safety profile and a wider spectrum of target tissues led to the development of recombinant lentiviral vectors (251).

Lentiviruses are single stranded RNA viruses that belong to the retrovirus class. Members of the lentiviral family include viruses such as the human HIV, feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV) and equine infectious anaemia virus (EIAV) (reviewed in (252)). Recombinant vectors derived from HIV type 1 (HIV-1) (251, 253), are the best characterised and remain the most commonly used lentiviral delivery vehicles to date (254).

Lentiviral vectors have emerged as versatile and efficient transfer tools as a consequence of their unique ability to mediate stable transduction of dividing and non-dividing cells thus allowing for persistent gene expression. Recombinant lentiviral vectors exhibit low immunogenicity, have improved capacity to accommodate large transgenes and can be pseudotyped with different glycoproteins allowing for transduction of a wide range of target cells and tissues (reviewed in (255, 256)). In addition, lentiviruses have a favourable safety profile in comparison to oncoretroviral vectors (257, 258). Infection with the wild-type HIV-1 virus has detrimental pathological consequences (259). Accordingly,

lentiviruses derived from HIV-1 have been meticulously engineered to ensure a high degree of bio-safety (260, 261). The following bio-safety precautions have been incorporated in the design of late generation lentiviruses: 1) deletion of promoter and enhancer elements in the 3' long terminal repeat (LTR) to generate self-inactivating (SIN) lentiviral vectors (262); 2) splitting genomic sequences necessary for virus propagation to reduce the likelihood of formation of replication-competent viruses during production; 3) pseudotyping- the use of heterologous viral envelopes to modify native tropism (reviewed in (263)); 4) inclusion of polyadenylation enhancer elements to circumvent read-through transcription into neighbouring genomic sequences (264); 5) recoding of splice sites in lentiviral constructs to limit aberrant splicing (265). In addition, synthetic DNA insulators may be potentially useful in ensuring insulation of the host-genome from internal promoters used to drive transgene expression (266-268), however the utility of DNA insulators in hepatocytes has not been assessed.

Replication defective, HIV-derived lentiviral vectors have been generated by deleting the *gag-pol-env* genes required for self-propagation while leaving sequences that are required in *cis* for successful packaging and integration of the viral genome (251). The viral structural proteins, enzymes (Integrase and Reverse Transcriptase), heterologous envelope glycoproteins as well as some accessory proteins (Rev, Tat), are provided by separate packaging constructs in *trans*. The split vector design (Figure 1.4) has been implemented to reduce the potential prospect of homologous recombination and subsequent generation of replication-competent species during virus production (269, 270). Nevertheless, recombination between sequences in the transfer lentiviral backbone and packaging constructs has not yet been observed (271). Accessory HIV-1 genes known to contribute to HIV-related pathology such as *nef*, the virion infectivity factor (*vif*) as well as genes encoding the viral proteins r and u, are not included in packaging plasmids used to

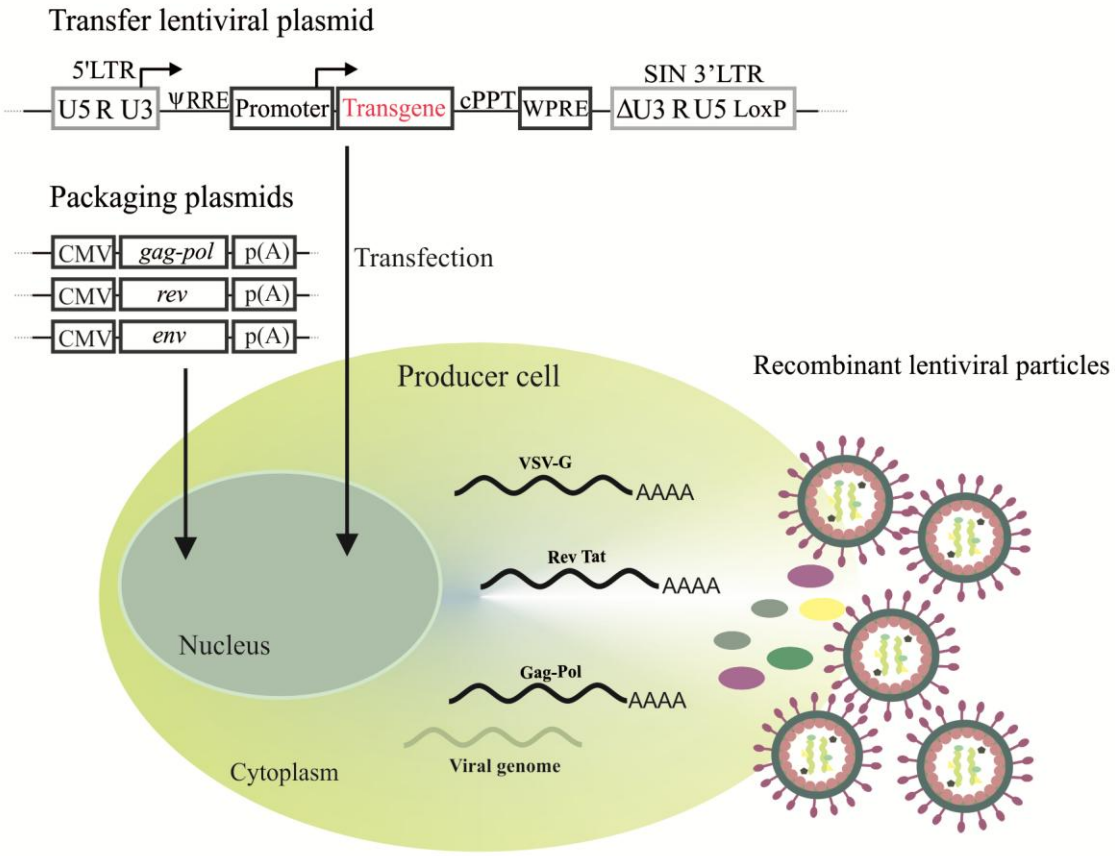


Figure 1.4: <u>Production of SIN recombinant lentivirus.</u>

Recombinant lentiviruses are produced by transient co-transfection of the transfer lentiviral plasmid and packaging constructs. The transfer lentiviral vector construct is flanked by the 3' SIN and 5' LTR sequences and encodes *cis*-acting sequences necessary for viral propagation. Heterologous envelope glycoproteins as well as accessory proteins (Rev, Tat), are provided by separate packaging constructs in *trans*. Infectious viral particles are harvested from the supernatant and subjected to additional purification and concentration steps depending on the intended downstream application. SIN 3' LTR (LTR with a deletion in the U3 region), WPRE (woodchuck hepatitis virus posttranscriptional regulatory element), LTR (long terminal repeat), ψ (HIV-1 packaging signal), RRE (rev-responsive element), cPPT (central polypurine tract).

propagate recombinant lentiviruses (272, 273).

The minimal *cis*-acting sequences typically encoded by late generation lentiviral transfer vectors include: the HIV-1 packaging signal (Ψ), the Rev responsive element (RRE), the woodchuck hepatitis regulatory element (WPRE) and the central polypurine tract (cPPT). The RRE interacts with rev to mediate nuclear export of unspliced genomic RNA (274, 275) and the packaging signal (Ψ) is required for encapsidation of genomic RNA into virions (276). Inclusion of the WPRE has been demonstrated to improve transgene expression (277).

The conversion of the lentiviral single stranded RNA genome into proviral DNA is mediated by the viral reverse transcriptase (252). Proviral DNA is complexed with viral factors, reverse transcriptase and integrase to form the pre-integration complex (PIC) which is capable of translocating across the nuclear membrane and mediating chromatin integration of lentiviral DNA (278). The cPPT sequence, typically included in recombinant lentiviral vectors, is derived from the *Pol* reading frame and it was initially proposed to enhance nuclear translocation of proviral DNA (279). A triple-strand structure, denoted as the central DNA flap, has been proposed to play a key role in cPPT-mediated nuclear translocation of the PIC and as a result transduction of non dividing cells including hepatocytes (280, 281). Novel mechanisms that could play a role in PIC-mediated nuclear translocation have been described (282, 283), however the exact mechanism underlying the above process remains to be elucidated.

The potential of retroviruses to induce aberrant expression of critical cellular genes upon proviral DNA integration and induce insertional mutagenesis is well documented (284-286). Consequently the engineering of late generation lentiviral vectors has involved deletion of promoter and enhancer elements in the 3' LTR to generate SIN lentiviral vectors (262) (Figure 1.4). The wild type LTR (U3-R-U5 sequence) contains several

elements responsible for transcriptional regulation. The U3 sequence comprises upstream regulatory elements as well as a core promoter while the R and U5 sequences comprise downstream regulatory elements including a polyadenylation signal (252). In SIN vector the deletion of promoter and enhancer elements in the 3' LTR is transferred to the 5' LTR during reverse transcription thus abolishing LTR-mediated transcription of the integrated lentiviral sequence. Typically, transcription from functional LTRs is activated by Tat which interacts with the TAR stem loop RNA structure and functions as a transcriptional activator (287). The SIN design of late generation lentiviral vectors has commonly included complete replacement of the 5' LTR with a heterologous viral promoter, such as the CMV or Rous sarcoma virus (RSV) promoters thus abolishing the need for Tat dependant, LTR-driven proviral transcription (261, 269).

Additionally, a SIN provirus with transcriptionaly inactive LTRs has a reduced risk of lentiviral mobilisation by wild-type HIV-1. Nevertheless, the risk of potential recombination with a wild-type virus is not fully eliminated as SIN lentiviral vectors have been shown to have low-level residual expression from the 5' LTR resulting in the production of full-length genomic transcripts competent for encapsidation (288). The above safety issue is pertinent to the use of recombinant lentiviruses that target haematopoietic tissues which are naturally permissive to wild-type HIV-1 infection.

Recombinant lentiviruses are typically propagated in HEK293T producer cells following transient co-transfection of the transfer plasmid encoding the vector genome and the corresponding *gag-pol*, *env* and *rev* packaging constructs (289) (Figure 1.4). Stable packaging cells lines for lentivirus production have also been developed (290, 291), however constitutive expression of viral protease and VSV-G is cytotoxic and accomplishing tight transcriptional control using inducible promoters has proven to be difficult. Despite further improvements of inducible packaging cell lines (292), virus

production through transient transfection of 293T cells remains the gold standard for large-scale lentivirus production for clinical use (293). A range of different techniques including ultracentrifugation, anion exchange chromatography as well as gel filtration can be utilised to purify and concentrate recombinant lentiviruses and these are contingent on the downstream application for which the virus is being generated (294, 295). Several titration methods including quantitative PCR, GFP quantitation by flow cytometry, p24 antigen ELISA and measuring reverse transcriptase activity or the concentration of genomic RNA can be used to determine the biological and physical titre of lentiviral preparations (296). As the use of lentiviruses is being increasingly explored for clinical application, stringent protocols following current Good Manufacturing Practices (cGMP) methods must be employed for the production of clinical-grade lentiviral preparations (297).

The tropism of lentiviruses can be broadened and their biodistribution modified by pseudotyping these viruses with heterologous viral envelopes. Vesicular Stomatitis Virus glycoprotein G (VSV-G) is the most widely used envelope protein for lentivirus pseudotyping as it confers broad tropism and stability (298). Various other viral envelopes have been used to extend the transduction range of lentiviral vectors, which include glycoproteins from measles virus, murine leukaemia virus, rabies, Ross River virus as well as Ebola. Baculovirus GP64 as well as hepatitis C virus and Sindai virus F proteins have been used to confer hepatotropism (reviewed in (263)). In addition, specific ligands or antibodies can be incorporated into the viral envelope and bind to corresponding receptors specific for the ligand or antibodies thus restricting lentivirus entry to specific cells (reviewed in (256)). The use of LV targeting has been employed successfully to enhance transduction of haematopoietic tissue. Nevertheless, the use of a desired envelope might not be feasible for therapeutic applications that require high titre lentivirus stocks as inclusion of novel envelope glycoproteins often leads to titre reduction.

The improved late generation lentiviral vectors with SIN LTRs have emerged as versatile transfer tools as a consequence of their improved safety profile (257), the ability to mediate efficient gene transfer to non-dividing cells as well as sustained expression of therapeutic sequences. In contrast to retroviruses which integrate near gene regulatory areas, lentiviral vectors have a preference for transcriptionaly active sites (299), thus reducing the risk of induction of insertional mutagenesis. *In vivo* genotoxicity studies, using tumour prone mice, have been utilised to compare the genotoxic potential of γ-retroviral vectors and late generation lentiviral vector with SIN LTRs (257, 300). These studies and others (301, 302), have shown a favourable safety profile for lentiviral vectors with a significantly reduced potential to cause insertional mutagenesis with respect to γ-retroviral vectors. Nevertheless, the potential of SIN lentiviral vectors to induce genotoxicity is not completely abolished as these vectors are capable of inducing aberrant splicing (265, 303, 304) as well as enhancing the expression of genes neighbouring the integration sites, particularly if they encode a strong, internal enhancer/promoter sequence (300-302). The use of endogenous cellular promoters to drive transgene expression may reduce the risk of lentiviral vector-mediated insertional mutagenesis (268, 305). In addition, the Cre recombinase-loxP system (306, 307) could be exploited to add an additional safety switch and allow for conditional deletion of integrated lentiviral sequences that comprise loxP sites in the LTRs. Thus far, lentivirus-mediated insertional mutagenesis involving proto-oncogene activation has not been reported in clinical trials assessing the safety and efficacy of recombinant lentiviruses for the treatment of Metachromatic leukodystrophy (308), Adrenoleukodystrophy (309), Wiskott-Aldrich syndrome (310) and HIV (311, 312). Nevertheless, the ability of these vectors to cause insertional mutagenesis remains a concern, and is subject to ongoing investigations (268). Currently, a total of 71 clinical trials involving lentiviral vector delivery are ongoing or have been completed worldwide,

supporting the clinical use of lentiviral vectors as a safer and less toxic alternative to retroviral vectors (http://www.abedia.com /wiley/ index.html). Most trials are based on *ex vivo* lentiviral gene modification of peripheral T lymphocytes or haematopoietic stem cells (HSC). Researchers at the City of Hope National Medical Centre in collaboration with Benitec, have recently completed a phase I clinical trial for the treatment of AIDS lymphoma using a lentiviral vector that induces three forms of anti-HIV RNA, including a shRNA targeting an exon in HIV-1 tat/rev (313), and the positive results from the trial have demonstrated the safety and feasibility of this approach. This trial represents an important milestone in the development of clinical, viral vector mediated RNAi technology and for the field of gene therapy overall. It was the first to demonstrate the use of an expressed RNAi activator in the clinic. In addition, the study highlights the feasibility of using lentiviral vectors as a delivery vehicle for expressed RNAi effecters in the setting of a chronic viral infection indicating a similar approach could be successfully applied for the treatment of other persistent viral infections such as HBV. Similar to *ex vivo* HSC modification, hepatocytes may be efficiently transduced with viral vectors in suspension and transfused back into the patient. A recently developed protocol has described efficient hepatocyte transduction with lentiviral vectors and immediate transplantation (SLIT) (314). The SLIT protocol is clinically applicable and it results in engraftment and proliferation of transduced hepatocytes while preserving their functionality. The feasibility and biosafety of this approach has been demonstrated in non human primates and the protocol was shown to be effective using human hepatocytes (315-317).

Efficient *in vivo* lentiviral gene transfer to hepatocytes following systemic administration has been demonstrated by several groups (281, 318, 319). Nevertheless, the transduction efficiency of recombinant LVs is influenced by the proliferation and cell cycle activation of targeted hepatocytes (320). Accordingly, low transduction efficiencies have

been observed following *in vivo* delivery of VSV-G pseudotyped lentiviruses via the tail vein in adult rodents, as most hepatocytes in the adult liver are quiescent. Various strategies can be used to induce hepatocyte proliferation and improve gene transfer in adult rodents, which include performing partial hepatectomy or administering hepatocyte proliferation-enhancing pharmacological agents (320, 321). In addition, the incorporation of a central DNA flap sequence in late generation lentiviral vectors has improved hepatocyte transduction efficiency of recombinant lentiviruses (319). Lentiviral transduction following systemic delivery of recombinant lentivirus has been reported to be significantly higher in young and newborn rodents as a result of high hepatocyte proliferation (180, 322-324). Naturally, newborn rodents do not have a functional immune system, thus lentivirus administration to newborns circumvents stimulation of an immune response to virus-encoded transgenes, typically observed in adult immunocompetent mice (325).

Several studies have demonstrated potential utility of lentiviral RNAi-based hybrid technology for the treatment of chronic viral infections and liver pathology-causing genes. Recombinant lentiviral vectors have been successfully used for the delivery of expressed RNAi activators targeting HCC-related genes to hepatocytes (326). In addition, efficient lentiviral vector delivery of expressed anti-HIV (327) and anti-HCV (328) RNAi effecters has been reported. A recent study has highlighted the importance of considering various factors pertaining to the engineering of efficient and functional RNAi-based lentiviral vectors encoding expressed pri-miR mimics (329). Careful consideration of these factors is required when designing RNAi-based lentiviral vectors, to ensure optimal vector design and downstream efficacy.

The use of lentiviral vectors to deliver anti-HBV RNAi effecters targeting HBV replication is an attractive option. Stable lentiviral transfer should be facilitated in the

context of an inflamed and regenerating liver (330) and enable sustained expression of the therapeutic anti-HBV molecules to counter viral persistence, providing a compelling rationale for advancement of lentiviral vector-based RNAi gene therapy for chronic HBV infection.

1.4 Models of HBV replication and infection

Preclinical evaluation of the safety and efficacy of novel RNAi-based anti-HBV gene therapy in appropriate *in vitro* and *in vivo* experimental models is a fundamental step in the development and clinical translation of potential HBV therapeutics. For many years the lack of a cell line permissive to HBV infection has hampered efforts to elucidate early events of the viral life cycle as well as gain an understanding of important aspects of virus-host interaction. Consequently, replication-competent plasmid vectors were commonly utilised to recapitulate viral replication following *in vitro* transfection of liver-derived cells. Replication-competent HBV plasmids typically encode a greater-than-genome-length sequence that upon transfection is capable of driving transcription of the pregenomic RNA. HBV replication cell lines, such as the HepG2.2.15 cell line have also been developed. The HepG2.2.15 cell line was generated by transforming the HepG2 human hepatoblastoma cell line with a greater-than-genome-length HBV sequence (331). HepG2.2.15 cells can be used for reliable evaluation of the efficacy of novel HBV therapies *in vitro*, however like replication-competent plasmids this system only recapitulates viral replication and does not represent a true model of HBV infection. Another potentially useful cell culture system includes the human hepatoma-derived HepaRG cells which are capable of sustaining HBV infection following differentiation into hepatocyte-like cells (332, 333). Nevertheless, specialised and lengthy cell culture protocols have to be followed to induce differentiation into biliary-like and liver-like cells. Additionally, only a minority of the differentiated

hepatocyte population is in fact permissive for HBV infection (334). The recent discovery that the virus uses the NTCP receptor to enter hepatocytes has accelerated the development of improved HBV permissive cell culture systems that could be exploited for routine assessment of the efficacy of novel antiviral therapies (8).

Similarly to *in vitro* models of HBV infection, the availability of small animal models of HBV infection is limited. HBV can be used to infect chimpanzees (335, 336) and the Asian tree shrew (337). Woodchucks (77, 338-340) and Peking ducks (341) can be infected with HBV-related viruses such as the woodchuck hepatitis virus and the duck hepatitis B virus. Although important insights into the life cycle, viral persistence, hepatocarcinogenesis as well as the antiviral efficacy of anti-HBV therapies have been obtained from these animal models, ethical and practical considerations have limited their routine use in the laboratory. Rather, researchers have focused on developing more convenient and well-characterised murine models. One such model employs the hydrodynamic tail vein injection procedure (342) to deliver a replication-competent HBV plasmid to the livers of mice thereby simulating viral replication *in vivo* (343). A drawback of this technique is that it is transient. Furthermore it induces cellular damage and elicits an immune response which can complicate the analysis of anti-viral effects. The hydrodynamic model of HBV replication however is a very convenient procedure that can be employed for short-term efficacy studies. Transgenic mice have been developed that contain an integrated replication-competent HBV sequence. Viral replication in HBV transgenic mice is therefore constitutive and continues for the duration of the animal's life thereby closely mimicking chronic HBV infection in humans (344, 345). The HBV transgenic mouse model has been extensively used to evaluate the antiviral efficacy of antiviral drugs including interferon (346), several nucleoside analogues (347, 348) and recently RNAi-based therapeutics (reviewed in (349)). Nevertheless, the hydrodynamic

procedure and HBV transgenic mouse models only simulate viral replication and not viral infection. In addition, cccDNA is not formed in the hepatocytes of the hydrodynamic injection model or transgenic HBV mice (344). Analysing the effects of anti-HBV therapies on this persistent HBV replication template would provide deeper insight into their efficacy. The uPA-SCID mouse model expresses the toxic *urokinase plasminogen activator* (*uPA*) gene under the transcriptional control of the albumin enhancer/promoter resulting in liver damage (350). Being immunocompromised these mice can be engrafted with human hepatocytes without rejection thereby establishing a chimaeric mouse with a humanised liver that can be infected with HBV. Transplanted hepatocytes have a growth advantage over endogenous hepatocytes in this mouse model which possibly contributes to efficient engraftment. Hepatocytes chronically infected with HBV are characterised by persistent expression of viral antigens as a result of episomal persistence of cccDNA. These mice have been successfully used for infection with both HBV (351) and the hepatitis C virus (352) and the model is very promising for evaluating potential antiviral therapy. Since delivery remains the greatest hurdle to clinical application of RNAi-based therapy, demonstrating efficient and safe delivery and antiviral efficacy in these models is likely to accelerate the use of anti-HBV RNAi-based gene therapy in the clinic.

1.5 AIMS

The objective of the present set of investigations has been the design and development of an original strategy to counter chronic HBV infection based on the use of lentiviral vectors to deliver RNAi-based anti-viral effecters. To advance the use of recombinant lentiviruses for HBV treatment, we have engineered second-generation lentiviral vectors to include the liver-specific Pol II cassettes that generate HBV-silencing pri-miR mimics. To overcome concerns of insertional mutagenesis, Lox P sites were

included in the vector LTRs to allow for excision of the integrated lentiviral sequence following *de novo* expression of Cre recombinase and conditional ablation of transduced hepatocytes.

Broad specific aims included the following:

1) Engineering second generation SIN recombinant lentiviral vectors for delivery of anti-HBV RNAi activators

This involved the initial generation of liver-specific, Pol II cassettes that express HBV-silencing pri-miR mimics. Previously characterised, effective anti-HBV pri-miR sequences (159, 160), designed to target single (monocistronic) or multiple (polycistronic) sites of the HBV genome, were further optimised by placing them under the control of liver-specific transcriptional elements with the aim of accomplishing controllable and tissue specific expression.

2) Characterisation of the RNAi-based HBV-silencing lentiviral vectors in cultured cells

Thorough assessment of the functionality and antiviral efficacy of the recombinant HBV silencing lentiviral vectors encoding mono- and poly- cistronic liver-specific pri-miR mimics was carried out in liver-derived cell culture systems.

3) Assessment of the antiviral efficacy of the HBV-silencing LVs *in vivo*

Once recombinant HBV-silencing lentiviruses were propagated and characterised *in vitro* their safety and ability to knockdown viral replication in an *in vivo* model of HBV replication was explored. Accomplishing efficient delivery and sustained expression of therapeutic RNAi-based anti-HBV effecters *in vivo* is a critical step in the process of bench to bedside translation of this promising new technology.

2 MATERIALS AND METHODS

2.1 Generation of expression vectors encoding liver-specific anti-HBV pri-miR mimics

2.1.1 Assessing functionality and tissue-specificity of the liver-specific mTTR promoter

2.1.1.1 Construction of the pmTTR Fluc expression vector

To assess the functionality and tissue specificity of the liver-specific murine transthyretin (mTTR) enhancer-promoter sequence, a vector expressing the *Firefly luciferase* transgene under the control of the hepatospecific promoter was constructed and designated as pmTTR Fluc. The strategy employed to construct the pmTTR Fluc vector involved several cloning steps. Initially a sequence encoding the mTTR promoter was amplified from the pRRLsin.cPPT.mTTR.WPRE lentiviral vector (353) using the Expand High Fidelity PCR^{PLUS} System (Roche Diagnostics GmbH, Germany). The oligonucleotides used were synthesised by standard phosphoramidite chemistry (Inqaba Biotech, South Africa) and had the following sequences: mTTR Forward 5'- GAT CAG ATC TGT CGA CAA AAT TTT ATC GAT CAC GAG ACT AGC -3' and mTTR Reverse 5'- GAT CGC TCA CCA TGG TGG TGG CAG GAG CTT -3'. The forward primer was designed to introduce *Bgl*II and *Sal*I sites at the 5' end of the amplicon.

Purified amplicons were ligated into a PCR cloning vector pTZ57R/T using the InsTAclone PCR cloning Kit (Thermo Scientific, MA, USA). Prepared plasmids were subjected to restriction enzyme digestion with *Dra*I and *Nhe*I to identify clones which contained the mTTR enhancer-promoter sequence inserted in the desired orientation. All

selected clones were sequenced (Inqaba Biotech, South Africa). A plasmid containing the correct promoter sequence was identified and designated as pTZ-mTTR.

Finally, the pmTTR Fluc expression vector was generated by replacing the CMV immediate-early enhancer promoter sequence within pCMV Fluc (154), a plasmid encoding a *Firefly luciferase* sequence downstream of the CMV promoter, with the mTTR promoter sequence. Briefly, the mTTR promoter sequence was excised with *Bgl*II and *Sac*I and the purified fragment was inserted into equivalent sites of the pCMV Fluc vector backbone. While the *Bgl*II restriction site was introduced at the 5' end of the amplified promoter sequence, the *Sac*I restriction site used was present in the multiple cloning site region of the pTZ57R/T vector. Prepared plasmids were subjected to a *Bam*HI and *Sca*I restriction enzyme digestion to select clones containing the desired insert.

2.1.1.2 Transfections of cultured liver- and kidney- derived mammalian cells

The human hepatoma-derived cell line, Huh7 (354), and HEK293T cells were maintained in complete Dulbecco's Modified Eagle Medium (DMEM): Gibco® DMEM (Life Technologies Inc., CA, USA) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Life Technologies Inc., CA, USA), penicillin (10 000 units/ml) and streptomycin (100 µg/ml) (Sigma-Aldrich, MO, USA).

Human hepatoblastoma-derived cells stably transfected with HBV DNA, HepG2.2.15 (331) were maintained in complete William's E culture medium: Gibco® William's E Medium GlutaMAX™ (Life Technologies Inc., CA, USA) supplemented with 10% heat-inactivated FCS, penicillin (10 000 units/ml), streptomycin (100 µg/ml) and Gibco® Geneticin selective antibiotic (400 µg/ml) (Life Technologies Inc., CA, USA). Huh7, HEK293T and HepG2.2.15 cells were seeded in 24 well culture dishes and transfected with 200 ng of pmTTR Fluc or the pCMV Fluc plasmid. In addition, a plasmid

that expresses *Renilla* luciferase constitutively, phRL-CMV (Promega, WI, USA) (200 ng) and an enhanced green fluorescent protein (eGFP) expressing plasmid pCI-neo eGFP (120 ng) were included in each transfection. Transfections were performed using Lipofectamine® 2000 (Life Technologies Inc., CA, USA) (Appendix A1-2).

2.1.1.3 Quantification of luciferase reporter enzyme activity

Luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega, WI, USA). Culture medium was aspirated from wells containing transfected cells and 100 µl of Passive lysis buffer was added to each well and lysis proceeded with shaking for 15 minutes at room temperature. Ten microlitres of the lysates were dispensed into individual wells of a Costar® 96-well assay plate (Corning Inc., NY, USA). A Veritas™ Dual Injection Luminometer (Turner BioSystems, CA, USA) was used to measure the activity of reporter enzymes, following the addition of 50 µl of their corresponding substrates. Firefly luciferase activity was measured 48 hours after transfection and normalised to *Renilla* luciferase activity.

2.1.2 Construction of liver-specific anti-HBV pri-miR expression vectors

To generate liver-specific anti-HBV pri-miR expression vectors, the CMV immediate-early enhancer promoter sequence was removed from the previously described pCMV pri-miR-31/5 (160) and pCMV pri-miR-31/5-8-9 (159) vectors and replaced with an mTTR promoter sequence (Figure 2.1).

Briefly, the mTTR promoter sequence was excised from the pTZ-mTTR plasmid with *Bgl*II and *Sac*I and the purified fragment was ligated to equivalent sites into the pCMV pri-miR-31/5 and pCMV pri-miR-31/5-8-9 vector backbones generating monocistronic (pmTTR pri-miR-31/5) and polycistronic (pmTTR pri-miR-31/5-8-9) pri-

miR expression vectors. Prepared plasmids containing the correct insert size were identified following a *Bam*HI and *Sal*I restriction enzyme digestion screen.

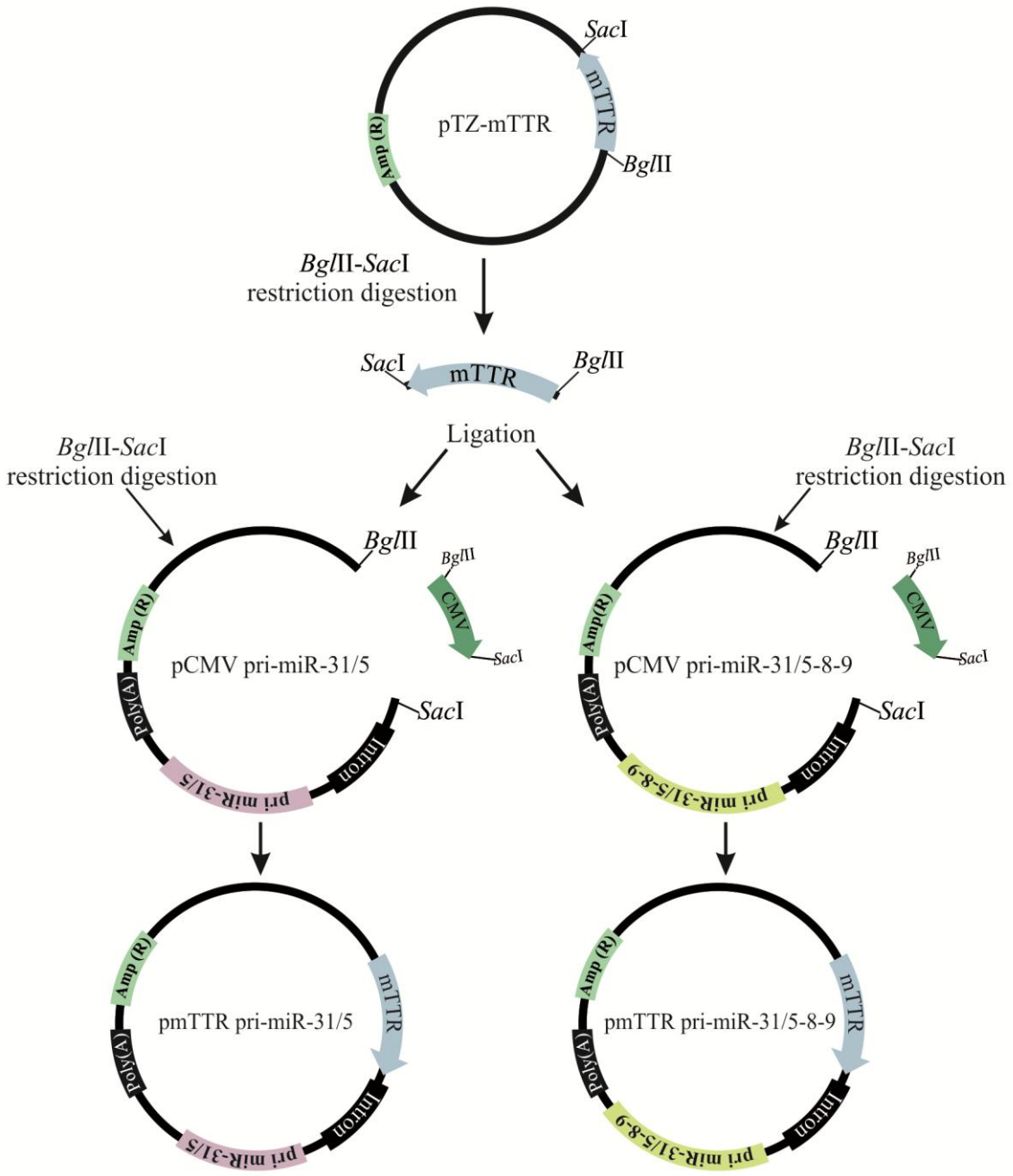


Figure 2.1: <u>Schematic illustration of the construction of liver-specific anti-HBV pri-miR expression vectors.</u>

The mTTR promoter sequence which had been inserted in the pTZ57R/T plasmid was excised with *Bgl*II and *Sac*I and inserted into corresponding sites of pCI-neo derived, CMV pri-miR expression vectors (pCMV pri-miR-31/5 and pCMV pri-miR-31/5-8-9) to generate the liver-specific pri-miR expression vectors pmTTR pri-miR-31/5 and pmTTR pri-miR-31/5-8-9

2.2 Construction of recombinant lentiviral vectors encoding liver-specific anti-HBV pri-miR mimics

The second generation lentiviral plasmid pLVTH (355) was used to generate recombinant HBV-silencing lentiviral vectors. To construct the pLVTH-derived vectors encoding liver-specific anti-HBV pri-miR mimics, the monocistronic (mTTR pri-miR-31/5) and polycistronic (mTTR pri-miR-31/5-8-9) pri-miR expression cassettes were excised from the pmTTR pri-miR-31/5 and pmTTR pri-miR-31/5-8-9 expression vectors with *Sal*I. The purified fragments were inserted into the *Sal*I site of the pLVTH backbone generating the pLVTH miR-31/5 and pLVTH miR-31/5-8-9 recombinant lentiviral vectors (Figure 2.2). Prepared plasmids were subjected to *Bam*HI and *Sal*I restriction enzyme digestion to select clones containing the desired insert.

2.3 Assessment of the efficacy of expressed liver-specific anti-HBV pri-miR mimics

2.3.1 Target and reporter plasmids

pCH-9/3091

The pCH-9/3091 HBV target plasmid has been described earlier (356). The plasmid contains an HBV genome sequence, encoding terminal repeats, under the control of the CMV immediate-early promoter enhancer. A greater that genome length pgRNA transcript is generated following transcription. Once the pgRNA is reverse transcribed viral propagation in transfected cells occurs simulating HBV replication.

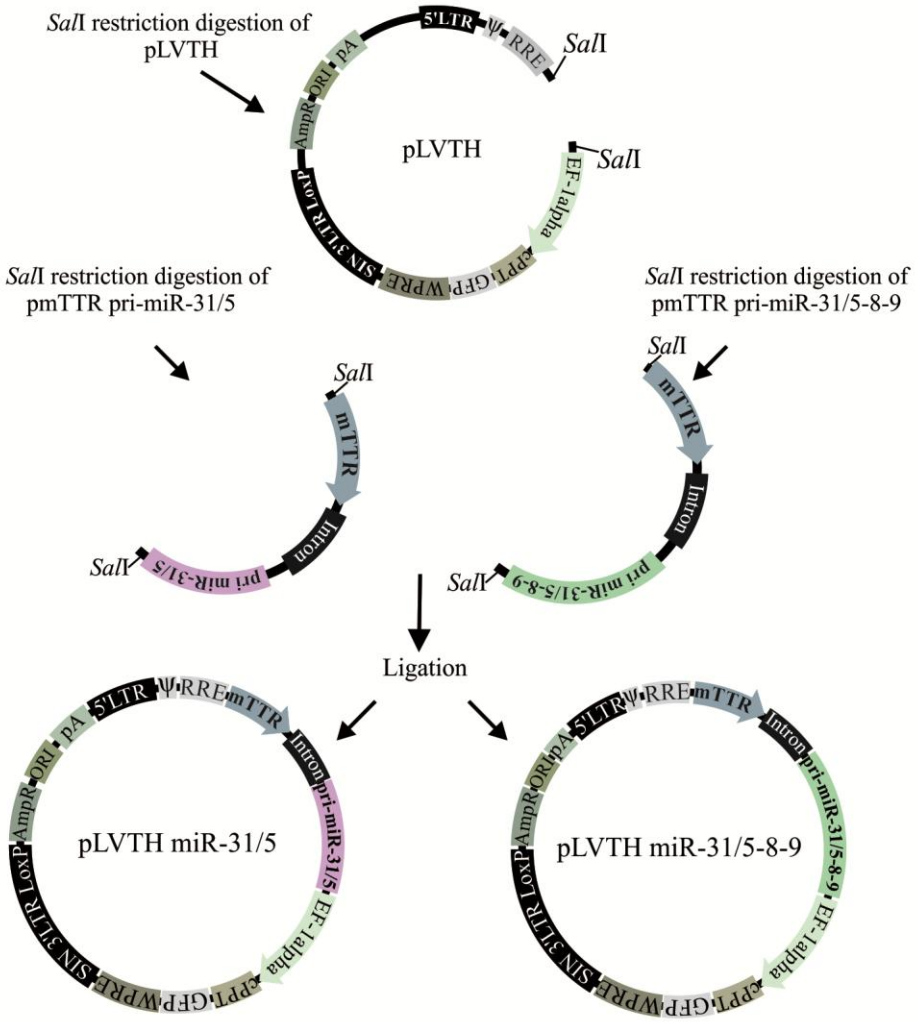


Figure 2.2: <u>Schematic illustration of the cloning strategy used to construct HBV-silencing lentiviral vectors encoding liver-specific pri-miR mimics.</u>

Expression cassettes encoding monocistronic (pri-miR-31/5) and polycistronic (pri-miR-31/5-8-9) pri-miR mimics placed under the control of the liver-specific mTTR promoter were excised from the pmTTR pri-miR-31/5 and pmTTR pri-miR-31/5-8-9 expression vectors with *Sal*I. The cassettes were inserted into the *Sal*I site of the second generation recombinant lentiviral vector pLVTH to generate anti-HBV lentiviral vectors carrying monocistronic (pLVTH miR-31/5) and polycistronic (pLVTH miR-31/5-8-9) pri-miR mimics. WPRE (woodchuck hepatitis virus posttranscriptional regulatory element), LTR (long terminal repeat), SIN 3'LTR (LTR with a deletion in the U3 region), ψ (HIV-1 packaging signal), RRE (rev-responsive element), cPPT (central polypurine tract).

***psiCHECK*-HBx**

Construction of the psiCHECK-*HBx* plasmid has been described before (357). The plasmid contains the complete *HBx* sequence downstream of the Renilla *luciferase* ORF within psiCHECK™-2 (Promega, WI, USA).

2.3.2 Assessing HBV knockdown efficacy and tissue-specificity of expressed liver-specific anti-HBV pri-miR mimics

Huh7 cells and HEK293T cells were maintained in complete DMEM (Section 2.1.1.2) and seeded in 24 well culture dishes the day before transfection. Antiviral efficacy and tissue-specific expression of the anti-HBV liver-specific pri-miR mimics were assessed by co-transfecting 100 ng of the psiCHECK-*HBx* reporter plasmid with 800 ng of individual pCI-neo derived, pmTTR or lentiviral vectors encoding monocistronic or polycistronic pri-miR sequences (pCMV miR-31/5, pCMV miR-31/5-8-9, pmTTR pri-miR-31/5, pmTTR pri-miR-31/5-8-9, pLVTH miR-31/5 or pLVTH miR-31/5-8-9). The transfection was performed using Lipofectamine® 2000 (Appendix A1-2). One hundred and twenty nanograms of an eGFP expressing plasmid (pCI-neo eGFP) was included in each transfection. The Dual Luciferase® Reporter Assay System described in section 2.1.1.3 was used to measure luciferase activity forty-eight hours after transfection and the ratio of *Renilla* luciferase to Firefly luciferase activity was determined.

2.3.3 Assessing inhibition of HBsAg secretion by expressed liver-specific anti-HBV pri-miR mimics

2.3.3.1 Transfections

Huh7 cells were maintained in complete DMEM (Section 2.1.1.2) and seeded in 24 well culture dishes the day before transfection. To assess the knockdown efficacy of the liver-specific anti-HBV pri-miR mimics, 100 ng of the HBV replication competent plasmid pCH-9/3091 were co-transfected with 800 ng of pCI-neo derived expression vectors (pmTTR pri-miR-31/5, pmTTR pri-miR-31/5-8-9) and lentiviral vectors (pLVTH miR-31/5, pLVTH miR-31/5-8-9) using Lipofectamine® 2000 (Appendix A1-2). One hundred nanograms of an eGFP expressing plasmid (pCI-eGFP) was included in each transfection to control for transfection efficiency.

2.3.3.2 HBsAg ELISA

HBsAg secretion was quantified using the Monolisa™ HBsAg ULTRA assay kit (Bio-Rad, CA, USA) which works on the principle of sandwich type ELISA. In brief, 48 hours after transfection with the pCH-9/3091 plasmid, culture medium was harvested from wells containing transfected cells and 100 µl of culture medium from each sample was added into custom microplate wells coated with mouse monoclonal anti-HBsAg antibodies. Fifty microlitres of Conjugate Solution, containing anti-HBsAg mouse monoclonal and goat polyclonal antibodies bound to peroxidase, were dispensed into each well. The sample-containing microplate was incubated at 37°C for 1 hour and 30 minutes (± 5 minutes).

Next, to remove the unbound conjugate solution, wells were washed a minimum of five times using the Washing Solution (Tris NaCl buffer, pH 7.4; 0.04% ProClin™ 300).

One hundred microlitres of Development Solution (Citric acid and Sodium acetate solution, pH 4.0; 0.015% H_2O_2; 4% dimethylsulphoxide [DMSO] and tetramethyl benzidine) was added to each well. After the plate was incubated for 30 minutes in the dark at room temperature and the enzymatic reaction had developed, 100 µl of Stopping Solution (1 N H_2SO_4) was dispensed into each well to stop the reaction. The optical density of the samples was measured at 420/690 nm within 30 minutes of addition of the Stopping solution using a microplate reader (Bio-Rad, CA, USA).

2.4 Production, concentration and titration of pLVTH-derived recombinant lentiviral vectors expressing liver-specific anti-HBV pri-miR mimics

2.4.1 Production of VSV-G pseudotyped recombinant lentiviruses

LV stocks (LVTH, LVTH miR-31/5 and LVTH miR-31/5-8-9) were produced by transient calcium phosphate transfection of HEK293T cells with individual lentiviral plasmids (pLVTH, pLVTH miR-31/5 or pLVTH miR-31/5-8-9 respectively) and the corresponding packaging plasmids (psPAX2 and pMD2.G). The pLVTH, psPAX2 and pMD2.G plasmids were made available from the Laboratory of Dr Didier Trono (Laboratory of Virology and Genetics, EPFL, Lausanne, Switzerland, http://tronolab.epfl.ch/). Additional information regarding the sequences encoded by the transfer and packaging recombinant lentiviral plasmids has been included in Table 2.1.

Low passage HEK293T cells were used for the production of LV stocks. A total of 1×10^6 HEK293T cells were seeded per one 15 cm^2 culture dish the day before transfection in complete DMEM (Section 2.1.1.2). Cell numbers and viability were

determined using a Neubauer haemocytometer and Trypan blue exclusion dye (Lonza, MD, USA). For a transient transfection of five 15 cm^2 culture dishes a multiplasmid transfection mix was prepared in a 50 ml sterile tube by adding 150 µg of transfer plasmid, 112.5 µg of psPAX2 and 75 µg of the pMD2.G plasmid. The final volume of the plasmid mix was adjusted to 3.75 ml with deionised water. An equal volume of 0.5 M $CaCl_2$ solution was added to the plasmid mix and the mixture was vortexed gently. The final transfection mixture was prepared by drop wise addition of the $CaCl_2$ - DNA mix to 7.5 ml of 2× HEPES-buffered saline while vortexing the solution. The final transfection mixture was prepared by drop wise addition of the $CaCl_2$ - DNA mix to 7.5 ml of 2× HEPES-buffered saline (50 mM HEPES, 1.5 mM Na_2HPO_4, 280 mM NaCl, 10 mM KCL; pH 7.1) while vortexing the solution. The mixture was incubated at room temperature for 15 minutes to allow formation of DNA precipitates. Three millilitre aliquots were distributed into each 15 cm^2 culture dish and the dishes were gently swirled. Cells were incubated for 18 hours at 37°C in the presence of 5% CO_2. Virus-containing supernatant was harvested at 18 and 48 hours after transfection. Transfection efficiency was assessed by examining the transfected cells under a fluorescence microscope. Pooled supernatants containing infectious lentiviral particles were centrifuged at 1,000 × g for 5 minutes at 4°C and subsequently filtered through a 0.45 µm membrane using AcroVac™ Filter Units (Pall Life Sciences, NY, USA). Clarified virus-containing medium was stored at 4°C for a maximum of 3 days before the virus was concentrated.

Table 2.1: Description of second-generation, recombinant lentiviral plasmids used for the production of HBV-silencing LVs.

Plasmid	Description
pLVTH	Second generation SIN recombinant lentiviral transfer vector encoding a GFP transgene expressed from an internal human elongation factor-1 alpha (EF-1 alpha) promoter
pLVTH miR-31/5	pLVTH-derived recombinant lentiviral vector encoding a monocistronic anti-HBV pri-miR sequence (pri-miR-31/5) expressed from an internal mTTR promoter
pLVTH miR-31/5-8-9	pLVTH-derived recombinant lentiviral vector encoding a polycistronic anti-HBV pri-miR sequence (pri-miR-31/5-8-9) expressed from an internal mTTR promoter
pMD2.G	Second generation packaging plasmid encoding VSV-G envelope glycoprotein
psPAX2	Second generation packaging plasmid encoding HIV-1 Gag, Pol, Tat and Rev proteins

2.4.2 Concentration of recombinant lentiviruses

2.4.2.1 Ultrafiltration

Lentivirus concentration by ultrafiltration involved diafiltration of virus-containing medium using a VivaFlow50 crossflow filtration cassette (Sartorius Stedim Biotech GmbH, Germany). Typically, to achieve 10 fold concentration of the lentivirus-containing medium, 200 ml of medium was ultrafiltered through the VivaFlow cassette run by a peristaltic pump at a flow rate of 15-30 ml/minute. The cassette was regenerated with 250 ml of 0.1 N NaOH followed by a rinse with one litre of deionised water. The regenerated cassette was stored in 70% ethanol. Fifty microlitre aliquots of the ultrafiltrate were dispensed in sterile microcentrifuge tubes and stored at -80°C.

2.4.2.2 Ultracentrifugation

High-titre LV stocks were prepared by concentrating harvested virus-containing medium by ultracentrifugation. Briefly, aliquots of clarified lentivirus medium were distributed into SW28 open-top conical centrifuge tubes (Beckman, CA, USA). The tubes were centrifuged at 50 000 × g for 90 minutes at 4°C in a Beckman ultracentrifuge using a SW28 ultracentrifuge rotor (Beckman, CA, USA). When centrifugation was completed the supernatant was poured off and the tubes were inverted and left to dry on a paper towel for five minutes. The remaining liquid droplets were aspirated. The translucent pellet from each centrifuge tube was resuspended in 30 µl of Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca(2+) and Mg(2+) (Life Technologies Inc., CA, USA) by gently pipetting up and down. Virus-containing aliquots were pooled and transferred into a sterile microcentrifuge tube. Vector stocks were prepared by aliquoting 25 µl fractions of the pooled virus-containing solution in sterile microcentrifuge tubes. All vector stocks were frozen and stored at -80°C.

2.4.3 Titration of recombinant lentiviruses

Viral preparations were titrated on human cervical cancer-derived HeLa cells using a previously described real-time quantitative PCR titration method (314), which allows for accurate estimation of the functional titre of a viral preparation i.e. the number of infection-competent viral particles in the vector stock.

To determine the functional titre of LV stocks, 5×10^4 HeLa cells, maintained in complete DMEM (Section 2.1.1.2), were seeded per well in a 24 well culture plate. The following day cells were infected in duplicate with a 100 fold, 10 fold and 0.1 fold dilution of a LV stock of unknown titre. After 24 hours, the cells were washed with DPBS without Ca(2+) and Mg(2+) and cultured for additional 3 days to decrease carryover plasmid contamination. Genomic DNA from these cells was extracted using the FlexiGene DNA Kit (Qiagen, GmbH, Germany).

The number of integrated vector copies per cell was determined using a quantitative PCR titration assay, based on amplification of a small fragment of the lentiviral vector-specific GAG sequence from genomic DNA of lentivirus transduced cells. The human *β-actin* (hBA) housekeeping gene was amplified to normalise the amount of genomic DNA in the individual samples. Vector copy numbers were determined using a standard curve, generated by preparing standards in which the pLVTH plasmid was present at 100, 10, 1, 0.1, and 0 copies in HeLa genomic DNA.

The quantitative PCR titration assay was carried out using the Roche Lightcycler v.2 (Roche Diagnostics GmbH, Germany) and the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Diagnostics GmbH, Germany) with the following parameters: a 10 minute hot start at 95°C, 50 cycles of denaturation at 95°C for 15 seconds, annealing at 63°C for 15 seconds and extension at 72°C for 15 seconds. The primer set, GAG Forward: 5'- GGA GCT AGA ACG ATT CGC AGT TA -3' and GAG

Reverse: 5’- GGT TGT AGC TGT CCC AGT ATT TGT C -3’ was used to amplify the GAG region from pLVTH and proviral DNA. The following primers were used to amplify *β-actin* DNA: BA Forward: 5'- TCC GTG TGG ATC GGC GGC TCC A -3' and BA Reverse: 5'- CTG CTT GCT GAT CCA CAT CTG -3'. The oligonucleotide sequences used were synthesised by standard phosphoramidite chemistry (Inqaba Biotech, South Africa).

To calculate the titre of the LVs, first the number of proviral genome copies per cell was interpolated from the standard curve using the ΔCt values of the samples (average Ct of GAG minus the average Ct of hBA). The standard curve was generated by plotting the ΔCt values of the pLVTH standards against the known copy numbers. The titre of the LV was then calculated as follows: ^{HeLa}TU/ml = [Number of HeLa cells at time of transduction × number of vector copies per cell (quantitative PCR result)] / [volume of diluted vector (ml) × vector dilution].

2.5 Transduction of Huh7 cells with recombinant lentivirus expressing liver-specific anti-HBV pri-miR mimics

The day before infection Huh7 cells were seeded in 6 well culture dishes in complete DMEM (Section 2.1.1.2). The following day the medium from wells containing the cells to be infected was removed and replenished with fresh growth medium. Concentrated lentivirus suspension was added to individual wells containing target cells at an MOI of 40 and the cells were incubated at 37°C in a humidified incubator with atmosphere of 5% CO_2 for 24 hours before the virus-containing medium was removed. Transduction efficiency was assessed by monitoring GFP expression under a fluorescence microscope. Stable transduction was accomplished, demonstrated by long-term persistence of GFP-positive hepatocytes.

2.5.1 Determination of the number of proviral lentiviral vector copies in transduced Huh7 cells

To determine proviral DNA copy numbers, genomic DNA was extracted from transduced cells using the FlexiGene DNA Kit according to the manufacturer's instructions. Previously described quantitative PCR assay (section 2.4.3) was used to determine the number of vector copies associated with genomic DNA extracted from stably transduced cells.

2.6 Assessment of anti-HBV pri-miR mimic processing and sequence rearrangement in stably transduced Huh7 cells

2.6.1 Polyacrylamide gel electrophoresis and Northern blot analysis

Total RNA was extracted from stably transduced Huh7 cells using Tri-Reagent (Sigma-Aldrich, MO, USA) according to the manufacturer's instructions. Samples containing 30 µg of RNA extracted from Huh7 cells which had been transduced with LVTH, LVTH miR-31/5 or LVTH miR-31/5-8-9 lentiviruses, were denatured at 95°C for 5 minutes and separated on a 15% denaturing polyacrylamide gel. Radiolabelled Decade™ RNA molecular weight markers (Life Technologies Inc., CA, USA) were run alongside the cellular RNA. RNA quality was verified by briefly staining the polyacrylamide gel in 0.5× Tris-Borate EDTA (TBE) solution which contained ethidium bromide at a final concentration of 0.5 µg/ml and examining the gel on a UV transilluminator.

The RNA was transferred to a positively charged nylon membrane (Amersham, NJ, USA) by electro-blotting (3 mA/cm^2) for one hour at 4°C using a Semi-Dry Electroblotting Unit Z34,050-2 (Sigma-Aldrich, MO, USA). When the transfer was completed the RNA-

containing membranes were cross-linked by UV irradiation at 200 000 μJ/cm^2 using a UV crosslinker (UVP Inc., CA, USA). The membranes were placed in hybridisation tubes and 1 ml of Rapid-hyb buffer (Amersham, NJ, USA) was added per 10 cm^2 of membrane. Next, the membranes were prehybridised by incubating the tubes with rolling agitation in a hybridisation oven for 30 minutes at 42°C.

Oligonucleotides designed to bind to putative guide sequences of pri-miR-31/5 and pri-miR-31/5-8-9 were radiolabelled with 20 μCi of [γ-^{32}P]-adenosine triphosphate (ATP) using T4 polynucleotide kinase (Thermo Scientific, MA, USA) and used as probes. The oligonucleotide probes, synthesised by standard phosphoramidite chemistry (Inqaba Biotech, South Africa), had the following sequences: Guide 5: 5'- CCG TGT GCA CTT CGC TTC -3', Guide 8: 5'- CAA TGT CAA CGA CCG ACC -3' and Guide 9: 5'- TAG GAG GCT GTA GGC ATA -3'. Complementary probes were diluted in hybridisation buffer and subsequently added to individual hybridisation tubes at a final concentration of 10 ng/ml. Hybridisation was performed overnight at 42°C. Following hybridisation, three washes were performed to eliminate excess probe as well as non-specific probe-RNA hybridisation. A low stringency wash was carried out using 5× SSC, 0.1% sodium dodecyl sulphate (SDS) solution at room temperature for 20 minutes. An additional two high stringency washes were carried out with a 1× SSC, 0.1% SDS solution at 42°C for 15 minutes. A 20× SSC stock buffer (3 M NaCl, 0.3 M sodium citrate; pH 7.0) was used to make the 5× and 1× SSC solutions. The probed membranes were exposed to imaging plates (Fujifilm, Japan) for 10 days. Analysis was carried out using a FLA-7000 PhosphorImager (Fujifilm, Japan). The probed membranes were stripped with 50 ml 0.1× SSC, 1% SDS solution at 80°C for 30 minutes and re-hybridised with a radiolabelled oligonucleotide sequence complementary to U6 small nuclear RNA (snRNA). The U6 oligonucleotide probe was synthesised by standard phosphoramidite chemistry (Inqaba

Biotech, South Africa) and had the following sequence: 5'- TAG TAT ATG TGC TGC CGA AGC GAG CA -3'. Densitometric analysis was carried out using GeneTools software version 4 (SynGene (A division of Synoptics Ltd.), Cambridge, UK).

2.6.2 PCR recombination analysis of integrated polycistronic pri-miR mimic sequences in stably transduced Huh7 cells

PCR-based recombination analysis was performed to verify if sequence recombination had occurred during lentivirus reverse transcription between equivalent sequences in the polycistronic pri-miR-31/5-8-9 cassette. The PCR was performed using genomic DNA extracted from Huh7 cells which had been stably transduced with LVTH miR-31/5-8-9 or control LVTH LV. DNA was extracted using the FlexiGene DNA Kit according to the manufacturer's instructions. PCR was carried out using the Thermo Scientific 2× PCR master mix (Thermo Scientific, MA, USA) with the following thermocycling parameters: a 2 minute hot start at 95°C and 30 cycles of denaturation at 95°C for 30 seconds annealing at 60°C for 30 seconds and extension at 72° for 10 seconds. The primer sequences used were: Recombination Forward: 5'- ACT GGA GAG GGG TGA AGC GA -3' and Recombination Reverse: 5'- AGA GTC GAC GAT AAG CTT TGC A -3' (Inqaba Biotech, South Africa).

2.7 Evaluation of the HBV knockdown efficacy of LV-delivered anti-HBV pri-miR mimics in stably transduced Huh7 cells

2.7.1 Target and reporter plasmids

*2.7.1.1 psiCHECK-m*HBx

The psiCHECK-m*HBx* luciferase reporter plasmid has been described previously (159). The plasmid contains a mutated *HBx* target sequence downstream of the Renilla *luciferase* ORF within psiCHECK™-2. The mutated *HBx* target sequence contains several point mutations in the region targeted by the putative guide 5 of pri-miR 31/5 and pri-miR 31/5-8-9 mimics.

2.7.1.2 psiCHECK-5T, psiCHECK-8T and psiCHECK-9T

Previously described psiCHECK-5T, psiCHECK-8T and psiCHECK-9T reporter plasmids (159) contain sequences targeted by putative guides 5 (psiCHECK-5T), 8 (psiCHECK-8T) and 9 (psiCHECK-9T) downstream of the Renilla *luciferase* ORF within the psiCHECK-2.2 plasmid.

2.7.2 Assessment of HBV knockdown efficiency

Huh7 cells were cultured in complete DMEM (Section 2.1.1.2). To determine *in situ* knockdown of HBV target gene expression, LVTH, LVTH miR-31/5 and LVTH miR-31/5-8-9-transduced Huh7 cells were seeded in 24 well culture dishes the day before transfection. The cells were transfected with a 100 ng of the psiCHECK-*HBx* plasmid using Lipofectamine® 2000 (Appendix A1-2). Forty-eight hours following transfection the

cells were lysed and *Renilla* luciferase activity was measured using the Dual-Luciferase® Reporter Assay System as described in section 2.1.1.3. *Renilla* luciferase activity was normalised to Firefly luciferase activity.

2.7.3 Assessment of knockdown efficiency against individual HBV target sequences

Huh7 cells were maintained in complete DMEM (Section 2.1.1.2) and seeded in a 24 well culture dish the day before transfection. To determine *in situ* knockdown efficiency against individual HBV target sequences, Huh7 cells which had been stably transduced with control LVTH or therapeutic LVTH miR-31/5-8-9 LV were transfected with a 100 ng of individual target plasmids (psiCHECK-5T, psiCHECK-8T or psiCHECK-9T) using Lipofectamine® 2000 (Appendix A1-2). Forty-eight hours following transfection the cells were lysed and *Renilla* luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Section 2.1.1.3) and the ratio of *Renilla* luciferase to Firefly luciferase activity was calculated.

2.7.4 Assessment of anti-HBV pri-miR mediated knockdown against a mutant *HBx* sequence

Huh7 cells were maintained in complete DMEM (Section 2.1.1.2) and seeded in a 24 well culture dish the day before transfection. To determine the efficacy of LV-delivered pri-miR mimics against a mutant *HBx* sequence, LVTH-, LVTH miR-31/5- and LVTH miR-31/5-8-9- transduced cells were transfected with a 100 ng of the psiCHECK-m*HBx* luciferase reporter plasmid using Lipofectamine® 2000 (Appendix A1-2). Forty-eight

hours after transfection, knockdown efficiency was assayed by measuring *Renilla* luciferase activity (Section 2.1.1.3) normalised to Firefly luciferase expression.

2.7.5 Assessment of inhibition of viral replication by integrated anti-HBV pri-miR mimics

Huh7 cells were maintained in complete DMEM (Section 2.1.1.2). To assess inhibition of HBV replication mediated by LV-delivered pri-miR mimics, Huh7 cells which had been stably transduced with LVTH, LVTH miR-31/5 and LVTH miR-31/5-8-9 were seeded in a 24 well culture dish the day before transfection and transfected with 100 ng of the HBV replication competent pCH-9/3091 target plasmid using Lipofectamine® 2000 (Appendix A1-2). The cells were incubated for forty-eight hours after transfection before the medium of each well was removed for further analysis. HBsAg secretion was measured using the Monolisa™ HBsAg ULTRA assay kit as described in Section 2.3.3.2.

2.8 Evaluation of saturation of the endogenous miR pathway by LV-delivered anti-HBV pri-miR mimics

2.8.1 Assessment of disruption of endogenous miR-16 function in stably transduced cells

2.8.1.1 Sponge and target plasmids

The U6-driven sponge plasmid- pTZ-U6-miR-16S×7 (159) contains seven copies of an imperfectly complementary target of miR-16. The miR-16 dual luciferase target

plasmid- psiCHECK-miR-16T×7 (159) contains seven miR-16 sites downstream of the Renilla *luciferase* ORF within psiCHECK™-2.

2.8.1.2 Saturation assay

To determine whether the LV-delivered liver-specific anti HBV pri-miR mimics induce saturation of the natural miR pathway and subsequently interfere with the function of endogenous miRs, a saturation assay was performed. The assay involved transfecting 100 ng of the psiCHECK-miR-16T×7 target plasmid using Lipofectamine® 2000 (Appendix A1-2) into stably transduced LVTH, LVTH miR-31/5 and LVTH miR-31/5-8-9 Huh7 cells which had been seeded in 24 well culture dishes the day before transfection. As a positive control for disruption of miR-16 function, LVTH-transduced Huh7 cells were co-transfected with 800 ng of the pTZ-U6-miR-16S×7 sponge plasmid and 100 ng of the psiCHECK-miR-16T×7 reporter plasmid using Lipofectamine® 2000 (Appendix A1-2). Disruption of endogenous miR-16 function was assayed by measuring *Renilla* to Firefly luciferase reporter gene activity 48 hours following transfection as described in Section 2.1.1.3.

2.9 Evaluation of the HBV knockdown efficacy of LV-delivered anti-HBV pri-miR mimics in stably transduced HepG2.2.15 cells

2.9.1 Transduction of HepG2.2.15 cells with recombinant LV expressing a liver-specific anti-HBV polycistronic pri-miR sequence

HepG2.2.15 cells were maintained in complete William's E culture medium (Section 2.1.1.2). The cells were transduced with LVTH or LVTH miR-31/5-8-9 lentiviral

suspensions at an MOI of 40 as described in Section 2.5. Transduction efficiency was assessed by monitoring GFP expression under a fluorescence microscope. The number of proviral vector copies in transduced cells was determined as described in Section 2.5.1.

2.9.2 Quantification of HBsAg and HBeAg secretion in stably transduced HepG2.2.15 cells

Five hundred thousand HepG2.2.15 cells which had been stably transduced with LVTH or LVTH miR 31/5-8-9 lentivirus were seeded in triplicate in 6 well culture dishes in complete William's E culture medium (Section 2.1.1.2). The medium was not replaced for the duration of the entire experiment. Five hundred microlitres of medium were harvested from individual wells containing LVTH or LVTH miR 31/5-8-9-transduced cells at 48, 72 and 96 hours after the cells had been seeded. Secretion of HBsAg, HBeAg as well as viral DNA quantity were assayed in the respective samples.

HBsAg was quantified as described in section 2.3.3.2. HBeAg secretion was quantitatively measured using a sandwich type ELISA method (Gentaur, France). Briefly, the protocol involved addition of samples of interest along with horseradish peroxidase conjugated HBeAg monoclonal antibodies to microwells coated with HBeAg specific monoclonal antibodies. In the presence of HBeAg an immunocomplex is formed between the antibody conjugated to horseradish peroxidase and HBeAg bound to HBeAg antibody. Several washes were performed to remove unbound antibody. After addition of a chromogenic solution containing a substrate for horseradish peroxidase, the substrate was hydrolysed resulting in colour change. Typically, colour intensity is proportional to the amount of HBeAg captured and was measured after the addition of a stopping solution using a microplate reader.

2.9.3 HBV DNA quantification in stably transduced HepG2.2.15 cells

Real-time quantitative PCR was performed to assay miR-mediated inhibition of viral replication in HepG2.2.15 cells which had been stably transduced with LVTH or LVTH miR-31/5-8-9 lentivirus. Virion DNA was measured in culture medium from samples harvested at 48, 72 and 96 hours after the cells had been seeded. The Total Nucleic Acid Isolation Kit and MagNA Pure LC system (Roche Diagnostics, GmbH, Germany) was used to extract HBV DNA from 200 µl of the individual samples according to manufacturer's instructions. Viral DNA quantification was performed by real-time quantitative PCR carried out using HBV-specific primers and the Roche Lightcycler v.2. The primers used were: HBV Forward 5'- TGC ACC TGT ATT CCA TC -3' and HBV Reverse 5'- CTG AAA GCC AAA CAG TGG -3'. PCR analysis was performed using the SensiMix™ Capillary Kit (Bioline, England, UK) and the following cycling parameters: a 30 second hot start at 95°C and 50 cycles of denaturation at 95°C for 10 seconds, annealing at 58°C for 10 seconds and extension at 72°C for 10 seconds. The HBV DNA quantification standard curve used was generated using EuroHep calibrators (358).

2.10 Assessment of long-term transgene expression mediated by the liver-specific mTTR promoter in vivo

2.10.1 Generation of a recombinant lentiviral vector expressing the *Firefly luciferase* transgene from an internal mTTR promoter

Invitrogen's Gateway® cloning technology (Life Technologies Inc., CA, USA) was used to generate a recombinant bicistronic lentiviral vector (pLNT-mTTR-JDG) encoding a *Firefly luciferase* transgene expressed by the mTTR liver-specific promoter. Briefly, the

Gateway® cloning system exploits the recombination properties of bacteriophage lambda to enable efficient transfer of DNA sequences, flanked by synthetic attachment sites (att), between different vectors of interest (359).

The cloning strategy employed to construct the pLNT-mTTR-JDG lentiviral vector, involved cloning of an mTTR promoter sequence into an entry vector (pENTR™1A) (Figure 2.3) and subsequent transfer of the promoter sequence into a destination vector (pLNT-GW-JDG) via site specific recombination (Figure 2.4). The entry (pENTR™1A) and destination (pLNT-GW-JDG) vectors used were kindly provided by the group of Dr Simon N. Waddington from University College London, UK.

In addition, both pENTR™1A-mTTR and pLNT-GW-JDG vectors contain a toxic/selection *ccd*B gene flanked by either attL or attR attachment sequences respectively. Following translation, the ccdB protein inhibits the growth of most *Escherichia coli* (*E. coli*) strains (360) allowing for efficient selection of desired clones that have successfully recombined to remove the suicide gene. The pENTR™1A and pLNT-GW-JDG plasmids were propagated in the DB3.1 *E. coli* strain which is resistant to the toxic effects of the *ccd*B gene.

Importantly, the pLNT-GW-JDG lentiviral vector used to derive the pLNT-mTTR-JDG construct comprises a 2A-linked Firefly luciferase-eGFP bicistronic expression cassette downstream of the *ccd*B selection gene (Figure 2.4). The presence of the 2A proteolytic cleavage site between the Firefly luciferase and GFP sequences should potentially allow for stoichiometric expression of both genes (361).

To clone the mTTR promoter sequence into the pENTR™1A vector, an mTTR promoter-encoding sequence was excised with *Sal*I-*Nhe*I from the previously described pmTTR pri-miR-31/5 vector (Section 2.1.2), and the purified fragment was ligated to compatible *Sal*I-*Xba*I sites of the pENTR™1A backbone, generating the pENTR™1A-

mTTR entry clone plasmid (Figure 2.3). Prepared plasmids were subjected to a *Nhe*I and *Xba*I restriction enzyme digestion to identify clones containing the correct insert.

To construct the pLNT-mTTR-JDG lentiviral vector a recombination reaction was performed using the following components: 150 ng of pENTR™1A-mTTR entry plasmid, 150 ng of pLNT-GW-JDG destination vector, 1× Gateway® LR Clonase™ II enzyme mix and TE buffer, pH 8.0 to a final volume of 20 µl. The sample was incubated at room temperature for one hour. The reaction was terminated by addition of 2 µl of Proteinase K Solution (2 µg/µl) (Life Technologies Inc., CA, USA) followed by an incubation step at 37ºC for 10 minutes. The sample was used to transform XL-1 blue *E.coli* bacteria and a correct expression clone was selected following a *Sal*I and *Nco*I restriction digestion of prepared plasmids.

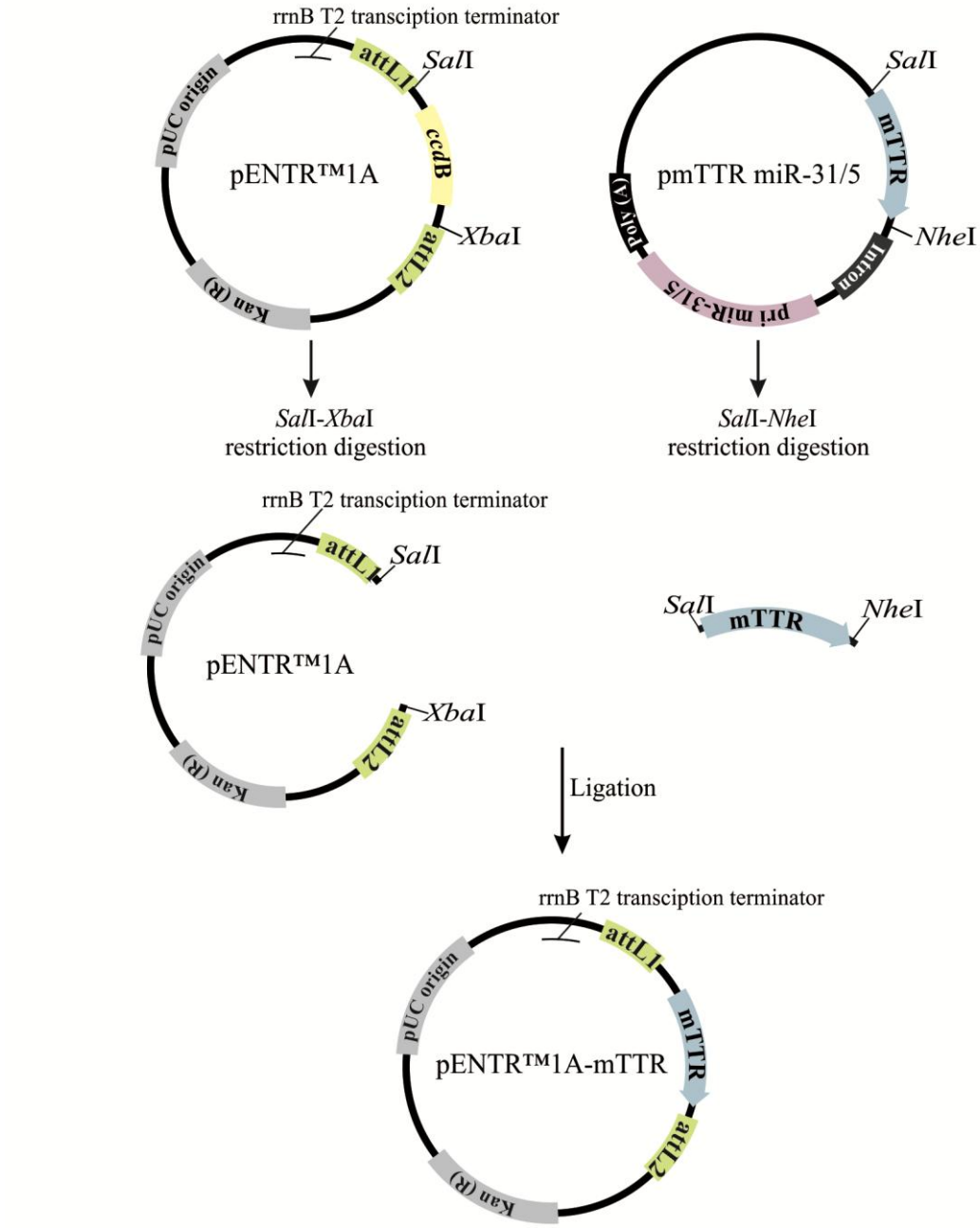


Figure 2.3: <u>Schematic representation of the cloning strategy used for the construction of the pENTR™1A-mTTR entry vector.</u>

The mTTR enhancer-promoter sequence was excised from the pmTTR pri-miR-31/5 expression vector with *Sal*I and *Nhe*I. The fragment was ligated to compatible *Sal*I-*Xba*I sites in the pENTR™1A cloning vector backbone to generate the pENTR™1A-mTTR entry clone vector containing an attL-flanked mTTR promoter sequence.

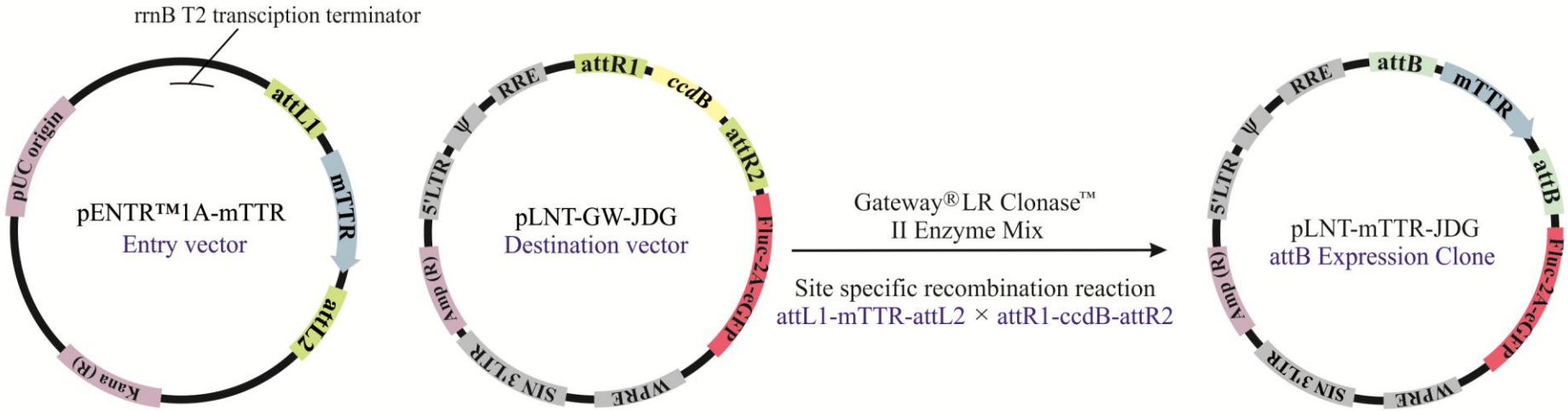


Figure 2.4: <u>Schematic representation of Gateway® cloning strategy for the generation of the pLNT-mTTR-JDG lentiviral vector.</u>

The Gateway® LR recombination reaction, catalysed by the Gateway® LR Clonase™ II enzyme mix was utilised to facilitate the transfer of the attL-flanked mTTR promoter sequence from the pENTR™1A-mTTR entry vector into an attR attachment site-containing destination lentiviral vector (pLNT-GW-JDG). The end product of the recombination reaction was the pLNT-mTTR-JDG lentiviral vector encoding an attB attachment site-flanked mTTR promoter sequence upstream of the *Firefly luciferase*-2A-eGFP ORF. WPRE (woodchuck hepatitis virus posttranscriptional regulatory element), LTR (long terminal repeat), SIN 3' LTR (LTR with a deletion in the U3 region), RRE (rev-responsive element), ψ (HIV-1 packaging signal).

2.10.2 Production and titration of recombinant pLNT-mTTR-JDG LV

The second-generation, recombinant lentiviral vector pLNT-mTTR-JDG has been described in Section 2.10.1. The Gag-Pol expressing pCMVΔR8.74 and the VSV-G expressing pMD.G2 packaging plasmids were kindly provided by the group of Dr Simon N. Waddington from University College London, UK.

Recombinant pLNT-mTTR-JDG LV was produced by transient co-transfection of the transfer lentiviral plasmid and the above mentioned packaging plasmids into HEK293T cells using polyethylenimine (PEI) (Polysciences, PA, USA) as a transfection reagent (Appendix A1-2). Virus-containing supernatant was harvested at 20 and 48 hours after transfection. Pooled virus-containing medium was clarified by centrifugation at 1,000 × g for 5 minutes at 4°C and subsequently filtered through a 0.45 µm AcroVac™ Filter Unit. Clarified virus-containing medium was concentrated using Centricon® Plus-70 Centrifugal Filter Devices 100,000 nominal molecular weight limit (Merck-Millipore, MA, USA) according to the manufacturer's instructions. Briefly, the filter devices were filled with 70 ml of 70% ethanol and centrifuged at 3500 × g for 5 minutes. The filters were further rinsed twice with 70 ml of DPBS. The remaining solution from the filter cup was recovered by centrifuging at 1000 × g for 2 minutes. Next, 70 ml of clarified lentivirus-containing medium was added to individual filter cups and the devices were centrifuged at 3500 × g for 35 minutes. Concentrated virus was recovered by centrifuging the concentrate cups at 400 × g for 2 minutes. The virus was dispensed in sterile microcentrifuge tubes and stored at -80°C. Concentrated pLNT-mTTR-JDG virus was titrated in HeLa cells using the Global UltraRapid Lentiviral Titer Kit (System Biosciences, CA, USA) according to the manufacturer's instructions.

2.10.3 Neonatal liver gene transfer of recombinant LV expressing a *Firefly luciferase* transgene

Animal experimentation was conducted in accordance with procedures and protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee (Appendix A3). Recombinant lentivirus expressing a *Firefly luciferase* transgene transcribed from an internal mTTR promoter was injected into the circulation of anaesthetised newborn mice via the superficial temporal vein (362). Gas anaesthesia with isoflurane (Safeline Pharmaceuticals (PTY) LTD, South Africa) was used to anaesthetise the animals prior to administering the injection. The injections were carried out under 4× magnification using a stereo microscope (Wild Heerbrugg, Switzerland) and a 33-gauge, 16 mm long needle attached to a 100 µl Gastight Hamilton syringe (Hamilton, NV, USA). A light guide connected to a KL1500 LED cold light source (Olympus, Japan) was used to transilluminate the head of animals and improve visualisation of the superficial temporal vein. Each newborn animal (0- to 1- days old) received two injections of a maximum injectate volume of 60 µl over two successive days. One additional animal, to be used as control, received two 60 µl PBS injections over two days.

2.10.4 *In vivo* bioluminescence imaging

In vivo bioluminescence imaging of animals was carried out using the IVIS® Kinetic imaging system (Caliper Life Sciences Inc., MA, USA). Briefly, animals received D-luciferin (Gold Biotechnology, MO, USA) at 150 mg/kg by intra-peritoneal injection 15 minutes before imaging. A stock solution of D-luciferin was prepared by diluting 1 g of D-luciferin in 66.6 ml of PBS to a final concentration of 15 µg/µl. The animals were anaesthetised with isoflurane. Images were acquired with an automatic exposure and analysed using the Living Image® Software (Caliper Life Sciences Inc., MA, USA).

2.11 In vivo assessment of the antiviral efficacy and toxicity of the therapeutic LVTH-LUC miR-31/5-8-9 lentivirus

2.11.1 Construction of recombinant pLVTH- and pLVTH miR-31/5-8-9-derived lentiviral vectors expressing a *Firefly luciferase* transgene

To construct pLVTH- and pLVTH miR-31/5-8-9-derived lentiviral vectors encoding a 2A-linked Firefly luciferase-eGFP bicistronic expression cassette, the existing GFP sequence was excised from the pLVTH and pLVTH miR-31/5-8-9 plasmids with *Pme*I and *Nde*I. The ends of the vector DNA backbones were made blunt using Klenow Fragment (Thermo Scientific, MA, USA) and subsequently dephosphorylated using FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific, MA, USA). The 2A-linked Firefly luciferase-eGFP expression cassette was excised from the pLNT-GW-JDG plasmid with *Bam*HI and *Mlu*I and the purified fragment was treated with Klenow Fragment to fill in the 5' DNA overhangs. The blunt sequence was ligated to the blunted and dephosphorylated pLVTH and pLVTH miR 31/5-8-9 vector backbones to generate the pLVTH-LUC and pLVTH-LUC miR 31/5-8-9 lentiviral vectors (Figure 2.5). Prepared plasmids were subjected to *Nco*I and *Eco*91I restriction enzyme digestion to select clones containing the insert in the desired orientation.

2.11.2 Production and titration of recombinant LVTH-LUC and LVTH-LUC miR-31/5-8-9 LV

Recombinant LVTH-LUC and LVTH-LUC miR-31/5-8-9 LV was produced by transient co-transfection of the transfer lentiviral plasmid and packaging plasmids (psPAX2 and pMD2.G) into HEK293T cells using PEI as a transfection reagent (Appendix

A1-2). Virus-containing supernatant was harvested at 20 and 48 hours after transfection. Pooled virus-containing medium was clarified by centrifugation at 1,000 × g for 5 minutes at 4°C and subsequently filtered through a 0.45 μm AcroVac™ Filter Unit. Clarified virus-containing medium was concentrated using Centricon® Plus-70 Centrifugal Filter Devices as described in Section 2.10.2. Concentrated lentivirus was titrated in HeLa cells using the Global UltraRapid Lentiviral Titer Kit according to the manufacturer's instructions.

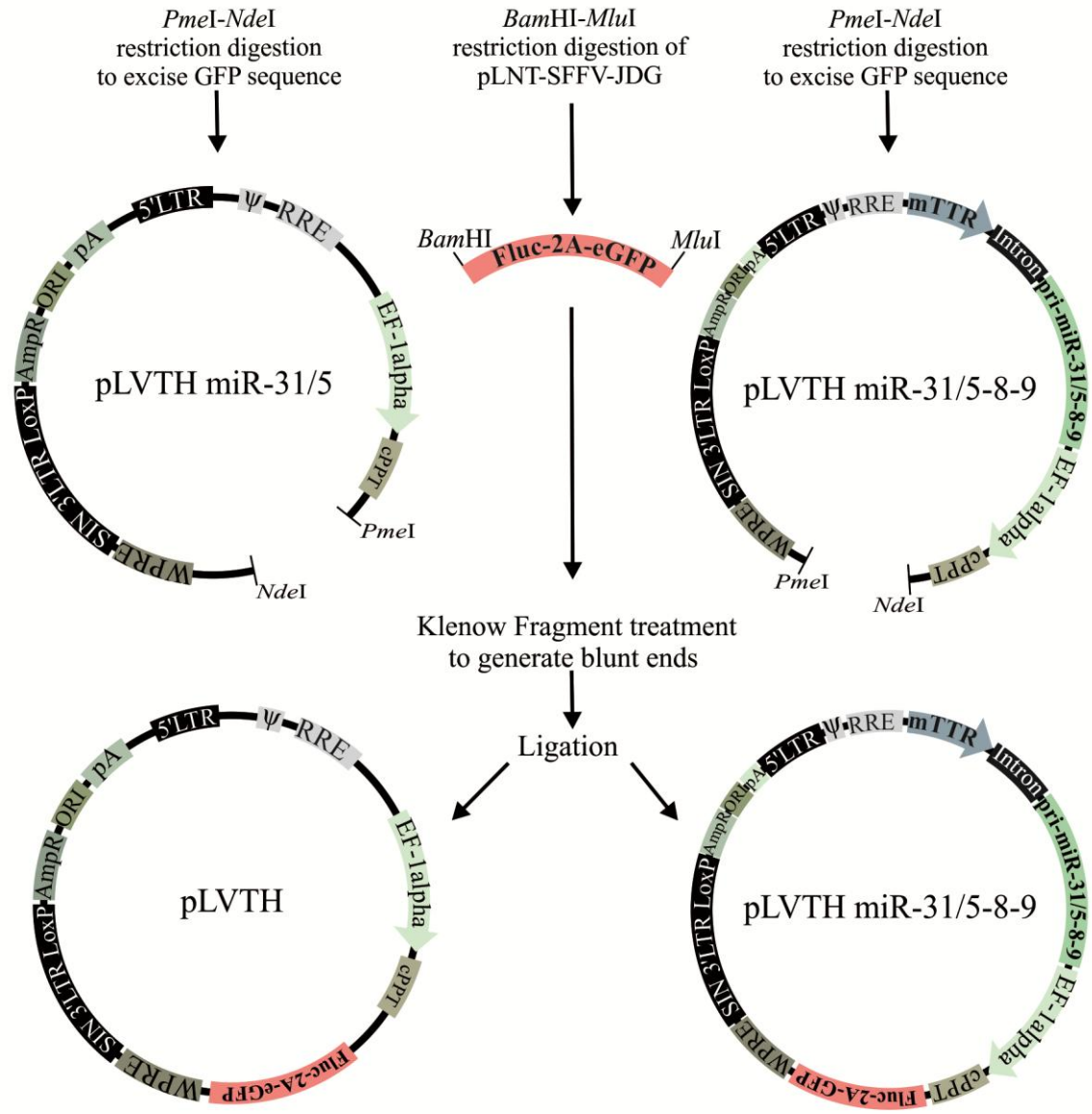


Figure 2.5: Schematic illustration of the cloning strategy used to construct the pLVTH-LUC and pLVTH-LUC miR-31/5-8-9 lentiviral vectors.

The GFP-encoding sequences were excised from pLVTH and pLVTH miR-31/5-8-9 lentiviral vectors with *Pme*I-*Nde*I and the backbones were treated with Klenow fragment to blunt DNA ends. A Klenow-treated GFP-2A-Firefly luciferase fragment was inserted into the respective vector backbones to generate the pLVTH-LUC and pLVTH-LUC miR-31/5-8-9 lentiviral vectors. WPRE (woodchuck hepatitis virus posttranscriptional regulatory element), LTR (long terminal repeat), SIN 3' LTR (LTR with a deletion in the U3 region), RRE (rev-responsive element), ψ (HIV-1 packaging signal).

2.11.3 LVTH-LUC and LVTH-LUC miR-31/5-8-9 LV gene transfer in neonatal and adult HBV transgenic mice

2.11.3.1 LV gene transfer via temporal vein injection in neonatal HBV transgenic mice

Recombinant LVs were delivered to the livers of newborn HBV transgenic mice via an intravenous temporal vein injection as described in section 2.10.3. Neonatal HBV transgenic animals received a single unilateral 60 µl injection of recombinant LVTH-LUC or LVTH-LUC miR-31/5-8-9 LV (8.7×10^7 TU per mouse).

2.11.3.2 LV gene transfer via tail vein injection in adult HBV transgenic mice

Recombinant LVTH-LUC and LVTH-LUC miR-31/5-8-9 LV was injected into the tail vein of 21 to 25 day old HBV transgenic mice. All animals that received a LV injection had medium to high HBV titres. The final injection volume was 0.5 ml of LVTH-LUC and LVTH-LUC miR-31/5-8-9 lentivirus (5.5×10^8 TU per mouse).

2.11.4 Quantification of serum alanine transaminase

Blood samples were collected at the end of the study from HBV transgenic animals that had received LV via a temporal vein injection. Mice that had received recombinant LV via a tail-vein injection, were bled at three different time points (48 hours post injection, one week post injection and at the end of the study). The samples were incubated at 4°C for at least 90 minutes to facilitate coagulation and were subsequently centrifuged at 10,000 × g for 10 minutes at 4°C. Mouse serum was extracted, diluted 1:2 with saline and the levels of serum alanine transaminase (ALT) in the samples were determined by the

National Health Laboratory Service at the Charlotte Maxeke Johannesburg Academic Hospital.

2.11.5 Quantification of serum HBsAg

Blood samples were collected weekly from all animals. Mouse serum was diluted 1:4 with saline and 100 µl of the diluted samples were used to quantify the level of HBsAg using the Monolisa™ HBsAg ULTRA assay kit as described in Section 2.3.3.2.

2.11.6 Quantification of serum HBV DNA

The Total Nucleic Acid Isolation Kit and MagNA Pure LC system was used to extract 50 µl of mouse serum extracted from blood samples which had been collected on a weekly basis. Viral DNA quantification was performed using real-time quantitative PCR carried out using HBV-specific primers, described in Section 2.9.3 and the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, CA, USA). PCR analysis was performed using the SsoFast™ EvaGreen® Supermix (Bio-Rad, CA, USA) and the following cycling parameters: a 30 second hot start at 95°C and 40 cycles of denaturation at 95°C for 10 seconds, annealing at 58°C for 10 seconds and extension at 72°C for 10 seconds. EuroHep standards were used to generate a standard curve for the quantification of viral particle equivalents (VPEs) in the individual samples (158).

2.11.7 Tissue collection

Mice were sacrificed at the completion of each study and the livers from individual animals were harvested. Livers were cut in three equal samples. The tissue samples were used to extract DNA and RNA.

2.11.8 Determination of vector copy number in mouse liver tissue

Genomic DNA was extracted from 25 mg of homogenised liver tissue using the QIAamp® DNA Mini Kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions. The Global UltraRapid Lentiviral Titer Kit was used to determine the number of vector copies associated with genomic DNA extracted from individual mouse liver samples.

2.11.9 Quantitative reverse transcriptase PCR of intrahepatic HBV RNA

Total RNA was extracted from mouse livers using Tri-Reagent and reverse transcribed with the QuantiTect® Reverse Transcription Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. The following primer sets mGAPDH F: 5'- TTC ACC ACC ATG GAG AAG GC -3' and mGAPDH R: 5'- GGC ATG GAC TGT GGT CAT GA -3', HBV Surface F 5'- TGC ACC TGT ATT CCA TC -3' and HBV Surface R 5'- CTG AAA GCC AAA CAG TGG -3', HBV Core F: 5'- ACC ACC AAA ATG CCC CTA T -3' and HBV Core R: 5'- TTC TGC GAG GCG GCG A -3' were used to amplify murine *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), HBV *surface* and *core* mRNA respectively. The oligonucleotide sequences used were synthesised by standard phosphoramidite chemistry (Inqaba Biotech, South Africa). PCR analysis was performed using the SsoFast™ EvaGreen® Supermix as described in Section 2.11.6. Specificity of the PCR products was verified by melting curve analysis. HBV *surface* and *core* gene mRNA levels were normalised to *GAPDH* mRNA.

2.11.10 Detection of mature miR-31/5 in liver tissue of lentivirus-injected HBV transgenic mice

The Custom TaqMan® Small RNA Assay (Life Technologies Inc., CA, USA) was used to detect and quantify the putative mature miR-31/5 guide sequences of pri-miR-31/5-8-9 in liver tissue samples. The assay involved performing reverse transcription with a stem-loop primer designed to bind specifically to the mature miR/31-5 sequence followed by a two step real-time PCR for detection of the mature miR sequence of interest. The basic principle of the assay is illustrated in Figure 2.6. Total RNA was extracted from mouse livers using Tri-Reagent and reverse transcribed with the TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies Inc., CA, USA) and a specific stem-loop reverse transcription primer according to the manufacturer's instructions. PCR analysis was performed using the products from individual reverse transcription reactions, TaqMan® Small RNA Assay custom designed for miR-31/5 or endogenous U6 snRNA and the TaqMan® 2× Universal PCR Master Mix with AmpErase® UNG (Life Technologies Inc., CA, USA) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Statistical difference was calculated using the Student's paired two-tailed t-test and was considered significant when P was less than 0.05. Calculations were performed with the GraphPad Prism software package (GraphPad Software Inc., CA, USA).

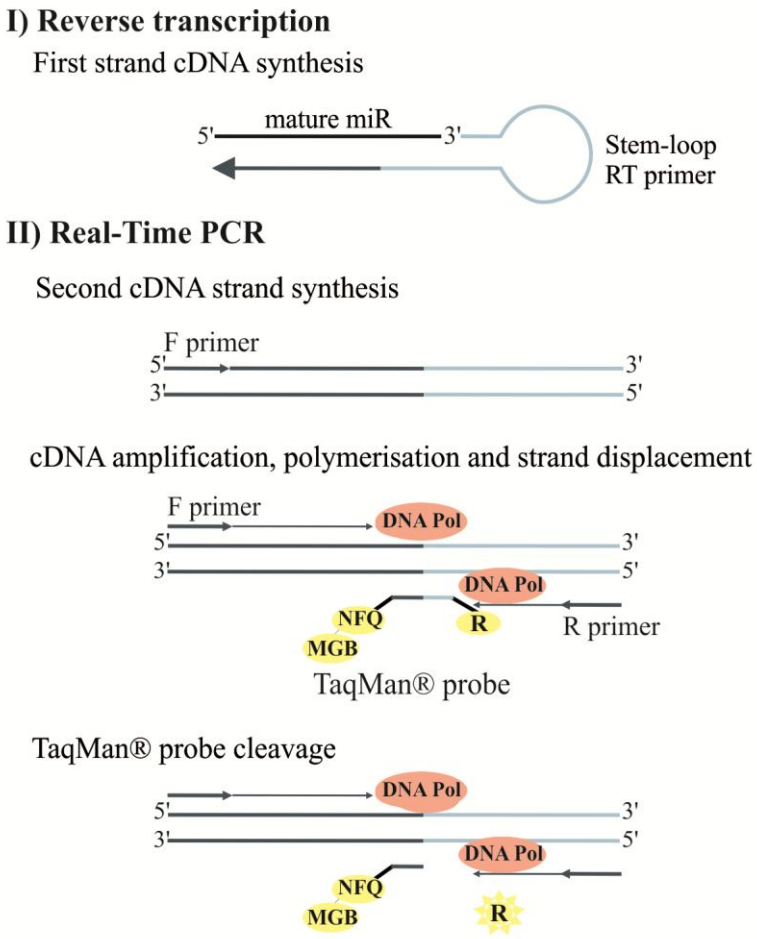


Figure 2.6: <u>TaqMan® Small RNA Assay-based quantification of mature miR sequences.</u>

The TaqMan® Small RNA Assay entails performing a two-step reverse transcription PCR procedure to detect specific small RNA sequences present in a sample of interest. A) Complementary DNA is transcribed from total RNA using a stem loop primer that has been designed bind specifically to a mature miR sequence of interest. B) During PCR the TaqMan® probe anneals to a sequence located between the forward and reverse primer binding sites. The probe includes a minor groove binder at its 3' end (MGB) and a non fluorescent quencher (NFQ) as well as a FAM reporter dye linked to its 5'end (R). If the target sequence is present the probe will specifically hybridise and will be subsequently cleaved by DNA polymerase (DNA Pol). Cleavage causes separation of the quencher from the reporter dye resulting in an increased fluorescence signal.

3 ENGINEERING SECOND GENERATION SIN RECOMBINANT LENTIVIRAL VECTORS FOR ANTI-HBV RNAI GENE THERAPY

3.1 Introduction

The use of expressed anti-HBV RNAi effecters has been shown to be a valuable therapeutic strategy to target viral replication and accomplish effective antiviral silencing (159, 160, 363, 364). Successful bench to bedside translation of anti-HBV RNAi-based gene therapy is contingent on accomplishing sustained and potent silencing of viral replication. Expressed RNAi effecters, allow for sustained production of therapeutic sequences from stable DNA templates and are compatible with both non-viral and viral vectors. In contrast to their non-viral counterparts, viral vectors are capable of mediating efficient transduction of target organs and long-term expression of therapeutic sequences, suited to countering persistent HBV infection. The selection of a particular viral vector for a specific application, is dependent on the nature of the disease as well as the desired therapeutic outcome. Stable integration of transgenes that may be achieved with recombinant lentiviruses makes these vectors useful for attaining sustained expression of antiviral RNAi activators.

To advance the use of recombinant lentiviruses for HBV treatment, SIN lentiviral vectors were engineered to express liver-specific, HBV silencing pri-miR-mimics. The expressed pri-miR mimics are derived from natural pri-miR-31 and have been engineered to generate anti-HBV RNAi guide sequences targeting single (monocistronic pri-miR sequences) or multiple (polycistronic pri-miR sequences) sites within the *HBx* ORF of the HBV genome (159, 160). Meticulous design of potentially effective therapeutics at the

bench is required to allow for future clinical application.The molecular engineering of HBV silencing lentiviral vectors involved careful design and consideration of various factors capable of influencing the efficacy and suitability of this strategy for anti-HBV RNAi-based gene therapy.

As mimics of naturally occurring pri-miRs, expressed anti-HBV pri-miR mimics are compatible with expression from Pol II promoters and have the associated advantages of controllable and tissue-specific expression. The design of previously characterised mono- and poly- cistronic anti-HBV pri-miR-31 expression cassettes (159, 160), was further optimised by replacing the promiscuous CMV promoter with the liver-specific mTTR promoter to facilitate hepatotropic expression and to ensure compatibility with the lentiviral delivery system. The use of a hepatospecific promoter, such as mTTR, allows for simultaneous expression of the therapeutic effecters in hepatocytes while regulating the dose of expressed therapeutic effecters. Liver-specific expression should also eliminate off-target effects that could arise from constitutive expression of the pri-miR mimics in non-targeted tissues. While the mTTR promoter has been successfully exploited to drive long-term expression of transgenes in the context of lentiviral vectors (180), shutdown of CMV promoter activity in the murine liver has been reported. (176, 177). Furthermore the CMV promoter has been reported to affect viral titres negatively when used to control miR expression in the context of lentiviral vectors (329).

The use of a moderate internal Pol II promoter, such as mTTR, (305) as well as the inclusion of LoxP sites in the 3' LTR of the SIN pLVTH lentiviral vector, allowing for conditional ablation of LV-transduced hepatocytes is likely to improve the biosafety profile of the HBV silencing lentiviral vectors. Additional features incorporated to improve functionality and efficacy of our strategy, included the exclusion of an internal polyadenylation signal downstream of the liver-specific pri-miR sequences in the lentiviral

vectors as well as placing the therapeutic expression cassettes in the sense orientation with respect to the lentiviral genome. Placing the a miR expression cassette in reverse orientation has been reported to hamper the expression of active miR sequences (329). The polyadenylation signal was excluded to prevent premature termination of the full length viral RNA genomic transcripts and consequently reduction in viral titre (365).

The aim of the next set of experiments was to evaluate the antiviral efficacy and tissue-specificity of the mono- and poly- cistronic pri-miR mimics expressed from the liver-specific mTTR promoter in cultured liver-derived cells. Furthermore, the compatibility of the novel, liver-specific pri-miR-31/5 and pri-miR-31/5-8-9 expression cassettes with the SIN second-generation pLVTH lentiviral vector was assessed.

3.2. Design and efficacy of monocistronic and polycistronic liver-specific anti-HBV pri-miR mimics

3.2.1 The mTTR promoter is capable of driving tissue-specific expression of the *Firefly luciferase* transgene

The mTTR enhancer-promoter sequence comprises the TTR enhancer and Pol II promoter to which a synthetic liver-specific enhancer sequence has been appended (366). The enhancer element has been designed to include binding sites for several hepatic transcription factors critical for initiating hepatotropic expression *in vitro* and *in vivo* (Figure 3.1 A).

To examine the tissue-specificity and functionality of the mTTR promoter the efficiency of mTTR driven expression of the *Firefly luciferase* transgene was assayed in liver-derived, Huh7 and HepG2.2.15 cells as well as kidney-derived HEK293T cells.

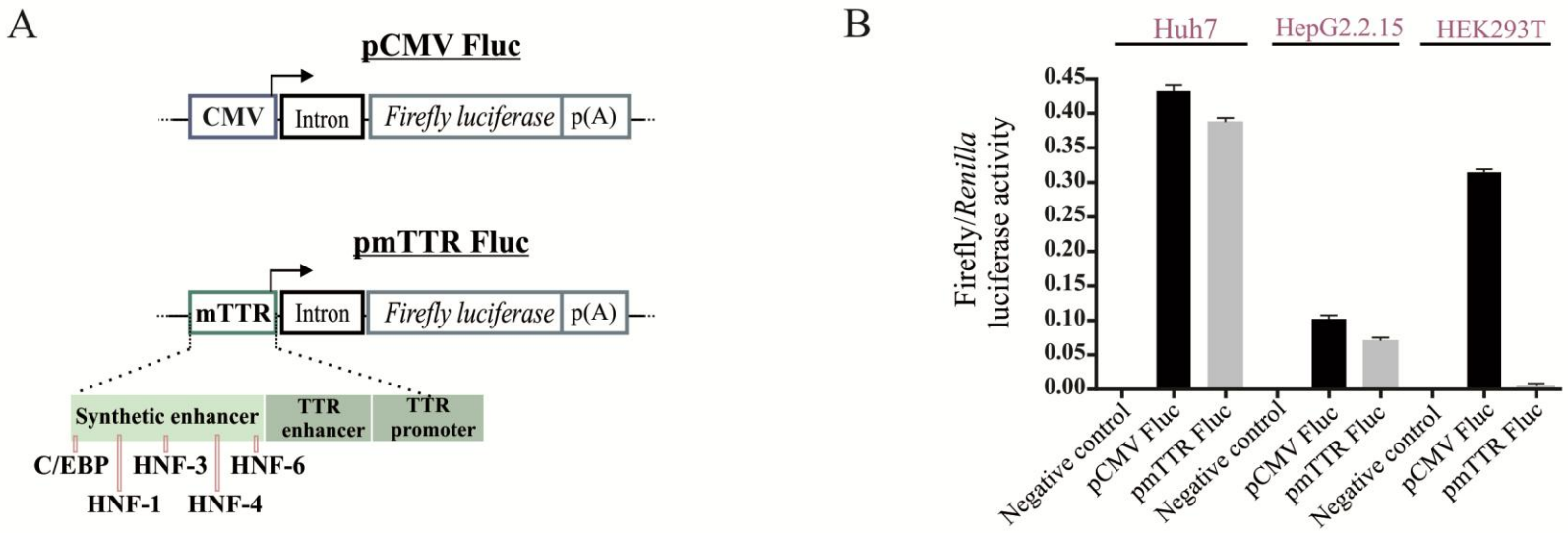


Figure 3.1: <u>Firefly luciferase expression from CMV and mTTR promoter sequences in liver- and kidney-derived cells.</u>

(A) Schematic representation of plasmids encoding the *Firefly luciferase* transgene under the control of a promiscuous CMV promoter (pCMV Fluc) or a liver-specific murine transthyretin (mTTR) promoter (pmTTR Fluc). The different elements of the mTTR promoter and transcription factor binding sites in the synthetic enhacer sequence - CCAAT/enhancer binding protein (C/EBP), hepatic nuclear factor (HNF) 1, HNF3, HNF4 and HNF 6 are indicated. (B) Firefly luciferase expression in liver (Huh7 and HepG2.2.15)- and kidney (HEK293T)-derived cells transfected with pCMV Fluc and pmTTR Fluc plasmids. A plasmid constitutively expressing *Renilla* luciferase (phRL-CMV) was included in all transfections. Data are represented as mean ratios of Firefly to *Renilla* luciferase activity and the bars represent the SEM from three independent experiments.

Firefly luciferase activity was measured in Huh7, HepG2.2.15 and HEK293T cells transfected with constructs encoding the *Firefly luciferase* transgene under the control of the promiscuous CMV immediate early promoter enhancer element (pCMV Fluc) or the liver-specific mTTR promoter (pmTTR Fluc). As anticipated, strong Firefly luciferase expression was observed in all cell populations transfected with the CMV expressing Firefly luciferase plasmid (Figure 3.1 B).

The capacity of the CMV promoter to drive constitutive, high-level albeit variable expression of transgenes in different mammalian cell lines has been well characterised (367, 368). Strong Firefly luciferase expression was also detected in Huh7 and HepG2.2.15 cells transfected with the mTTR expressing plasmid demonstrating that the mTTR promoter sequence is functional (Figure 3.1 B). In contrast, particularly low Firefly luciferase activity was measured in kidney-derived cells transfected with the mTTR expressing plasmid, confirming the tissue-specific expression profile of the mTTR promoter sequence.

3.2.2 Pri-miR mimics expressed from the mTTR promoter mediate tissue-specific HBV knockdown

Following demonstration of functionality as well as tissue-specific expression (Section 3.2.1), the mTTR Pol II transcriptional sequence was identified as a suitable promoter that could be utilised to drive liver-specific expression of the monocistronic and polycistronic anti-HBV pri-miR mimics. Liver-specific pri-miR expression cassettes were generated by placing monocistronic and polycistronic anti-HBV pri-miR sequences, originally embedded in CMV expression cassettes (159, 160), under the control of the mTTR promoter. The HBV-silencing pri-miR mimics are derived from natural pri- miR-31 and have been designed to target single (monocistronic pri-miR-31/5) or multiple

(polycistronic pri-miR-31/5-8-9) sites within the *HBx* ORF of the HBV genome (159, 160). The conserved *HBx* sequence is common to the 3' end of all HBV transcripts. As a result targeting *HBx* enables simultaneous knockdown of all viral mRNAs (158). A dual luciferase reporter gene assay was used to assess the knockdown efficacy of the liver-specific monocistronic and polycistronic pri-miR mimics (Figure 3.2). CMV-driven monocistronic (pCMV miR-31/5) and polycistronic (pCMV miR 31/5-8-9) pri-miR expression constructs which have been previously shown to mediate efficient knockdown (159, 160) were also included in the experiment.

Potent knockdown of HBV reporter gene expression was mediated by CMV-expressed pri-miR mimics in both liver- (Huh7) and kidney-derived (HEK293T) cells (Figure 3.2). Similarly, significant HBV knockdown was observed in liver-derived cells transfected with expression plasmids encoding mTTR-driven pri-miR mimics (pmTTR miR-31/5 and pmTTR miR-31/5-8-9) (Figure 3.2 B). However, a less pronounced silencing effect was observed in kidney-derived cells transfected with plasmids encoding mTTR-expressed pri-miR sequences (Figure 3.2 C). The low level of mTTR-driven pri-miR expression, observed in HEK293T kidney-derived cells, is likely to be attributable to the availability of a subset of the transcription factors required for optimal mTTR expression (369, 370). Overall these data indicate that pri-miR mimic sequences expressed from the mTTR promoter are capable of mediating potent and tissue-specific knockdown of HBV gene expression.

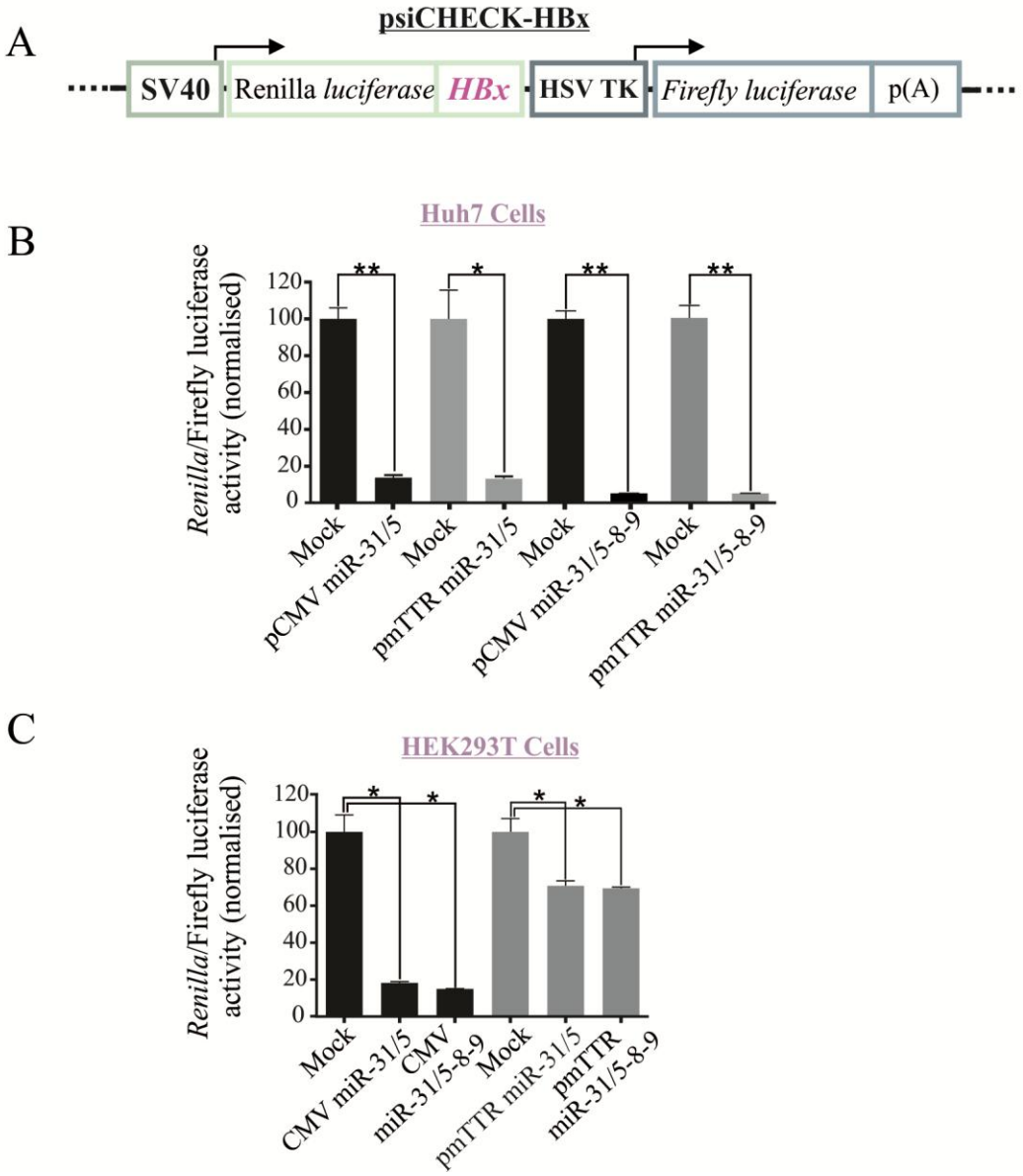


Figure 3.2: <u>HBV knockdown efficacy of liver-specific pri-miR expression cassettes.</u>

(A) The psiCHECK-*HBx* dual luciferase reporter vector contains the complete wild-type *HBx* target sequence (indicated in pink) downstream of the Renilla *luciferase* ORF within psiCHECK^{TM}-2. The psiCHECK-*HBx* reporter vector was co-transfected with plasmids encoding monocistronic and polycistronic pri-miR expression cassettes. Forty eight hours after transfection, *Renilla* and Firefly luciferase activity was measured in lysates of Huh7 (B) and HEK293T cells (C) cells. Data are represented as mean ratios of *Renilla* to Firefly luciferase activity (±SEM., n=3) and are normalised relative to mock-treated cells. $*p < 0.05$, $**p < 0.01$.

3.3 Design of the second generation SIN pLVTH-derived lentiviral vectors encoding liver-specific anti-HBV pri-miR mimics

A number of intricate factors were taken into account when designing the HBV-silencing lentiviral vectors to ensure optimal transgene functionality and downstream efficacy. To generate recombinant RNAi-based anti-HBV lentiviral vectors, expression cassettes encoding monocistronic and polycistronic liver-specific pri-miR mimics (Figure 3.3) were incorporated into the second generation SIN lentiviral vector, pLVTH (Figure 3.4). The HIV-1 derived minimal *cis*-acting elements encoded in the pLVTH lentiviral vector backbone include: HIV-1 5' LTR, a splice donor site, HIV-1 encapsidation signal (ψ), a truncated *gag* sequence, RRE, a splice acceptor site, an internal EF-1 alpha promoter, cPPT, *GFP* reporter transgene, WPRE and a 3' SIN LTR encompassing LoxP sites. Incorporation of additional sequences such as the WPRE, cPPT and RRE generally improves the overall vector efficiency by stimulating nuclear import of the PIC and cytoplasmic export of lentiviral mRNA as well as trafficking of unspliced genomic RNA to the cytoplasm (274, 277, 279). In addition, the cPPT facilitates efficient transduction of quiescent hepatocytes (281).

The VSV-derived glycoprotein G was selected to pseudotype the anti-HBV pri-miR-encoding pLVTH-derived LVs as it confers broad vector tropism as well as increased stability of lentiviral particles which allows for efficient large-scale LV production as well as efficient cell transduction (298). In addition to the importance of successfully propagating recombinant lentivirus capable of efficient transduction, it is critical to ensure the lentiviral construct used has a high-degree of predictable biosafety. The SIN configuration of pLVTH markedly reduces the risk of potential LV mediated insertional mutagenesis (311) as the vector encompasses a 3' LTR devoid of viral enhancer sequence (262).

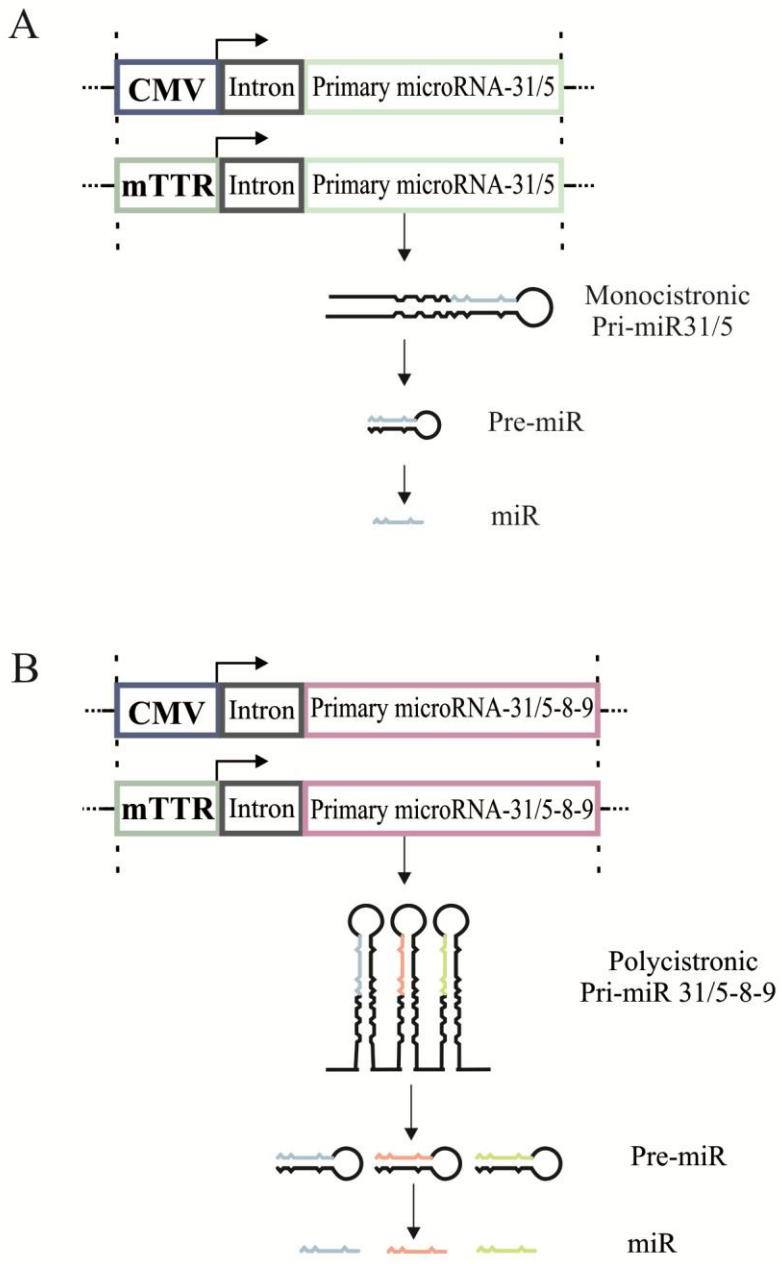


Figure 3.3: <u>Schematic illustration of the mono- and poly- cistronic liver-specific pri-miR expression cassettes.</u>

Monocistronic (A) and polycistronic (B) anti-HBV pri-miR sequences, originally embedded in CMV expression cassettes, were placed under the control of the enhanced mTTR promoter to generate liver-specific pri-miR expression cassettes. Putative processing of pri-miR mimics to generate mature anti-HBV miR sequences is shown.

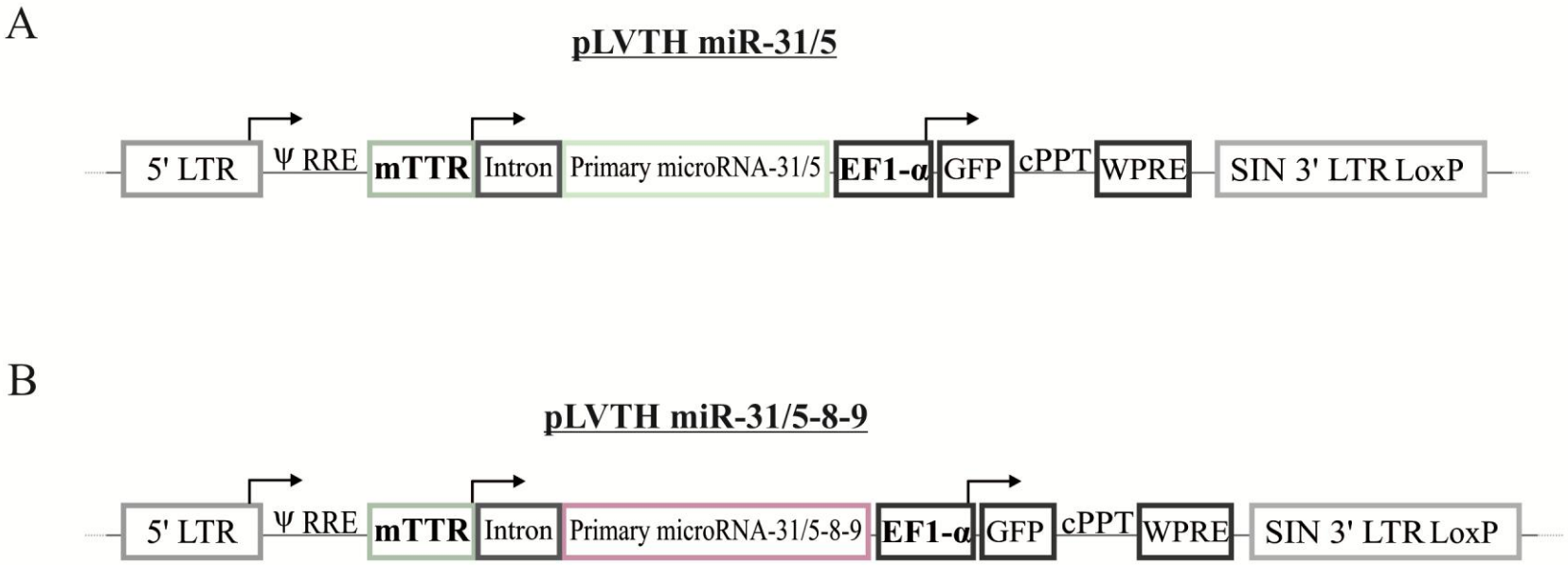


Figure 3.4: Schematic illustration of the second-generation SIN pLVTH- derived HBV-silencing lentiviral vectors.

Schematic of the pLVTH miR-31/5 (A) and pLVTH miR-31/5-8-9 (B) lentiviral vectors encoding monocistronic (pri-miR-31/5) and polycistronic (pri miR-31/5-8-9) liver-specific anti-HBV pri-miR mimics, respectively. The arrangement of the mTTR pri-miR expression cassettes in the context of the lentiviral construct is indicated. LTR (long terminal repeat), ψ (HIV-1 packaging signal), RRE (rev-responsive element), cPPT (central polypurine tract), WPRE (woodchuck hepatitis virus posttranscriptional regulatory element), SIN 3' LTR (long terminal repeat with a deletion in the U3 region).

Moreover, a LoxP site has been included in the 3' pLVTH LTR to add an additional safety-switch and enhance the biosafety profile of pLVTH-derived recombinant lentivirus. During reverse transcription the 3’ LTR sequence is copied to the 5’ end simultaneously copying the LoxP site. Consequently the integrated LV sequence would be flanked by LoxP sites allowing it to be excised following Cre-recombinase treatment of transduced cells (371, 372).

The design of the transgene expression cassette intended for insertion into an RNAi-based anti-HBV lentiviral construct can influence the titre of the lentivirus as well as the expression of the therapeutic RNAi molecule. The inclusion of a promiscuous CMV Pol II promoter in the context of miR-based lentiviral vector expression systems has been demonstrated to result in severe LV titre reduction (329), indicating that the CMV promoter sequence or activity is incompatible with the lentiviral system. The inclusion of the Pol II mTTR promoter driving pri-miR mimic expression did not result in any significant titre reduction when compared to the titre obtained for LV propagated using the pLVTH lentiviral backbone (Table 3.1).

Incorporating a polyadenylation signal immediately downstream of the transgene could improve expression (373), however the presence of an additional polyadenylation signal in a lentiviral construct would result in premature termination of the full length, transduction-competent genomic transcripts and a severe reduction in viral titre (365). Placing the a miR expression cassette in reverse orientation has been reported to hamper the expression of active miR sequences (329) possibly as a result of the absence of a polyadenylation signal which is typically located in the 3' LTR of lentiviral vectors. The pri-miR expression cassette was inserted in the pLVTH backbone in the forward (sense) orientation without a polyadenylation signal. The 3' LTR polyadenylation signal would contribute to improved stability of the pri-miR transcript produced from the liver-specific

encoded in the pLVTH lentiviral vector in the forward orientation, resulting in robust expression of mature miR sequences. In addition, Drosha-mediated recognition and processing of miR precursors encoded in the sense orientation of the viral genome, can potentially result in the production of truncated, non-functional genomic transcripts and reduced viral titres (329). Nevertheless, efficient production of anti-HIV LVs expressing miR precursors has been reported (182, 185).

Table 3.1: Average viral titres obtained for pLVTH and pLVTH-derived HBV-silencing LVs.

Lentivirus	Viral titre ^{HeLa}TU/ml
LVTH	~ 1.5×10^7
LVTH miR-31/5	~ 1.3×10^7
LVTH miR-31/5-8-9	~ 1.3×10^7

3.3.1 Lentiviral vector-encoded liver-specific pri-miR mimics mediate effective knockdown of viral replication

To assess the functionality of the anti-HBV mTTR monocistronic and polycistronic pri-miR expression cassettes in the context of the RNAi-based lentiviral vector system, Huh7 cells were co-transfected with a replication competent HBV plasmid (pCH-9/3091) (Figure 3.5) and lentiviral vectors encoding the mTTR pri-miR-31/5 (pLVTH miR-31/5) or mTTR pri-miR-31/5-8-9 (pLVTH miR-31/5-8-9) expression cassette. An HBsAg ELISA assay was performed to measure the inhibition of HBsAg secretion in transfected cells. Significant inhibition of HBsAg secretion (~90%) was observed in cells transfected with

the pLVTH miR-31/5 and pLVTH miR-31/5-8-9 lentiviral vector plasmids (Figure 3.5). The expressed, liver-specific pri-miR sequences, now encoded in the pLVTH-derived lentiviral constructs, were demonstrated to mediate effective inhibition of HBV replication (Figure 3.5). These data corroborate the potent HBV knockdown mediated by liver-specific anti-HBV pri-miR mimics encoded in mammalian expression vectors (Section 3.2.2).

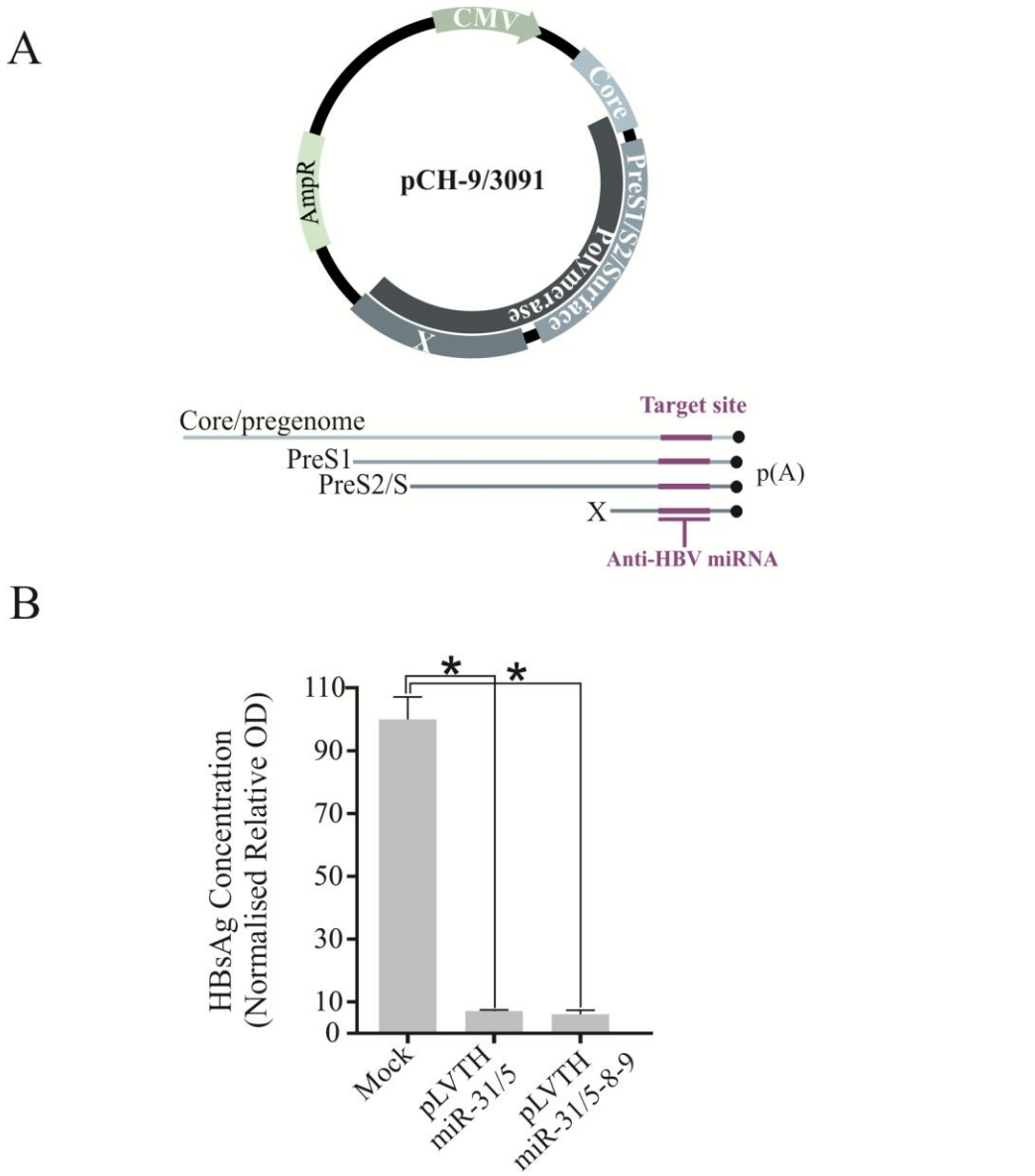


Figure 3.5: <u>Assesement of inhibition of HBsAg secretion by lentiviral vector encoded liver-specific anti-HBV miR mimics.</u>

(A) Schematic illustration of HBV ORFs organisation within the pCH-9/3091 target vector. HBV transcripts terminating at a common 3' end have been indicated in puple and include miR-31/5 and miR-31/5-8-9 targets. (B) HBsAg secretion was measured in Huh7 cells co-transfected with the pCH-9/3091 HBV target vector and pLVTH-derived lentiviral vectors encoding liver-specific mono- and poly- cistronic pri-miR mimics. HBsAg measurements from quantitative ELISA are given as normalised means (±SEM., n=3) relative to mock-treated cells. $*p < 0.05$

3.4 Conclusion

To advance the use of recombinant lentiviruses for HBV treatment, we engineered RNAi-based HBV silencing lentiviral vectors encoding mono- and poly- cistronic pri-miR sequences expressed from the liver-specific mTTR promoter. Initial evaluation of the functionality and tissue-specificity of the murine mTTR enhancer-promoter sequence, revealed that the promoter is capable of driving robust and tissue-specific expression of the *Firefly luciferase* transgene in liver-derived Huh7 cells. Following validation of the mTTR promoter, the anti-viral efficacy and tissue-specific expression of the monocistronic and polycistronic pri-miR mimics, placed under the control of the mTTR promoter, was assessed. Similarly to their CMV promoter counterparts, the monocistronic and polycistronic pri-miR mimics expressed from the liver-specific mTTR promoter were equally capable of mediating efficient knockdown of HBV gene expression.

Following demonstration of antiviral efficacy of the liver-specific pri-miR mimics, the sequences were incorporated into the pLVTH lentiviral backbone in the forward orientation without a polyadenylation signal. Finally, the antiviral efficacy of the anti-HBV pri-miR mimics in the context of the pLVTH-derived lentiviral vectors was assessed in liver-derived Huh7 cells. The liver-specific monocistronic and polycistronic pri-miR mimics encoded in the pLVTH lentiviral vector backbone were demonstrated to mediate efficient knockdown of HBV replication. Accomplishing robust viral knockdown indicated that the design of the HBV silencing lentiviral vectors was functional and efficient.

The above findings highlight potential utility of the novel RNAi-based HBV silencing lentiviral vector for the development of safe and effective therapy for chronic HBV. The efficacy of currently available treatments for chronic HBV infection is limited, as treatment using these therapeutic agents does not allow for durable silencing of HBV gene expression and it is associated with the emergence of viral escape mutants (374). In

addition, the risk of developing HCC remains undiminished in the majority of chronic carriers receiving currently licensed treatments (43). Globally, chronic HBV infection remains a significant cause of public health problems and the development of improved anti-HBV therapies is therefore a priority. Advancing the use of lentiviruses for RNAi-based anti-HBV gene therapy could be a potentially valuable strategy to allow for durable silencing and ultimately effective eradication of chronic viral infection.

In the following chapter the usefulness of the pLVTH-derived HBV silencing lentiviral vectors for the production of functional and potentially effective LVs to counter chronic HBV infection is explored.

4 CHARACTERISATION OF THE RNAi-BASED HBV-SILENCING LENTIVIRAL VECTORS *IN VITRO*

4.1 Introduction

Lentiviral vector delivery allows for sustained, long-term expression of therapeutic anti-HBV pri-miR mimics, making this delivery method particularly suited to countering persistent HBV infection. Several groups have reported efficient lentiviral vector delivery and sustained expression of expressed RNAi effecters (313, 326, 327). Data described in the previous chapter demonstrated that the design of the tailor-made RNAi-based HBV-silencing lentiviral vectors allows for optimal expression of the antiviral pri-miR mimics. Nevertheless, the functionality and efficacy of HBV silencing LVs, propagated using the SIN pLVTH-derived lentiviral vectors encoding monocistronic (LVTH miR-31/5) and polycistronic (LVTH miR-31/5-8-9) liver-specific anti-HBV pri-miR mimics, requires thorough assessment of transduction efficiency, antiviral efficacy and safety in pertinent cell culture systems.

Stable LV integration can be easily assessed by monitoring the expression of marker transgenes, such as GFP, frequently incorporated into recombinant lentiviral vectors including pLVTH. Additionally, genomic quantitative PCR analysis can be performed to determine the number of integrated lentiviral copies in stably transduced cells. Furthermore it is important to determine whether the integrated pri-miR sequences are being processed by the cellular RNAi machinery according to their intended design and whether recombination is occurring between equivalent sequences in the polycistronic pri-miR transcript during lentivirus transduction. Recombination-mediated deletion of pri-miR sequences in the context of a polycistronic scaffold has been reported previously (183,

185) and deletion events seem to be more prominent when an increasing number of hairpins, four hairpins or more, is included in the polycistronic pri-miR transcript (185). Nevertheless, a multitargeted RNAi approach, such as the use of polycistronic anti-HBV pri-miR mimics, is likely to be valuable in preventing the emergence of viral escape mutants while simultaneously enabling long-term sustained silencing of chronic HBV infection.

Recent evidence of saturation of the natural miR biogenesis pathway following AAV-8-mediated anti-HBV shRNA delivery has highlighted the need for careful analysis of unintended toxic effects following the delivery of exogenous RNAi effecters (165). The anti-HBV pri-miR mimics encoded in pLVTH-derived lentiviral vectors are expressed by the liver-specific mTTR Pol II promoter which allows for controllable and tissue specific expression. Although, saturation of the endogenous miR biogenesis pathway using the respective pri-miR mimics is not likely to occur (127), assessing potential saturation of the endogenous miR pathway following expression of LV-delivered anti-HBV pri-miR mimics is important.

Finally, evaluating the antiviral efficacy of HBV silencing LVs in relevant cell culture systems is an important objective in the preclinical development of potential lentiviral vector-based anti-HBV RNAi gene therapy. Antiviral efficacy can be evaluated in liver-derived cells that had been stably transduced with HBV silencing LVs and the subsequent transfection of replication-competent HBV plasmids. Additionally, HBV replication cell lines, such as the HepG2.2.15 cell line which had been generated by transforming the HepG2 human hepatoblastoma cell line with HBV DNA (331), can be used for rigorous evaluation of the efficacy of novel HBV therapies *in vitro*.

The aim of the next set of experiments was to assess the delivery efficiency and antiviral efficacy of pLVTH-derived VSV-G-pseudotyped RNAi-based HBV silencing LVs *in vitro*.

Characterisation of the RNAi-based HBV silencing lentiviral vectors *in vitro* entailed thorough assessment of: transduction efficiency, processing of integrated pri-miR mimics, potential saturation of the endogenous miR biogenesis pathway, utility of the integrated polycistronic anti-HBV pri-miR mimics in improving effectiveness against viral escape mutants and finaly evaluating antiviral efficacy in liver-derived Huh7 and HepG2.2.15 cell lines.

4.2 Propagation of HBV silencing LVs and processing of LV-delivered anti-HBV pri-miR mimics in stably transduced cells

VSV-G pseudotyped LVTH and HBV silencing, LVTH miR-31/5 as well as LVTH miR-31/5-8-9 LV were produced by transient transfection in HEK 293T cells. Similar titres were obtained for LVTH LV and the HBV silencing, LVTH-derived LVs, indicating that Drosha-mediated processing of the miR precursors in the HBV silencing LVs did not affect viral titres significantly.

Liver-derived Huh7 cells were transduced with LVTH, LVTH miR-31/5 and LVTH miR-31/5-8-9 lentiviral preparations at a MOI of 40. Genomic quantitative PCR analysis revealed that the LVTH and LVTH miR-31/5 lentiviruses were integrated at a copy number of 1.8 per cell, while LVTH miR-31/5-8-9-transduced cells had 1.2 copies of integrated LV per genome. To determine whether the integrated pri-miR sequences are being processed according to the intended design, low molecular weight Northern blot analysis was carried out.

Effective processing of the integrated pri-miR mimics by the cellular RNAi machinery was revealed by the presence of 20-22 nucleotide putative guide sequences (Figure 4.1). Bands corresponding to pri-miR and pre-miR precursor transcripts were not detectable, indicating that the exogenous pri-miR mimics are efficiently processed by the

cellular RNAi machinery. A single guide sequence, corresponding to the intended miR-31/5 guide, was detected in Huh7 cells which had been stably transduced with the LVTH miR-31/5 lentivirus. Hybridisation with probes complementary to anticipated miR-31/5, miR-31/8 and miR-31/9 guide sequences confirmed the formation of three individual guide sequences in LVTH miR-31/5-8-9 transduced cells.

Quantification of relative band intensities showed variation in the amounts of individual guides, processed from the integrated polycistronic pri-miR-31/5-8-9 scaffold. The miR-31/5 guide sequence was present in high quantity in stably transduced cells, followed by guide 8, while miR-31/9 guide sequence was the least abundant.

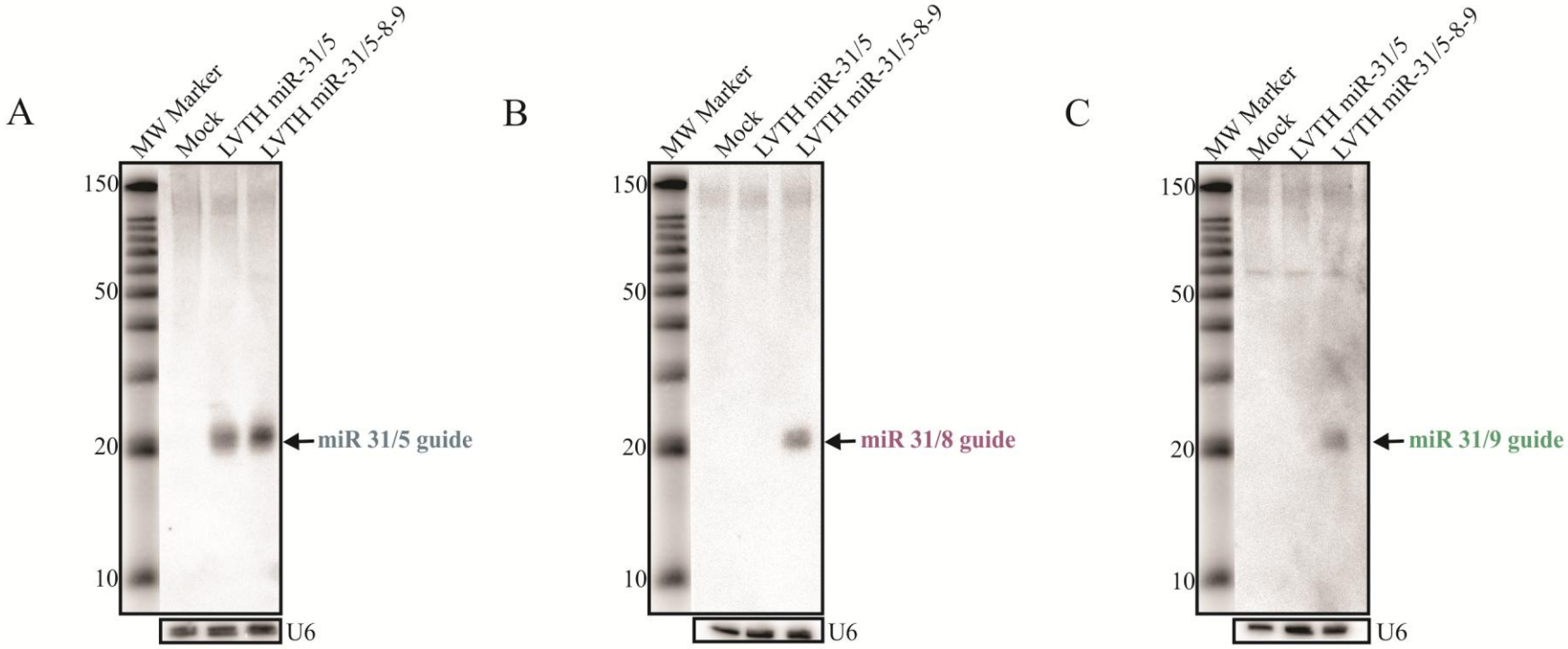


Figure 4.1: <u>Processing of lentiviral vector-delivered liver-specific pri-miR mimics in stably transduced Huh7 cells.</u>

Northern blot analysis was carried out using RNA extracted from Huh7 cells which had been stably transduced with LVTH, LVTH miR-31/5 and LVTH miR-31/5-8-9 LV preparations. Blots were probed using radiolabelled oligonucleotides complementary to the putative mature guide sequences of pri-miR-31/5 (A), pri-miR-31/8 (B) or pri-miR-31/9 (C). Bands equivalent to the guide sequence are indicated by an arrow. Molecular weight (MW) marker indicating fragment sizes of radiolabelled RNA is shown on the left of the autoradiographs. Blots were stripped and re-hybridised to a probe complementary to endogenous small nuclear U6 RNA to confirm equal loading of cellular RNA.

4.3 Recombination between repeated pri-miR scaffold sequences is not detectable in LVTH miR-31/5-8-9-transduced Huh7 cells

Recombination events between repeated sequences in the RNA genome can potentially occur at certain stages in the progression of reverse transcription as the viral reverse transcriptase processes templates with sequence repeats poorly (375, 376). Recombination-mediated deletion of pri-miR sequences in the context of a polycistronic scaffold has been reported previously (183). To investigate whether recombination had occurred between equivalent sequences in the polycistronic pri-miR-31/5-8-9 cassette during lentivirus transduction, genomic DNA extracted from LVTH miR-31/5-8-9 transduced Huh7 cells was analysed by PCR (Figure 4.2). PCR was performed using primers that span the mTTR pri-miR-31/5-8-9 cassette. A single 493 base pair (bp) band, corresponding to the size of the mTTR pri-miR-31/5-8-9 cassette, was amplified from LVTH miR-31/5-8-9 genomic DNA. Additional smaller size bands which could be indicative of sequence rearrangements in the integrated pri-miR 31/5-8-9 scaffold were not observed. However, minor background smearing is noticeable and this could be interpreted as evidence for some recombination occurring during LV transduction. Nevertheless, the intended band is dominant indicating that an intact polycistronic pri-miR-31/5-8-9 cassette is present in the genome of most transduced cells.

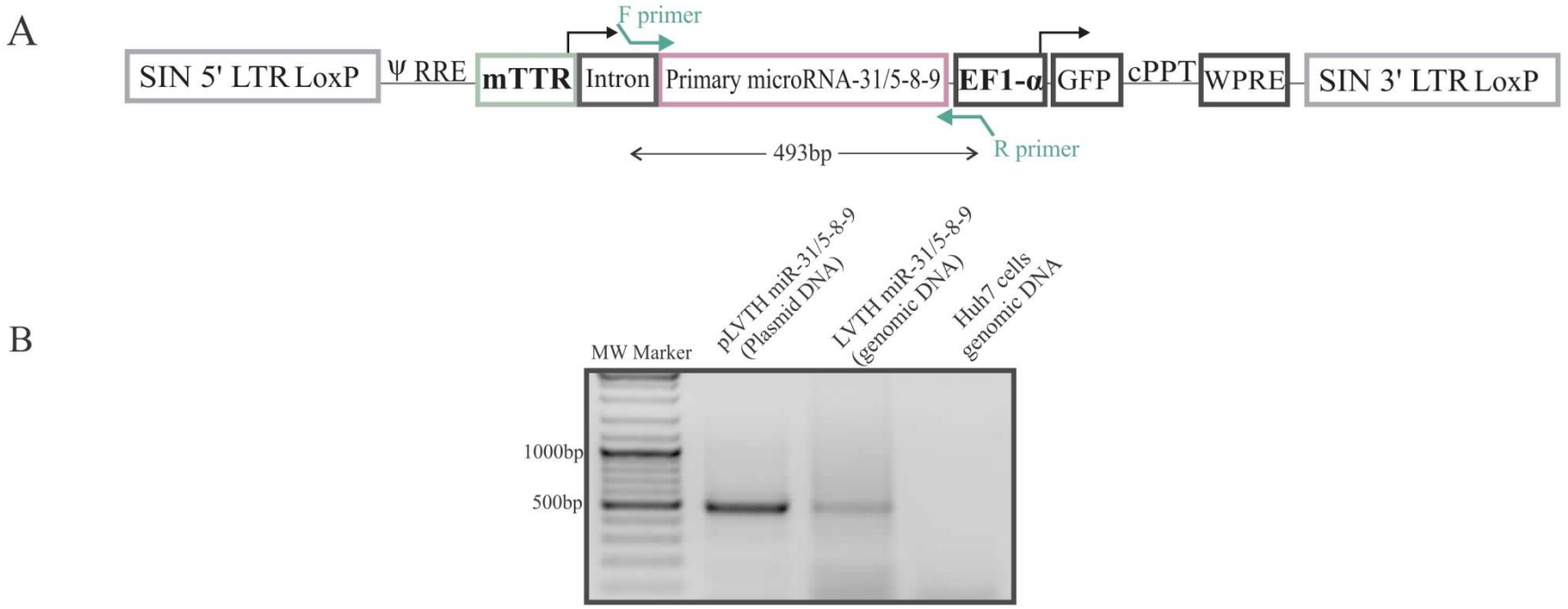


Figure 4.2: <u>PCR analysis of integrated anti-HBV pri-miR expression cassettes.</u>

(A) A schematic of the pLVTH miR-31/5-8-9 lentiviral vector in proviral form. The position of the primers (depicted as green arrows) relative to the pri-miR cassette in proviral DNA integrated in stably transduced cells is indicated. (B) Genomic DNA extracted from LVTH miR-31/5-8-9-transduced Huh7 cells (lane 2) was analysed for pri-miR sequence recombination by PCR, using an intronic forward primer and EF-1 alpha reverse primer. pLVTH miR-31/5-8-9 plasmid DNA (lane 1) and genomic DNA extracted from untransduced Huh7 cells (lane 3) were included as positive and negative controls respectively. MW (molecular weight).

4.4 Expression of lentiviral vector-delivered anti-HBV pri-miR mimics does not disrupt the endogenous miR pathway

In a pivotal study, Grimm and colleagues demonstrated saturation of the natural miR biogenesis pathway following AAV-8-mediated anti-HBV shRNA delivery (165). The high transduction efficiency achieved with the AAV-8 vector coupled with the strong expression of shRNA from the U6 promoter was shown to contribute to severe toxicity *in vivo* as a consequence of disruption of endogenous miR biogenesis. A saturation assay, which involved the assessment of miR-16 translational repression, was performed to verify that the LV-delivered anti-HBV pri-miR mimics do not saturate the endogenous miR pathway and effect perturbations in cellular miR function (159). The wide tissue expression profile of miR-16 allows for convenient assessment of potential disruption of endogenous miR function. A psiCHECK-derived reporter vector that encodes seven copies of an imperfect miR-16 target downstream of the Renilla *luciferase* ORF (psiCHECK-miR-16T×7) was used to measure cellular miR-16-mediated knockdown (159) (Figure 4.3). A sponge plasmid encoding seven partly complementary miR-16 target sites (pTZ-U6-miR-16S×7) was used as a control for loss of miR-16 silencing activity (Figure 4.3). Incorporation of partly complementary miR targets in sponge vectors, instead of perfectly complementary miR target sites, allows for effective competition with natural miR targets and subsequent association with activated RISCs, as most endogenous miRs are partly complementary to their targets and mediate silencing by translational repression and/or deadenylation (377).

As expected, co-transfection of the psi-miR-16T×7 vector and the pTZ-U6-miR-16S×7 sponge plasmid in LVTH-transduced Huh7 cells revealed disruption of miR-16 silencing function (Figure 4.4). In contrast, no disruption of miR-16 function was observed in LVTH, LVTH miR-31/5 and LVTH miR-31/5-8-9-transduced cells transfected with the

psi-miR-16T×7 vector (Figure 4.4). These data indicate that LV- delivered anti HBV pri-miR mimics do not have a repressive effect on endogenous miR-16 silencing activity and are not likely to cause saturation of cellular miR function. Accomplishing controllable and tissue-specific expression of potentially therapeutic pri-miR sequences is of great importance. Selection of a tissue-specific Pol II promoter, such as mTTR, allows for limited expression of the pri-miR mimics in non target tissues and tighter regulation of the dose of expressed pri-miR molecules in hepatocytes. As a result Pol II-mediated expression of pri-miR mimics reduces the potential of exogenously introduced pri-miR mimics to saturate components of the endogenous miR pathway and consequently disrupting natural miR biogenesis.

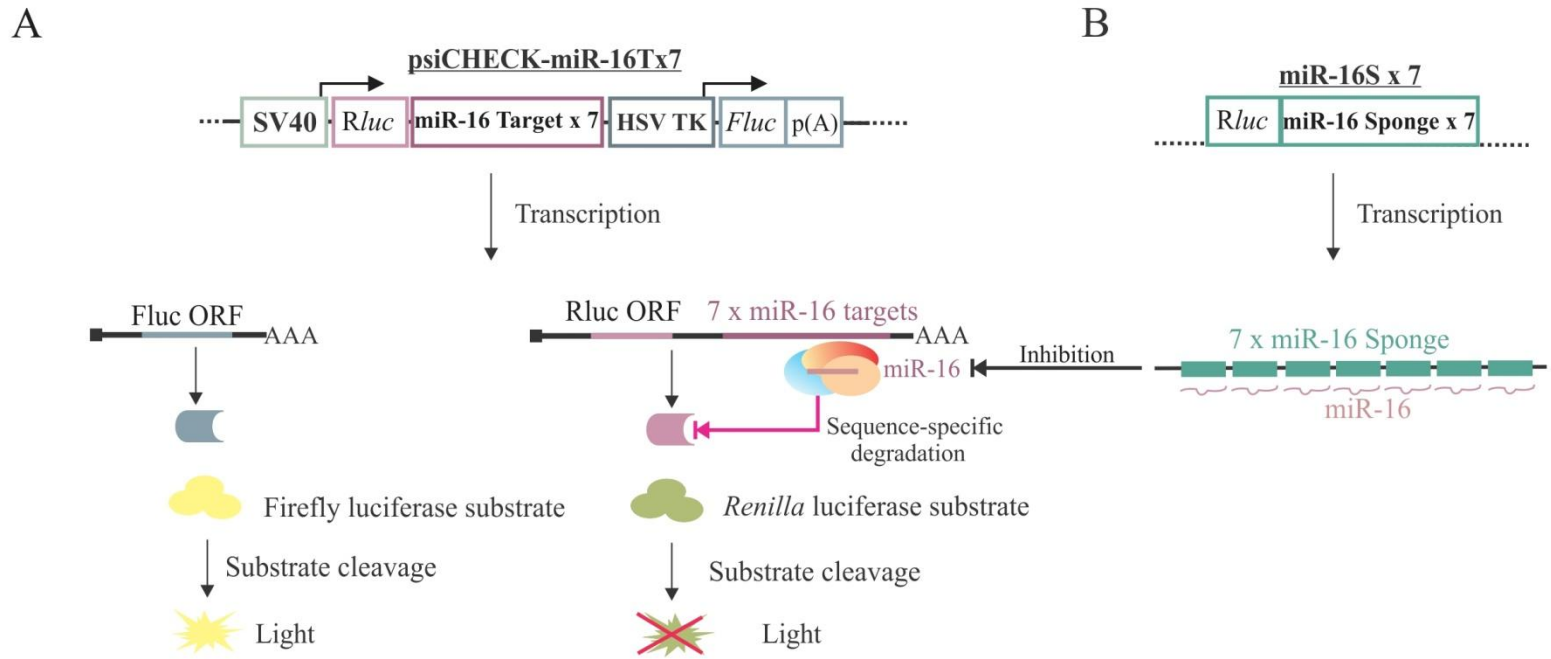


Figure 4.3: Illustration of constructs used to assess saturation of endogenous miR-16 function.

(A) Schematic of psiCHECK-miR-16T×7 dual luciferase reporter vector. The plasmid comprises seven miR-16 targets downstream of the Renilla *luciferase* ORF. (B) Schematic of miR-16 sponge expression cassette encoded within the pTZ-U6-miR-16S×7 plasmid. The cassette is expressed by a U6 Pol III promoter and generates a transcript that contains seven imperfectly matched miR-16 targets.

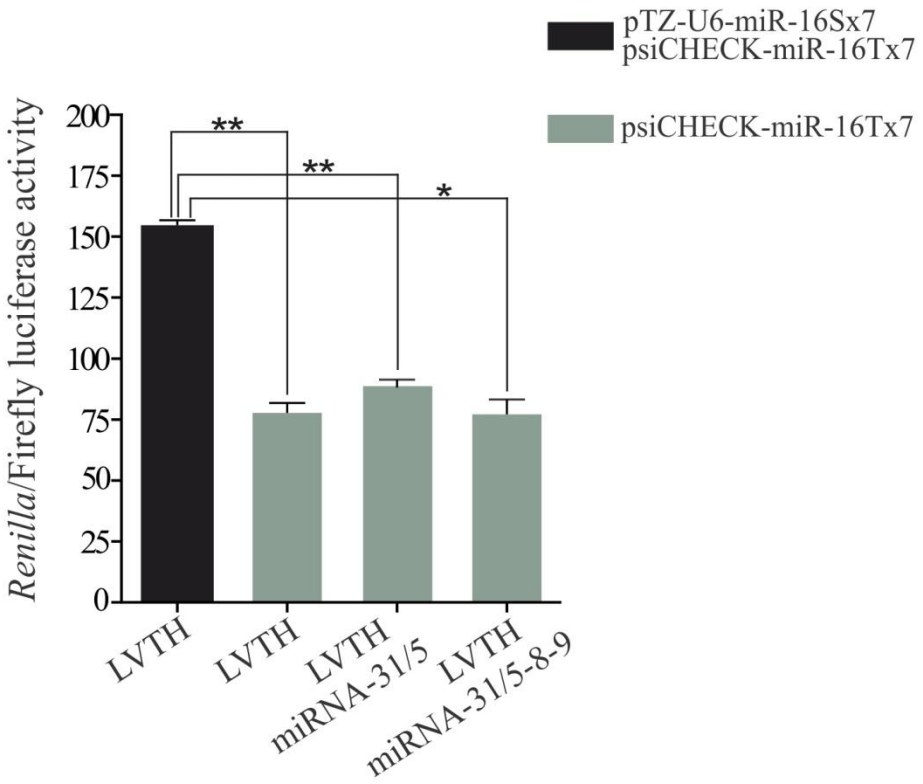


Figure 4.4: <u>Assessment of off target effects of integrated anti-HBV pri-miR mimics on endogenous miR-16 function.</u>

Stably-transduced Huh7 cells were transfected with the psiCHECK-miR-16T×7 reporter vector. As a control, LVTH-transduced Huh7 cells were transfected with both psiCHECK-miR-16T×7 target and pTZ-U6-miR-16S×7 sponge plasmids. *Renilla* luciferase activity was measured in the lysates of transfected cells forty-eight hours following transfection. Firefly luciferase constitutively expressed from psiCHECK-miR-16T×7 was used to normalise data. Data are presented as mean ratios of Renilla to Firefly luciferase activity (±SEM., n=3, ∗p < 0.05, ∗∗p < 0.01).

4.5 Silencing efficacy of the lentiviral vector-based anti-HBV miR system in stably transduced Huh7 cells

4.5.1 LV-delivered anti-HBV pri-miR mimics silence HBV gene expression and viral replication

The preceding data demonstrate that the LVs encoding liver-specific anti-HBV pri-miR mimics are capable of effectively transducing liver-derived cells and mediating stable integration of the therapeutic pri-miR sequences into the genome of transduced hepatocytes. Furthermore, Northern blot and saturation analysis showed that the pri-miR mimics are effectively processed to intended mature miR guide sequences without saturating the endogenous miR pathway. Next, a dual luciferase HBV reporter gene assay and a HBsAg ELISA was performed to assess the antiviral efficacy of LV-delivered monocistronic and polycistronic pri-miR mimics. HBV reporter gene expression was measured in Huh7 cells which had been stably transduced with LVTH miR-31/5 and LVTH miR-31/-5-8-9 lentiviral preparations at a MOI of 40. Significant inhibition of *Renilla* luciferase activity (Figure 4.5 A, B) was observed in transduced cells which had been transfected with the psiCHECK-HBx reporter vector (Section 3.2.2). Furthermore, potent inhibition of HBsAg secretion was measured in LVTH miR-31/5 and LVTH miR-31/5-8-9-transduced Huh7 cells (Figure 4.5 C, D) following a transfection of the pCH-9/3091 HBV replication competent plasmid. These findings confirm that the LV-delivered anti-HBV pri-miR mimics are capable of mediating powerful suppression of viral replication.

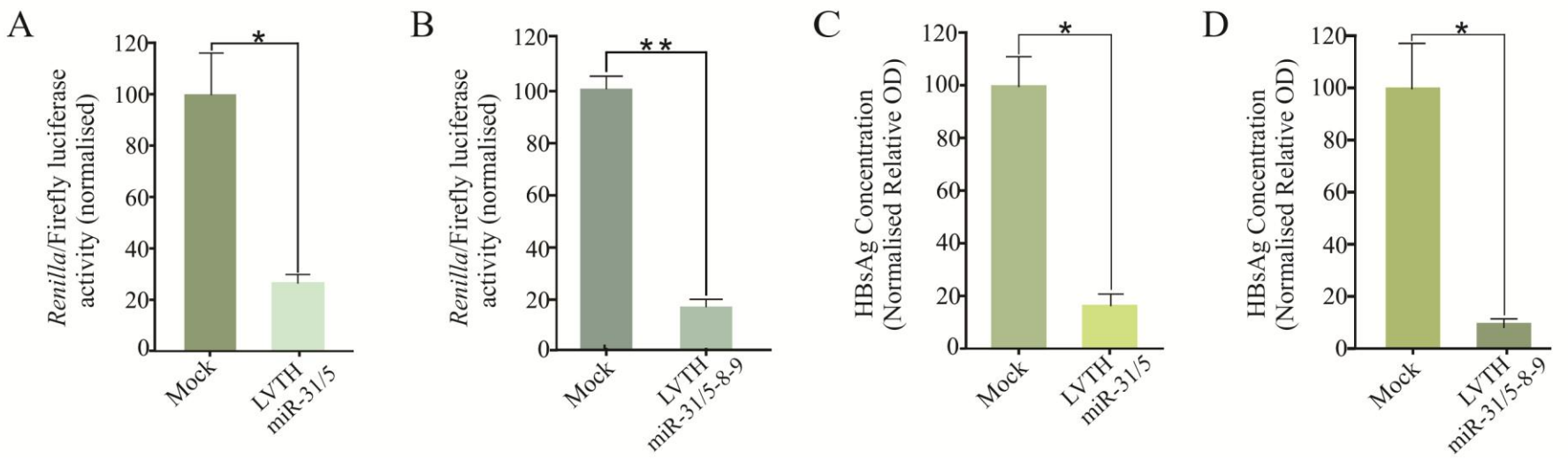


Figure 4.5: <u>HBV knockdown in stably transduced Huh7 cells.</u>

HBV knockdown was measured in Huh7 cells that had been stably transduced with LVs encoding monocistronic pri-miR-31/5 (A, C) and polycistronic pri-miR-31/5-8-9 (B, D) pri-miR mimics using a dual luciferase reporter gene assay (A, B) and a HBsAg ELISA (C, D).

(A, B) Huh7 cells were transfected with the dual luciferase HBV reporter vector psiCHECK-*HBx*. Forty-eight hours after transfection, *Renilla* luciferase and Firefly luciferase activities were measured in lysates of transfected cells. Data are presented as mean ratios of *Renilla* to Firefly luciferase activity (±SEM., n=3, $*p < 0.05$, $**p < 0.01$) and are normalised relative to LVTH-transduced cells. (C, D) HBsAg secretion was measured in stably transduced Huh7 cells, forty-eight hours after transfection with the pCH-9/3091 HBV target vector. Quantitative ELISA HBsAg measurements are given as a normalised mean (±SEM., n=3, $*p < 0.05$, $**p < 0.01$) relative to LVTH-transduced cells.

4.5.2 Integrated polycistronic anti-HBV pri-miR mimics mediate effective knockdown of individual viral target sequences

Northern blot analysis carried out on genomic DNA extracted from LVTH miR-31/5-8-9-transduced Huh7 cells revealed the presence of three putative mature miR guide sequences (Section 4.2). Nevertheless, variation was observed in the quantities of the individual mature miR guide sequences detected.

To assess the silencing efficacy of the individual miR effecters generated from a polycistronic miR scaffold, a dual luciferase reporter assay was performed. Vectors comprising individual sites targeted by putative miR-31/5 (psiCHECK-5T), miR-31/8 (psiCHECK-8T) and miR-31/9 (psiCHECK-9T) guide sequences downstream of the Renilla *luciferase* ORF (Figure 4.6 A) were transfected in LVTH miR-31/5-8-9 transduced Huh7 cells.

Renilla luciferase activity was measured in transfected cells (Figure 4.6 B) to determine the knockdown efficacy of each miR effecter. Although *Renilla* luciferase expression from each reporter vector was significantly inhibited, variable knockdown efficacy was observed against the different HBV targets. Knockdown of the psiCHECK-5T mediated by the pri-miR-31/5 sequence was the most potent, while moderate silencing activity was observed by the miR-31/9 sequence. The silencing activity of the individual guide sequences corresponded to the quantity of guide sequence detected by Northern blot analysis.

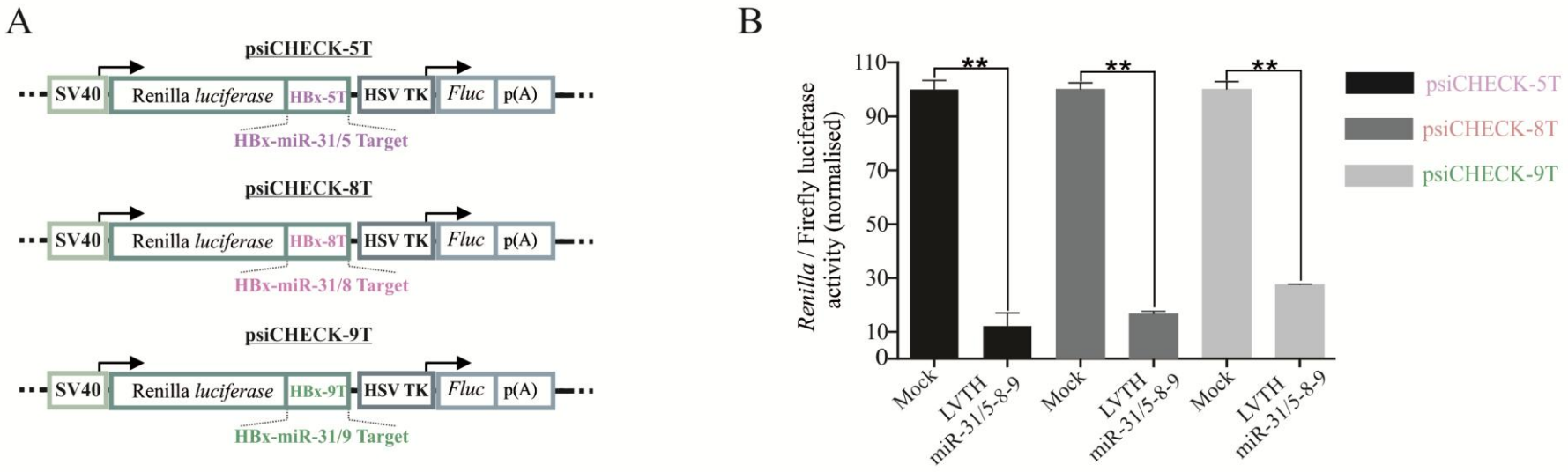


Figure 4.6: <u>Silencing of individual HBV targets by polycistronic LV-delivered anti-HBV pri-miR mimics.</u>

(A) Schematic illustration of dual luciferase reporter vectors comprising target sequences complementary to putative miR guides 5 (psiCHECK-5T), 8 (psiCHECK-8T) and 9 (psiCHECK-9T) downstream of the Renilla *lucuferase* ORF in psiCHECK^{TM}-2. (B) Huh7 cells that had been stably transduced with LVTH miR-31/5-8-9 recombinant lentivirus were transfected with individual dual luciferase reporter vectors (psiCHECK-5T, psiCHECK-8T and psiCHECK-9T). Forty eight hours after transfection *Renilla* and Firefly luciferase activities were measured and the efficacy of miR-mediated knockdown of the individual target sequences was determined. Data are presented as mean ratios of *Renilla* to Firefly luciferase activity (± SEM., n=3, $**p < 0.01$) and are normalised relative to LVTH-transduced cells.

4.5.3 Integrated polycistronic anti-HBV pri-miR mimics mediate knockdown of a mutant *HBx* target sequence

Simultaneous expression of several anti-HBV miR sequences from polycistronic cassettes could be potentially valuable in limiting the emergence of viral escape mutants. A dual-luciferase reporter assay using a psiCHECK-derived reporter vector encoding an *HBx* sequence with mutations in the site targeted by miR-31/5, was performed to assess the ability of the LV-delivered monocistronic and polycistronic pri-miR mimics to inhibit viral replication of a mutated HBV sequence (Figure 4.7 A & B). While the monocistronic anti-HBV pri-miR-31/5 sequence was capable of mediating potent inhibition of wild-type *HBx* gene expression, only modest suppression of mutant *HBx* gene expression was observed in cells transfected with the psiCHECK-m*HBx* vector (Figure 4.7 C). In contrast, LV-delivered polycistronic pri-miR-31/5-8-9 sequences effectively silenced the expression of both wild-type and mutated *HBx*, indicating that the polycistronic anti-HBV pri-miR configuration could be potentially useful in improving effectiveness against viral escape mutants.

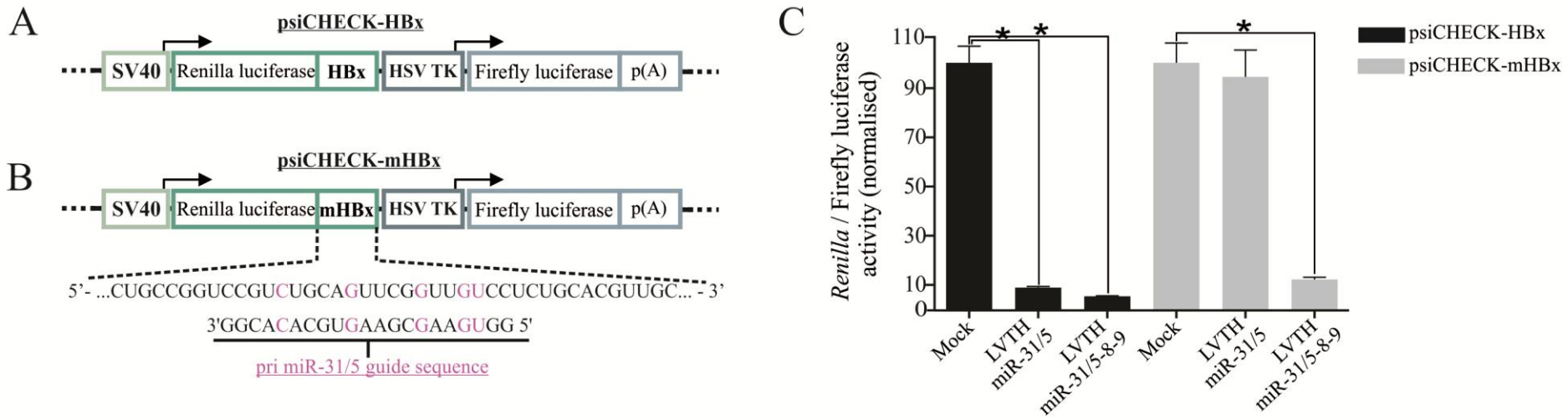


Figure 4.7: <u>Pri-miR-mediated silencing of wild-type *HBx* and mutant *HBx* target gene expression.</u>

Schematic illustration of the psiCHECK-*HBx* (A) and psiCHECK-m*HBx* (B) dual luciferase reporter vectors. psiCHECK-m*HBx* comprises an *HBx* target sequence with point mutations in the pri-miR-31/5 target site (highlighted in pink), downstream of the Renilla *luciferase* ORF in psiCHECK^{TM}-2. (C) Huh7 cells which had been stably transduced with LVTH miR-31/5 or LVTH miR-31/5-8-9 recombinant lentivirus were transfected with the psiCHECK-*HBx* or psiCHECK-m*HBx* reporter vectors. A dual luciferase reporter gene assay was performed to assess knockdown efficiency against the mutated *HBx* sequence. Data are presented as mean ratios of *Renilla* to Firefly luciferase activity (±SEM., n=3, $*p < 0.05$) and are normalised relative to LVTH-transduced cells.

4.6 Evaluation of the knockdown efficacy of the lentiviral vector-based anti-HBV miR system in stably transduced HepG2.2.15 cells

To evaluate the knockdown efficacy of LV-delivered polycistronic pri-miR sequence in the liver-derived HepG2.2.15 HBV replication cell line, HepG2.2.15 cells were stably transduced with LVTH or LVTH miR-31/5-8-9 virus at a MOI of 40. Stable integration was confirmed by genomic quantitative PCR analysis and monitoring GFP reporter gene expression.

4.6.1 Integrated anti-HBV pri-miR mimics mediate powerful inhibition of HBsAg and HBeAg secretion in HepG2.2.15 cells

To assess the antiviral effects of the polycistronic anti-HBV pri-miR mimic in HepG2.2.15 cells, HBsAg and HBeAg secretion in the supernatant of LVTH- and LVTH miR-31/5-8-9-transduced cells was measured by a quantitative ELISA at 48, 72 and 96 hours after an equal number of cells was seeded for each cell population (Figure 4.8). Significant inhibition of both HBsAg and HBeAg secretion was observed in LVTH miR-31/5-8-9-transduced HepG2.2.15 cells. While a similar trend in HBsAg and HBeAg HBV knockdown was observed, there was a more marked effect on HBsAg knockdown.

The LV-delivered pri-miR-31/5-8-9 therapeutic sequence mediated stable inhibition of HBsAg and HBeAg secretion in spite of the fast rate of HepG2.2.15 virion secretion. It is possible that not all individual cells in the LVTH miR-31/5-8-9-transduced population had been stably transduced with the pri-miR-31/5-8-9 encoding LV, which could result in an imbalanced virion secretion-miR mediated knockdown rate leading to an observation of an underestimated antigen knockdown.

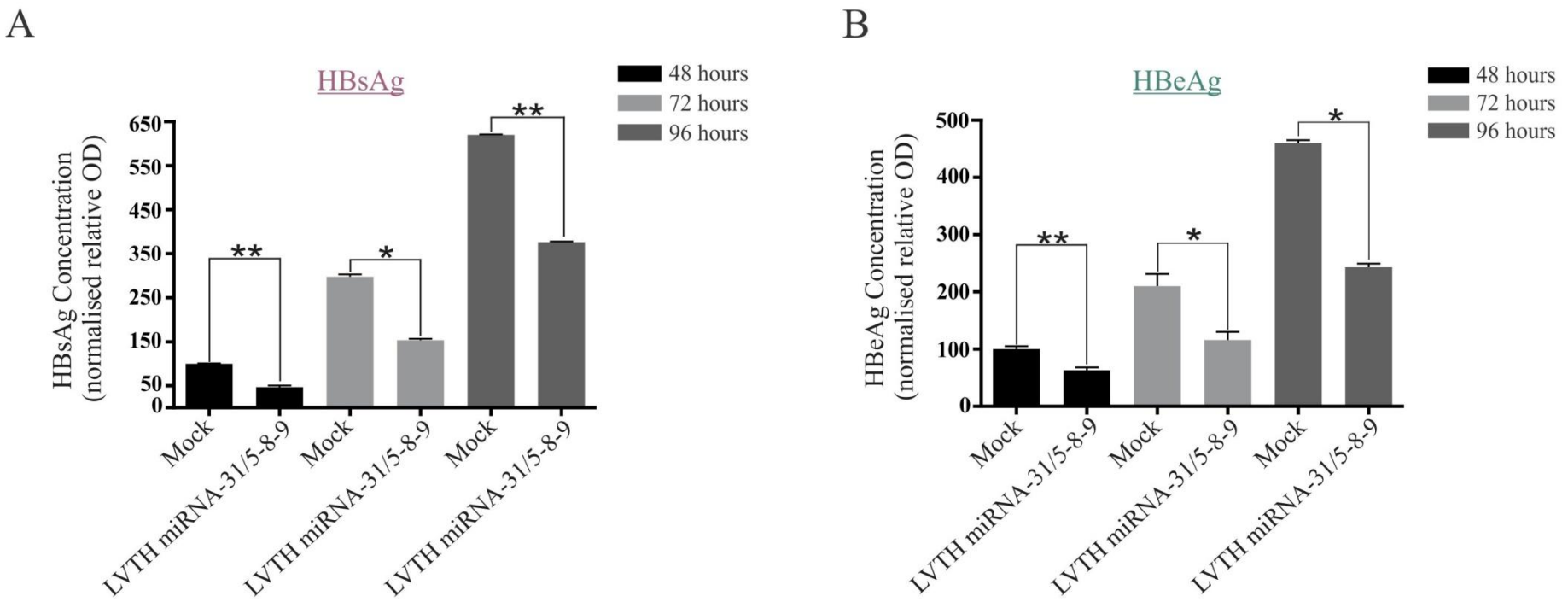


Figure 4.8: HBV antigen quantification in HepG2.2.15 culture supernatants.

Inhibition of HBsAg (A) and HBeAg (B) secretion over a time period of 96 hours was assessed in HepG2.2.15 cells which had been stably transduced with LVTH miR-31/5-8-9 LV. HBsAg and HBeAg measurements from quantitative ELISA are given as a normalised mean relative to LVTH-transduced cells. Graphs indicate mean ± SEM from three independent experiments, $*p < 0.05$, $**p < 0.01$.

4.6.2 Integrated anti-HBV pri-miR mimics mediate effective reduction of viral particle equivalents in HepG2.2.15 culture supernatants

Real-time quantitative PCR was performed to quantify HBV DNA in the supernatant of LVTH- and LVTH miR-31/5-8-9-transduced cells (Figure 4.9). HBV DNA was measured at three different time points (48 hours, 72 hours and 96 hours) to assess the antiviral effect of pri-miR-31/5-8-9 over an extended time period.

A significant reduction in the number of HBV VPEs was observed in HepG2.2.15 cells which had been transduced with the LVTH pri-miR-31/5-8-9 encoding lentivirus. The reduction in VPEs was maintained over the 96 hour time period, indicating that the pri-miR-31/5-8-9 sequence is capable of mediating stable suppression of HBV replication in a cell culture model characterised by particularly high viral replication. These data corroborate previously obtained results in HepG2.2.15 and Huh7 cells and highlight the antiviral efficacy of the lentiviral vector-based anti-HBV miR system.

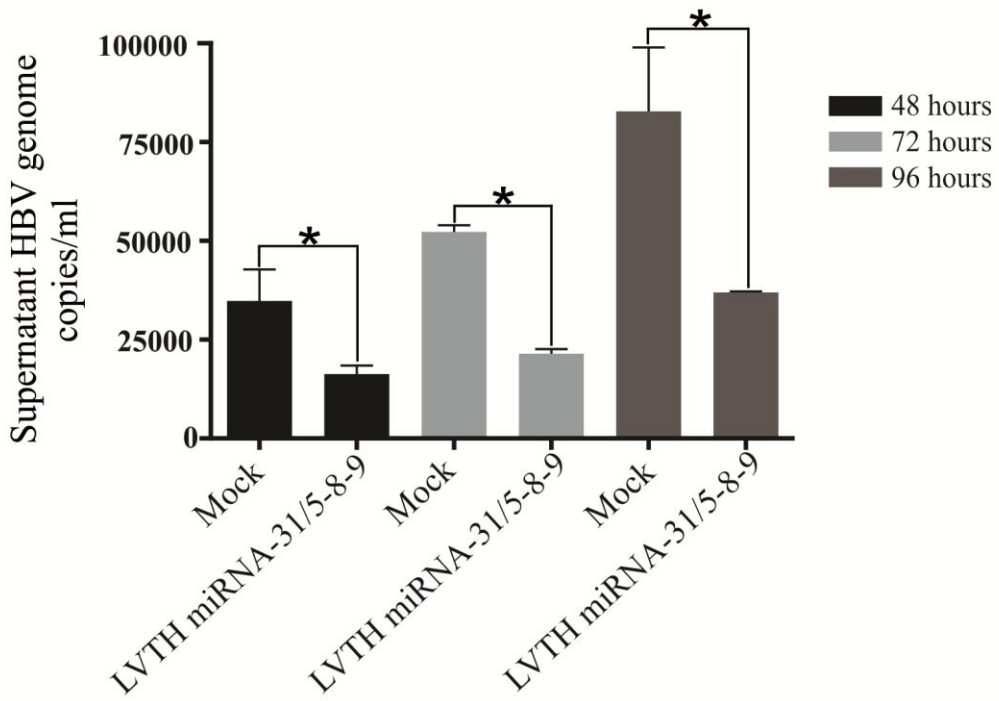


Figure 4.9: <u>HBV DNA quantification in stably transduced HepG2.2.15 cells.</u>

HBV viral load was measured in LVTH and LVTH miR-31/5-8-9-transduced HepG2.2.15 cells at 48 hours, 72 hours and 96 hours. HBV DNA secreted from stably transduced cells was determined by real-time quantitative PCR using HBV-specific primers (mean ± SEM., n=3, $*p < 0.05$). Virion copy numbers were determined using EuroHep HBV standards.

4.7 Conclusion

The design of transgene expression cassettes intended for incorporation into RNAi-activating lentiviral vector constructs may influence LV titre as well as expression of the therapeutic RNAi molecule. HBV silencing expression cassettes encoding mono- and poly-cistronic pri-miR mimics were inserted in the second-generation SIN pLVTH lentiviral vector in the forward (sense) orientation without a polyadenylation signal. The pLVTH and pLVTH-derived HBV silencing lentiviral vectors were used to propagate VSV-G pseudotyped recombinant LV. Significant differences in the titre obtained for pLVTH and pLVTH-derived LVs encoding pri-miR mimics were not observed. This could be attributed to Drosha saturation in the producer cells and/or RRE-Rev mediated nuclear export of unspliced and singly-spliced genomic lentiviral RNA (183, 274)**.**

Stable transduction of liver-derived Huh7 and HepG2.2.15 cells was achieved using the therapeutic LVs and this was evident from the long-term sustained expression of the *GFP* marker transgene in transduced cells. In addition, genomic quantitative PCR analysis revealed that the LVTH and LVTH miR 31/5 lentiviruses were integrated at a copy number of 1.8 per cell, while LVTH miR-31/5-8-9-transduced cells had 1.2 copies of integrated LV per genome. Northern blot analysis of RNA from stably transduced Huh7 cells verified that the integrated anti-HBV pri miR sequences were efficiently processed to form RNAi-activating guide strands according to the intended design. Consistent with previous work (159), variation in the amounts of individual guides processed from the integrated polycistronic pri-miR-31/5-8-9 scaffold was notable, with the miR-31/5 guide sequence being the most abundant sequence present in stably transduced cells. Saturation assays revealed that there was limited disruption of endogenous cellular miR function in cells which had been stably transduced with HBV silencing LVs, indicating that the therapeutic LVs are safe and not likely to induce toxic effects resulting from saturation of the

endogenous miR biogenesis pathway. PCR analysis of genomic DNA extracted from Huh7 cells which had been stably transduced with the polycistronic HBV silencing LV, revealed that recombination had not occurred between equivalent sequences in the polycistronic pri-miR-31/5-8-9 cassette during lentivirus transduction, in contrast to previous reports (183, 186). The extent of recombination occurring may be influenced by the particular pri-miR scaffolds as well as the configuration of polycistron used (185).

Following demonstration of stable integration and expression of the therapeutic pri-miR mimics with limited disruption of endogenous miR function, the antiviral efficacy of the RNAi-based HBV silencing LVs was evaluated. When stably transduced Huh7 cells were transfected with a HBV replication-competent plasmid, potent inhibition of markers of viral replication was achieved. In addition, the silencing activity of the individual guide sequences corresponded to the quantity of guide sequence detected by Northern blot analysis. Assessing silencing of a mutated HBV sequence revealed that the polycistronic mTTR-expressed pri-miR-31/5-8-9 sequence has superior silencing activity and could be potentially useful in limiting the emergence of viral escape mutants. Finally, powerful knockdown of markers of viral replication and circulating VPEs was demonstrated in HepG2.2.15 cells which had been stably transduced with a HBV silencing LV encoding the polycistronic pri-miR-31/5-8-9 sequence. The profile of HBV replicative intermediates in this cell line was found to closely mimic the profile observed in individuals chronically infected with the HBV virus (378), making the HepG2.2.15 cell line a stringent system for *in vitro* evaluation of the efficacy of novel antiviral therapies.

Thorough characterisation of the HBV silencing LVs in liver-derived Huh7 cells and demonstration of potent antiviral efficacy in the HepG2.2.15 HBV replication cell line lay the foundation for further *in vivo* evaluation of our strategy. In the next chapter we set

to explore whether the *in vitro* efficacy of the RNAi-based HBV-silencing LVs can be translated to efficient HBV knockdown in an animal model of viral replication.

5 COUNTERING VIRAL REPLICATION *IN VIVO* WITH HBV-SILENCING LENTIVIRUSES

5.1 Introduction

Comprehensive data from *in vitro* characterisation of the HBV-silencing LVs presented in the preceding chapters, has demonstrated potent inhibition of markers of viral replication as well as sustained, hepatospecific HBV knockdown with limited off-target effects. The above findings prompted further evaluation of the antiviral efficacy of the HBV-silencing LVs *in vivo*.

Preclinical evaluation of safety and efficacy of the novel antiviral therapeutic strategies in appropriate *in vivo* experimental models, is a fundamental step in the development and clinical translation of potential HBV therapeutics. HBV transgenic mice contain an integrated replication-competent HBV sequence, consequently viral replication in this murine model is constitutive and continues for the duration of the animal's life thereby mimicking chronic HBV infection of humans (345, 379). Accordingly, the HBV transgenic mouse model is suitable for rigorous evaluation of the antiviral efficacy of novel therapies.

Inclusion of the *Firefly luciferase* transgene in recombinant lentiviral vectors allows for non-invasive, long-term follow up of transgene expression as well as vector persistence in individual animals following LV administration. Although the use of reporter transgenes is convenient and useful for the assessment of transduction efficiency as well as biodistribution of therapeutic viruses, both *Firefly luciferase* and e*GFP* reporter genes encoded in the HBV silencing recombinant LVs can induce transgene-specific immune responses following systemic administration (380-382). Humoral and cellular

antigen-specific immune responses to LV-encoded GFP have been reported to induce clearance of transduced cells thus curtailing long-term expression of therapeutic transgenes (325).

Several groups have demonstrated stable transfer of therapeutic sequences to the liver following systemic delivery of recombinant LVs (180, 281, 318, 321, 382, 383). However, the transduction efficiency of recombinant LVs is influenced by the proliferation and cell cycle activation of targeted hepatocytes (320). Transduction following systemic delivery of recombinant lentiviruses has been reported to be significantly higher in young and newborn rodents as a result of high hepatocyte proliferation (180, 322-324). Highly efficient liver transduction and long-term transgene expression has been reported following LV delivery via an intravenous injection into the superficial temporal vein in neonatal mice (180, 322, 324). Successful transduction when using this approach is not surprising as vector genomes increase in number with the proliferating hepatocytes following LV gene transfer to neonatal mice. In addition, the immune-system of the newborn animal is not fully functional circumventing the induction of an immune response to reporter gene products and allowing for sustained expression of the anti-HBV pri-miR molecules. The distinct benefits of the above approach deemed it suitable for the delivery of HBV-silencing LVs and long-term evaluation of antiviral efficacy in the HBV transgenic mouse model.

The aim of the next set of experiments was to evaluate preclinical proof of principle of the efficacy of the novel anti-HBV lentiviral vector RNAi-hybrid technology in the HBV transgenic mouse model.

5.2 The mTTR promoter is capable of driving long-term stable transgene expression in vivo

To evaluate the efficiency and tissue-specificity of the mTTR promoter *in vivo*, a lentiviral vector encoding a mTTR-driven *Firefly luciferase* transgene (pLNT-mTTR-JDG) was generated (Figure 5.1 A). LVs expressing Firefly luciferase under the control of the mTTR promoter were administered to neonatal mice via an intravenous injection in the superficial temporal vein (Figure 5.1 B). Firefly luciferase expression can be sensitively detected in live animals using a bioluminescence imaging system following the administration of a substrate for the reporter enzyme. The bioluminescence imaging system typically comprises a charge-coupled device camera which captures light photons generated upon substrate oxidation (384). Bioluminescence imaging was used to analyse Firefly luciferase expression in mice that had received LVs encoding the *Firefly luciferase* transgene. Stable expression was recorded in the livers of imaged animals over a period of 12 months (Figure 5.2). These data corroborate previous reports demonstrating efficiency and functionality of the mTTR promoter *in vivo* (180, 385, 386) and indicate that the mTTR promoter is capable of driving sustained and hepatospecific gene expression, making it a valuable accessory for the development of liver-directed gene therapy.

A



B

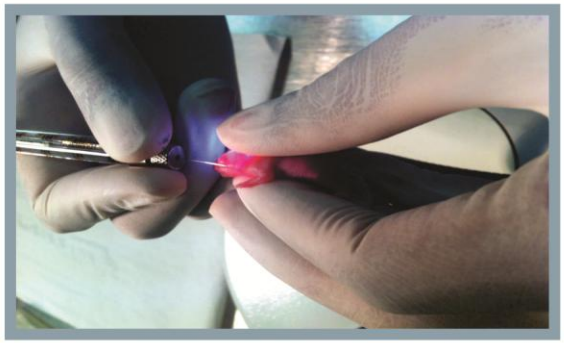


Figure 5.1: LNT-mTTR-JDG LV transfer to neonatal HBV transgenic mice.

(A) Schematic of the pLNT-mTTR-JDG lentiviral vector. The vector comprises a 2A-linked *Firefly luciferase*-e*GFP* bicistronic expression cassette (*FLuc*-2A-e*GFP*) downstream of an internal mTTR promoter. LTR (long terminal repeat), ψ (HIV-1 packaging signal), RRE (rev-responsive element), attB (synthetic attachment site), cPPT (central polypurine tract), WPRE (woodchuck hepatitis virus posttranscriptional regulatory element), SIN 3' LTR (long terminal repeat with a deletion in the U3 region). (B) LV transfer to neonatal HBV transgenic mice via intravenous injection into the superficial temporal vein.

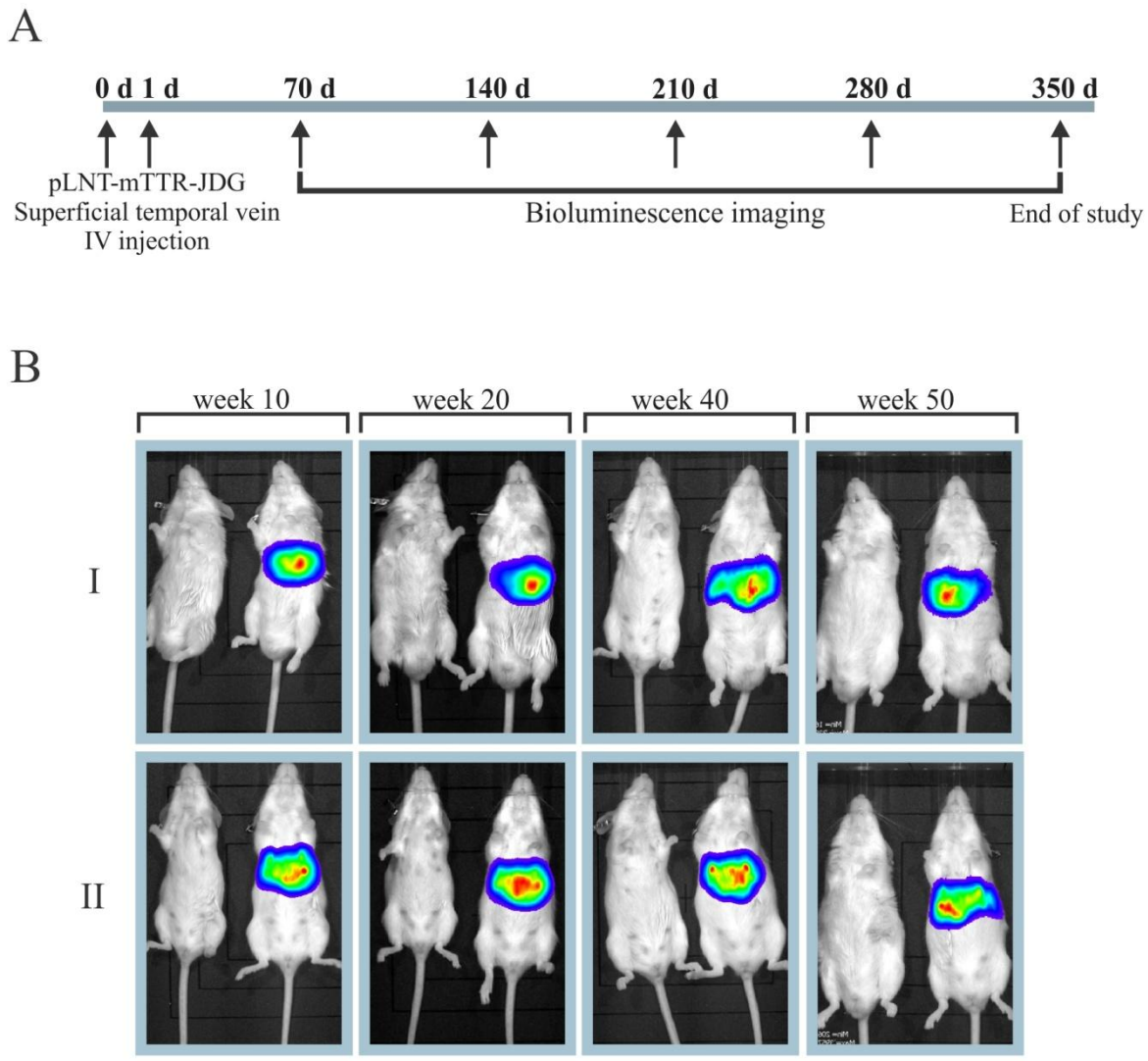


Figure 5.2: <u>Long-term follow-up of Firefly luciferase expression in LNT-mTTR-JDG LV-injected mice.</u>

(A) Experimental outline. Neonatal HBV transgenic mice received two bilateral superficial temporal vein intravenous injections of LNT-mTTR-JDG LV (6×10^7 TU per mouse) expressing Firefly luciferase or PBS at day 0 and 1 after birth. Firefly luciferase expression was analysed by bioluminescence imaging using the IVIS® Kinetic imaging system. (B) Images of two LNT-mTTR-JDG LV-injected mice (right) and PBS-injected control animals (left) are shown in the top (I) and bottom (II) panels. Mice were imaged at 10 week intervals for the duration of the study.

5.3 pLVTH- and pLVTH miR-31/5-8-9- derived LVs encoding the Firefly luciferase *transgene mediate stable transgene delivery* in vivo

The HBV-silencing lentiviral vector encoding the polycistronic pri-miR molecule, selected for *in vivo* evaluation of viral efficacy, was further modified to include a 2A-linked Firefly luciferase-eGFP bicistronic expression cassette generating the LVTH-LUC miR-31/5-8-9 lentiviral vector (Figure 5.3). The above modification allows for simultaneous assessment of transfection efficiency by fluorescence imaging as well as delivery efficiency by bioluminescence imaging. Control LVTH-LUC or HBV-silencing LVTH-LUC miR-31/5-8-9 LVs were administered to neonatal and adult HBV transgenic mice via a superficial temporal vein or tail vein injection respectively. LV administration via the tail vein was performed in groups of young adult mice (~ 3 weeks old) to improve transduction efficiency. In contrast to neonatal animals, sera of adult animals can be collected and analysed before LV administration allowing for convenient monitoring of markers of HBV replication in individual mice prior to and after administration of therapeutic virus. Bioluminescence imaging was performed to analyse Firefly luciferase expression in neonatal and adult mice that had received recombinant LV.

Stable LV-mediated liver transduction was achieved as evidenced by the variable but sustained *Firefly luciferase* expression observed in the livers of LV-injected animals (Figure 5.4 and 5.5). It is likely that the variability in expression between individual mice is as a result of the varying efficiency of the superficial temporal vein injection procedure. Nevertheless, the level of *Firefly luciferase* expression was fairly consistent in the livers of individual mice for the duration of the study. In several animals transgene expression was also evident at the site of LV injection, specifically in the cheek region of animals that

received temporal-vein LV injection as well as the tail and hind limbs of mice that received LV via their tail-veins (Figure 5.4 and 5.5). LV leakage and subsequent transduction of the surrounding tissue is likely to account for the above. Furthermore, the content of vector DNA in the livers of LV-injected mice that received recombinant LV was quantified by real-time PCR. Average vector DNA levels in the livers of mice that received tail vein injection or neonatal superficial temporal vein injection were 0.25 copies per genome and 0.45 copies per genome respectively, indicating that the LVs are capable of mediating efficient transduction of the liver following *in vivo* administration.

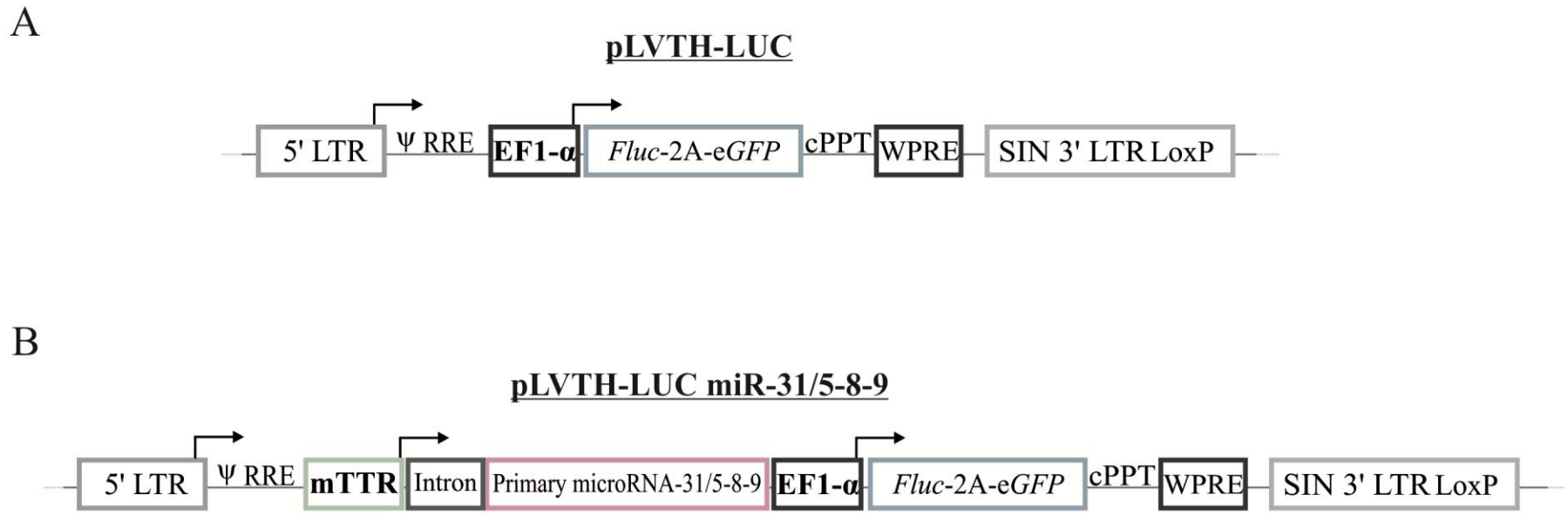


Figure 5.3: Schematic of pLVTH-LUC and pLVTH-LUC miR-31/5-8-9 lentiviral vectors.

pLVTH-LUC (A) and pLVTH-LUC miR-31/5-8-9 (B) lentiviral vectors were derived from the parental pLVTH and pLVTH miR-31/5-8-9 plasmids respectively. The vectors encode a 2A-linked *Firefly luciferase*-e*GFP* bicistronic expression cassette (*FLuc*-2A-e*GFP*) downstream of an internal EF1-α promoter. LTR (long terminal repeat), ψ (HIV-1 packaging signal), RRE (rev-responsive element), cPPT (central polypurine tract), WPRE (woodchuck hepatitis virus posttranscriptional regulatory element), SIN 3' LTR (long terminal repeat with a deletion in the U3 region).

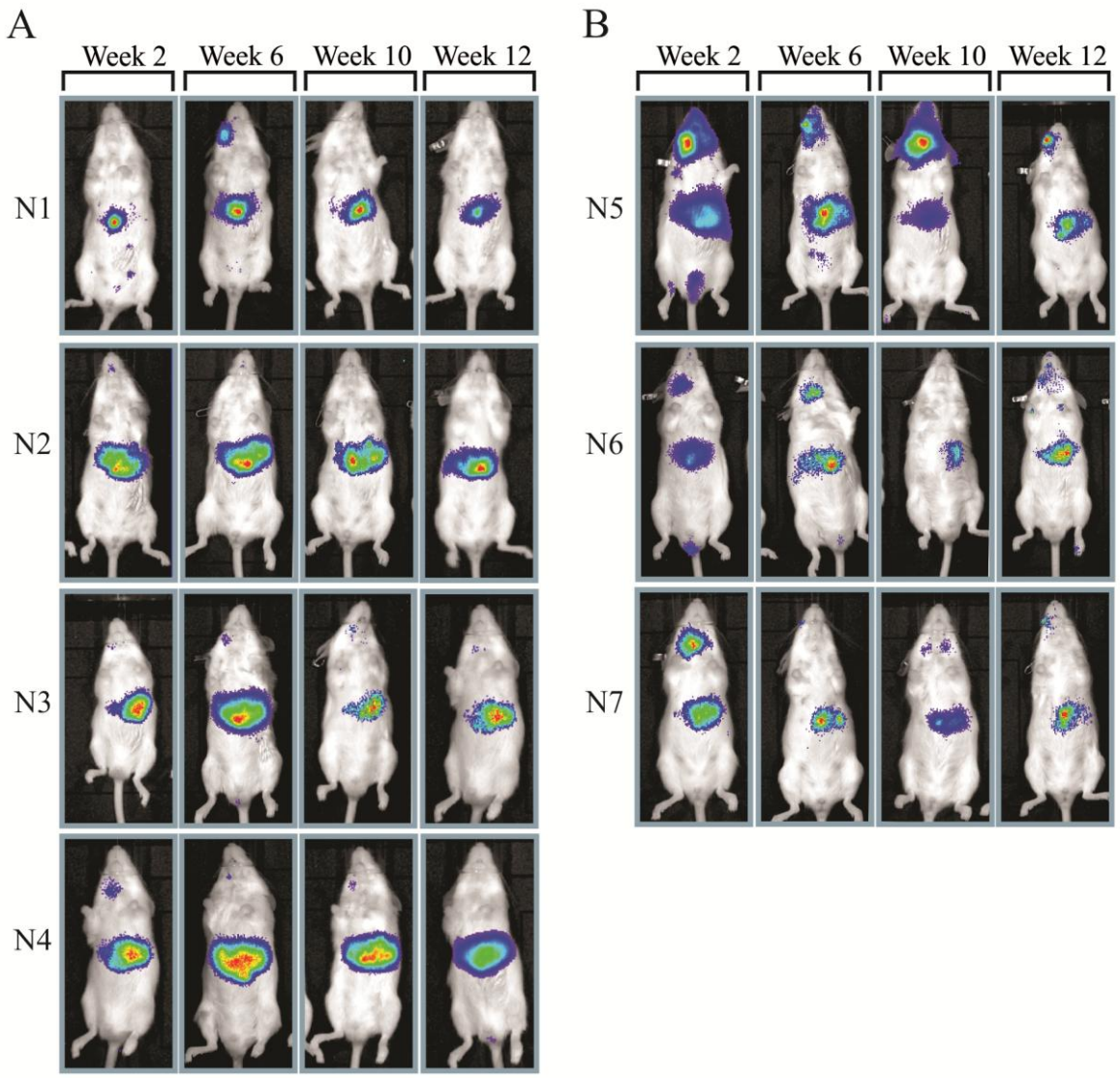


Figure 5.4: <u>Long-term follow-up of Firefly luciferase expression following LVTH-LUC and LVTH-LUC miR-31/5-8-9 LV delivery to neonatal HBV transgenic mice.</u>

Neonatal HBV transgenic mice received a single unilateral injection of LVTH-LUC miR-31/5-8-9 (N1-N4) or LVTH-LUC (N5-N7) LV (2.7×10^8 TU per mouse) into the superficial temporal vein. *Firefly luciferase* expression was analysed by bioluminescence imaging using the IVIS® Kinetic imaging system. Bioluminescence images from LVTH miR-31/5-8-9- (A) and LVTH-injected (B) mice imaged at 2 week intervals for a period of 12 weeks are shown.

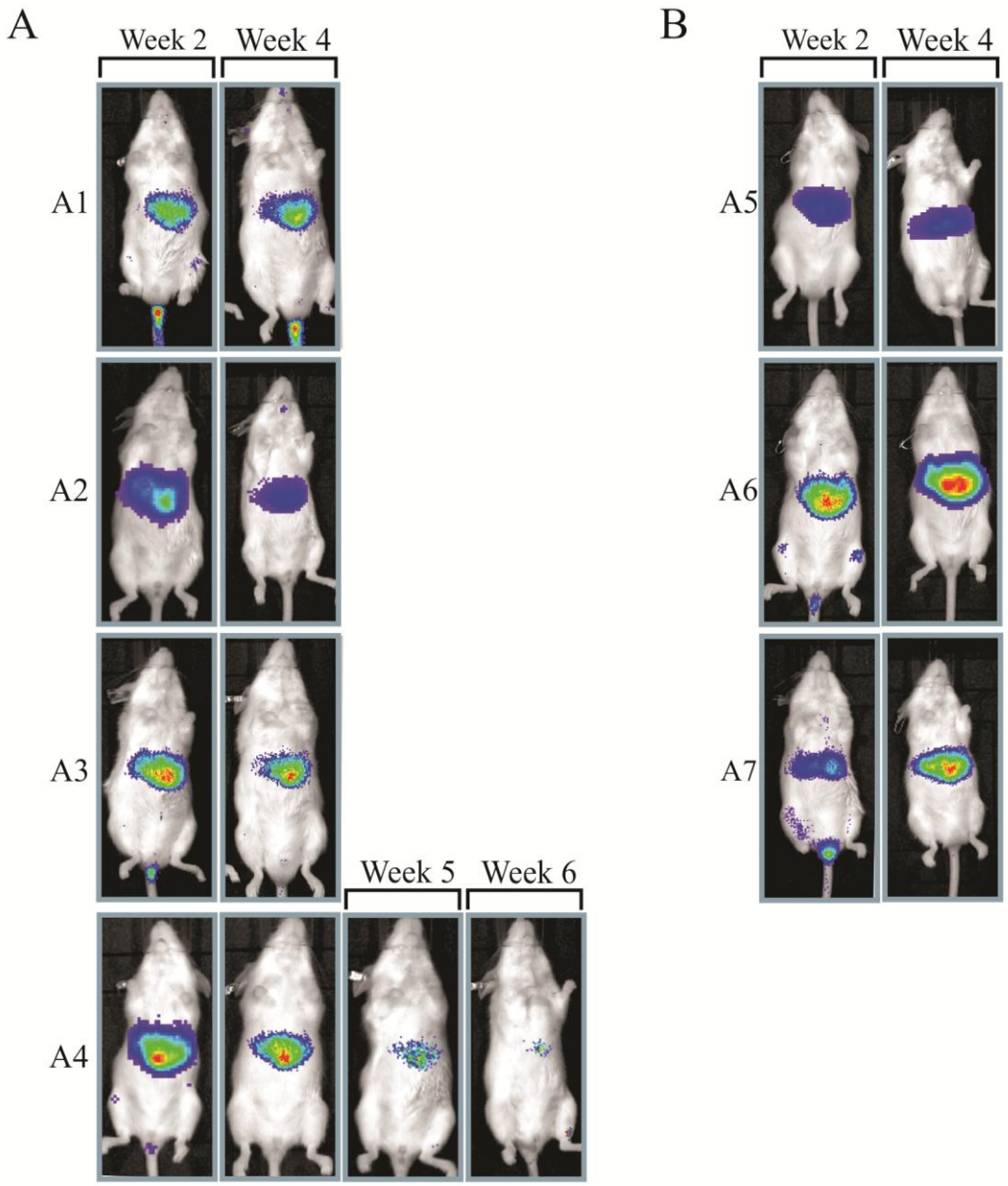


Figure 5.5: <u>Bioluminescence imaging of Firefly luciferase expression following systemic LV delivery to adult HBV transgenic mice.</u>

LVTH-LUC miR-31/5-8-9 (A1-A4) or LVTH-LUC (A5-A7) LV (8.5×10^8 TU per mouse) was delivered to adult HBV transgenic mice via tail vein injection. *Firefly luciferase* expression was analysed by bioluminescence imaging using the IVIS® Kinetic imaging system. Bioluminescence images from LVTH miR-31/5-8-9- (A) and LVTH-injected (B) mice imaged at 2 week intervals are shown.

5.4 The LVTH-LUC miR-31/5-8-9 LV silences HBV replication in vivo

To determine the antiviral efficacy of the LVTH-LUC miR-31/5-8-9 LV following *in vivo* administration to neonatal and adult HBV transgenic mice, the concentration of HBsAg was analysed in sera of LV-injected animals. Significant reduction of serum HBsAg was achieved in animals that received neonatal temporal vein or adult tail vein injection of LV expressing the polycistronic pri-miR-31/5-8-9 molecule compared to mice injected with control LVTH LV (Figure 5.6 A and 5.7 A).

While long-term stable inhibition of HBsAg secretion was maintained until the end point of the study in neonatal mice that received a temporal vein injection (Figure 5.6 A), only a transient inhibition of HBV replication was observed in mice that received the HBV-silencing LV systemically (Figure 5.7 A). Similarly, quantitative real-time PCR analysis of circulating VPEs revealed sustained long-term reduction in the level of serum HBV DNA in the neonatal cohort that received the anti-HBV LV via the temporal vein (Figure 5.6 B) compared to the short-term attenuation of viral replication observed in tail-vein injected adult HBV transgenic animals (Figure 5.7 B). Inhibition of HBsAg secretion and circulating VPEs in adult tail vein-injected mice was apparent over the first four weeks after systemic LV administration (Figure 5.7). Significant inhibition of markers of viral replication, observed at weeks 2, 3 and 4 following LV delivery was followed by a rapid increase in HBsAg levels and circulating virion counts (Figure 5.7 A; A4) as well as a sudden diminution in the level of reporter transgene expression (Figure 5.5 A; A4).

The above observation was not unexpected and is consistent with reports of limited LV expression as a result of the development of transgene product-specific immune responses and clearance of transduced cells following systemic LV administration in immunocompetent mice (382, 387, 388). Typically, widespread LV transduction can be

observed in the liver, bone marrow and spleen, after systemic LV administration (319). *De novo* expression of proteins in LV-transduced cells results in antigen presentation by professional APCs (Kupffer cells, dendritic cells) and non-professional APCs (liver sinusoidal endothelial cells) subsequently initiating a transgene product-specific immune response and elimination of transduced cells (382, 389). The HBV-silencing LV expresses both *GFP* and *Firefly luciferase* transgenes from an internal constitutive EF1-α promoter. It is likely that presentation of the GFP and Firefly luciferase antigens by hepatocytes and liver APCs or expression of these proteins in LV-transduced haematopoietic APCs (281) initiated the development of an immune response and subsequent elimination of LV-transduced cells in the liver.

In conclusion, the neonatal temporal vein injection was demonstrated to be a suitable approach for the evaluation of long-term antiviral efficacy of the HBV-silencing LVs. Data generated from the neonatal temporal injection model show that the HBV-silencing LVTH-LUC miR-31/5-8-9 LV is capable of mediating stable delivery of therapeutic anti-HBV pri-miR molecules and sustained silencing of viral replication in an *in vivo* model that simulates chronic HBV replication.

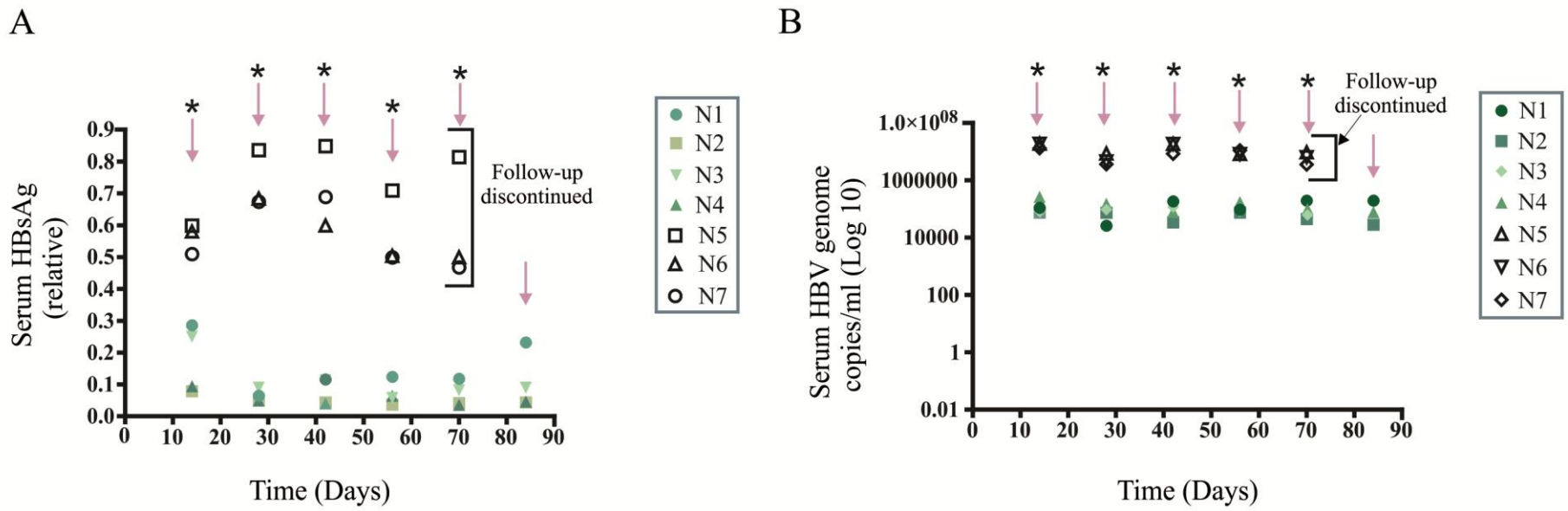


Figure 5.6: <u>Evaluation of long-term effects of HBV-silencing LV on HBsAg production and circulating viral particle equivalents *in vivo*.</u>

One day-old HBV transgenic mice received LVTH-LUC or LVTH-LUC miR-31/5-8-9 LV (2.7×10^7 TU per mouse) intravenously via a temporal vein injection. Twenty one days later, blood samples were collected from all animals at two week intervals (time points are indicated by pink arrows) and analysed by quantitative HBsAg ELISA (A) and real-time quantitative PCR (B). A time course of relative serum HBsAg concentrations (A) and viral loads (B) is shown (mean ± SEM., n≥3, $*p < 0.05$). EuroHep standards were used to determine virion copy numbers.

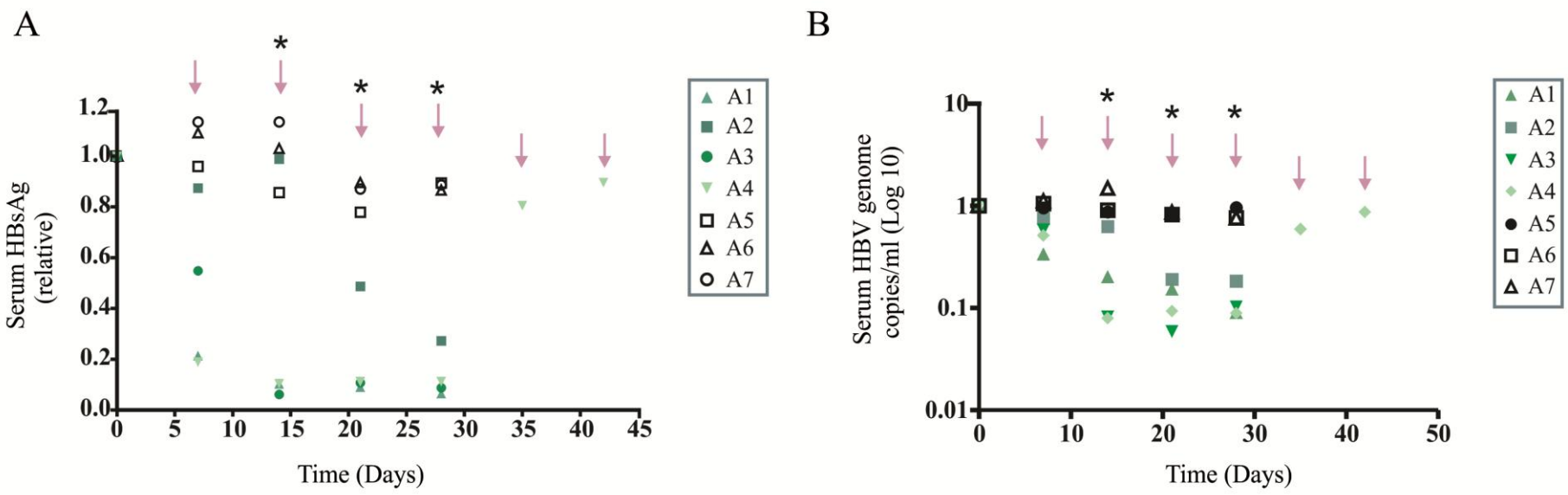


Figure 5.7: <u>Assessment of the effects of HBV-silencing LV on HBsAg production and circulating viral particle equivalents after LV administration to adult HBV transgenic mice.</u>

LVTH-LUC or LVTH-LUC miR-31/5-8-9 LV (2.5×10^8 TU per mouse) were delivered to adult HBV transgenic mice via a tail-vein injection. Blood samples were collected from all animals at two week intervals (time points are indicated by pink arrows) and analysed by quantitative HBsAg ELISA (A) and real-time quantitative PCR (B). A time course of relative serum HBsAg concentrations (A) and viral loads (B) is shown (mean ± SEM., n≥3, $*p < 0.05$). Data points were normalised relative to baseline HBsAg and viral load values obtained 2 days before LV administration. EuroHep standards were used to determine virion copy numbers.

5.5 LV-delivered polycistronic pri-miR sequences mediate intrahepatic knockdown of HBV gene expression

Following demonstration of knockdown of HBV gene expression at protein and DNA levels *in vivo*, the effects of the HBV-silencing LV encoding the polycistronic pri-miR-31/5-8-9 mimic on viral RNA levels in the liver of transgenic animals were assessed by a quantitative reverse transcriptase PCR.

A considerable reduction in intrahepatic HBV *surface* mRNA concentration relative to *GAPDH* mRNA was observed in transgenic mice that had received anti-HBV LV via a temporal vein or adult tail-vein injection compared to control injected mice (Figure 5.8 B and 5.9 B). However, the concentration of *core* HBV mRNA in both neonatal and adult HBV transgenic animals that received therapeutic LVTH-LUC miR-31/5-8-9 LV was not significantly diminished when compared to the LVTH-injected mouse cohort (Figure 5.8 A and 5.9 A). The above is consistent with the findings of a previously published report evaluating the efficacy of RNAi-based anti-HBV gene therapy in the HBV transgenic mouse model (157). Uprichard and colleagues observed that despite effective shRNA-mediated knockdown of HBV replication, the 3.5 kb pregenomic HBV mRNA transcript, encoding the *core* sequence, appeared to be more resistant to RNAi-mediated degradation. Nonetheless, the above findings indicate that the HBV-silencing LV encoding the polycistronic pri-miR-31/5-8-9 sequence mediates considerable intrahepatic knockdown of HBV gene expression and the degree of RNA inhibition achieved is sufficient to effect significant knockdown of HBV replication *in vivo*.

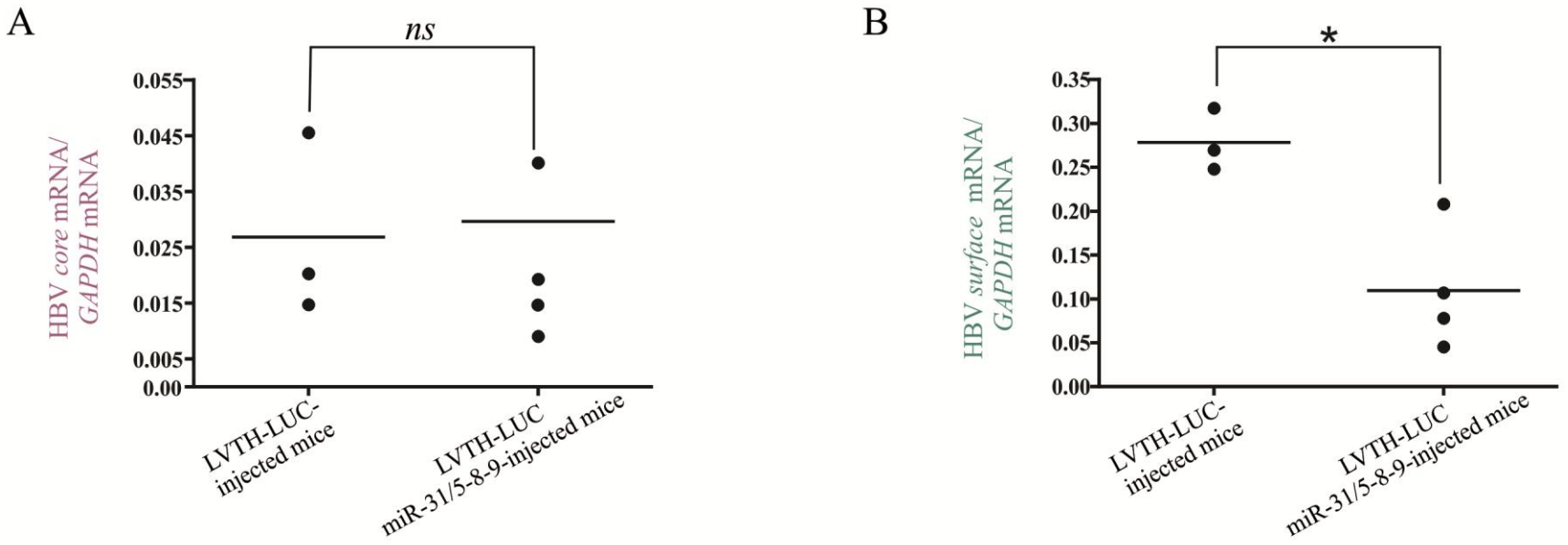


Figure 5.8: Concentration of *core* and *surface* HBV mRNA in the livers of temporal vein LV-injected HBV transgenic mice.

Total RNA was isolated at necropsy from the livers of HBV transgenic mice which had been injected with LVTH-LUC or LVTH-LUC miR-31/5-8-9 LV via the temporal vein. The concentration of *core* and *surface* HBV mRNA was determined by a quantitative reverse transcriptase PCR. Average *core* and *surface* mRNA levels relative to *GAPDH* mRNA from individual mice are shown (mean ± SEM., n≥3, $*p < 0.05$). ns (not significant).

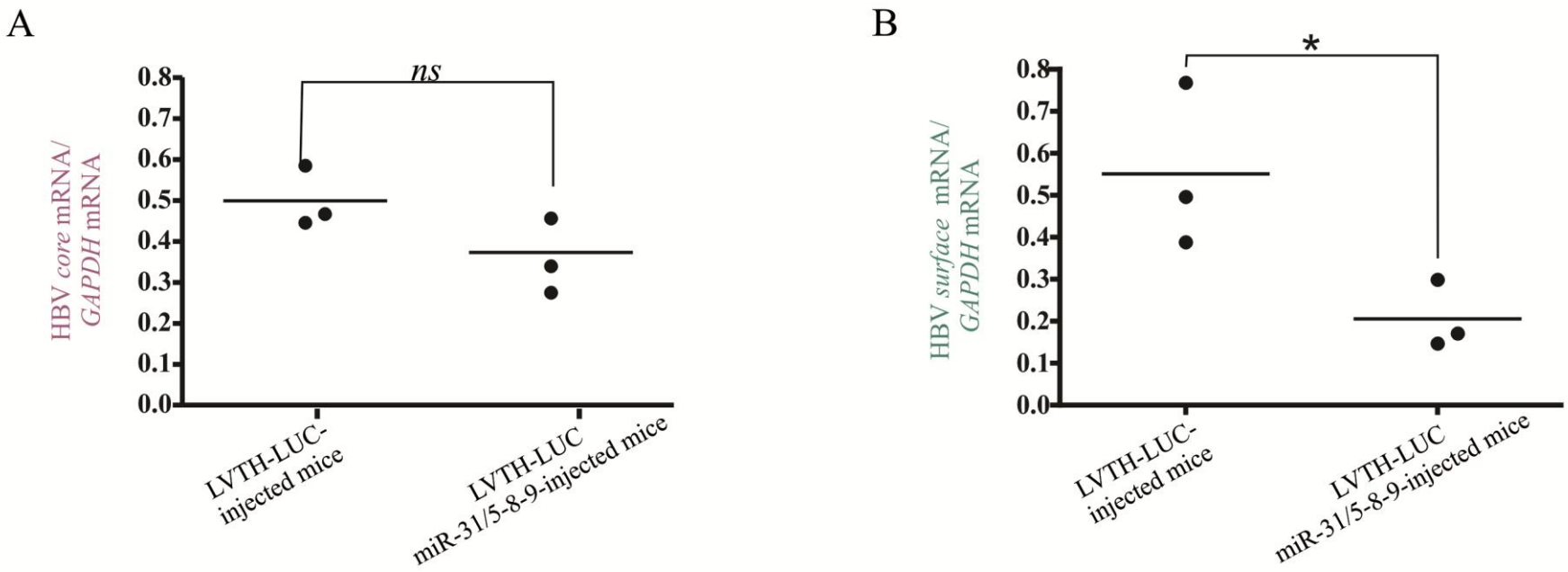


Figure 5.9: <u>Concentration of *core* and *surface* HBV mRNA in the livers of tail vein LV-injected HBV transgenic mice.</u>

Total RNA was isolated at necropsy from the livers of HBV transgenic mice which had been injected with LVTH-LUC or LVTH-LUC miR-31/5-8-9 LV via the tail-vein and subjected to quantitative reverse transcriptase PCR to determine the concentration of *core* and *surface* HBV mRNA. Ratios of the levels of *core* and *surface* mRNA normalised to *GAPDH* mRNA from individual mice are shown (mean ± SEM., n=3, $*p < 0.05$). ns (not significant).

5.6 HBV-silencing lentiviruses do not induce hepatotoxicity

To determine whether LVTH-derived LVs induce toxic responses in the liver following intravenous administration, ALT levels were analysed in sera collected from HBV transgenic animals that had received LVTH-LUC and LVTH-LUC miR-31/5-8-9 LV via a tail vein or superficial temporal vein injection. Serum ALT levels in mice that had received LV via a temporal vein injection were within the normal range at necropsy (Table 5.1) indicating that the LVs did not cause chronic hepatotoxicity. Similarly, ALT levels in adult HBV transgenic mice that received LVTH-LUC and LVTH-LUC miR-31/5-8-9 LV via a tail-vein injection were not raised following LV administration or at necropsy except for a minor increase observed in a single mouse (A1) (Table 5.1).

Table 5.1: Alanine transaminase (U/L) in sera of HBV transgenic mice that received LV intravenously.

Mouse ID	LV[1]	ALT 48 hours	ALT * 1 week	ALT at necropsy
N1	LVTH-LUC miR-31/5-8-9	-	-	34
N2	LVTH-LUC miR-31/5-8-9	-	-	34
N3	LVTH-LUC miR-31/5-8-9	-	-	58
N4	LVTH-LUC miR-31/5-8-9	-	-	46
N5	LVTH-LUC	-	-	70
N6	LVTH-LUC	-	-	56
N7	LVTH-LUC	-	-	50
A1[1]	LVTH-LUC miR-31/5-8-9	48	75	124
A2	LVTH-LUC miR-31/5-8-9	48	66	64
A3	LVTH-LUC miR-31/5-8-9	40	64	86
A4	LVTH-LUC miR-31/5-8-9	52	60	54
A5	LVTH-LUC	40	48	42
A6	LVTH-LUC	60	64	72
A7	LVTH-LUC	66	44	56

1 HBV transgenic mice received LVTH-LUC and LVTH-LUC miR-31/5-8-9 LV via an intravenous temporal (N1-N7) or tail- vein (A1-A7) injection.

* ALT values below100 (U/L) are considered normal for laboratory mice.

5.7 The polycistronic pri-miR-31/5-8-9 sequence is efficiently processed in the livers of LV-injected mice

To verify that the LV-delivered polycistronic miR sequence is being processed according to the intended design, total RNA extracted from mouse livers at necropsy was analysed by a two step reverse-transcriptase real-time PCR assay (Illustrated in Section 2.11.10) for the presence of the putative mature miR-31/5 guide sequences of pri-miR-31/5-8-9. Young adult mice that received LV via tail vein injection were sacrificed prior to induction of immune-mediated clearance of LV-transduced cells (A1-A3). Expression of the mature guide sequence was normalised to an endogenous U6 snRNA control, to correct for variation in the amount of RNA in the different samples. Mature miR-31/5 guide sequence was detected in the livers of HBV transgenic mice that received the LVTH-LUC miR-31/5-8-9 LV via a temporal or tail vein injection and was undetectable in the livers of mice that received LVTH-LUC LV (Table 5.2).

Table 5.2: Detection of mature miR-31/5 sequence in liver tissue of LV-injected HBV transgenic mice.

Mouse ID	Average Ct miR-31/5	Average Ct U6 snRNA	ΔCt (Ct U6 snRNA-Ct miR-31/5)
N1-N4[1]	19.65	21.41	1.76
N5-N7[2]	-	21.62	-
A1-A3[1]	21.17	21.68	0.51
A5-A7[2]	-	21.96	-

1 HBV transgenic mice that had received LVTH-LUC miR-31/5-8-9 LV via a temporal vein (N1-N4) or tail-vein (A1-A3) injection.

2 HBV transgenic mice that had received LVTH-LUC LV via a temporal (N5-N7) or tail-vein (A5-A7) injection.

5.8 Conclusion

Preclinical proof of principle of the efficacy of the novel RNAi-based lentiviral vector system was determined by administration of HBV silencing LVs in transgenic mice. Incorporation of the *Firefly luciferase* transgene in previously characterised HBV-silencing lentiviral vectors (Chapter 4) allowed for convenient assessment of transduction efficiency and stable expression of integrated sequences in live animals. The mTTR promoter, selected to drive expression of anti-HBV pri-miR mimics, was demonstrated to mediate stable expression of the *Firefly luciferase* transgene over a period of 12 months (study end point). These data validate previous reports demonstrating efficiency and functionality of the mTTR promoter *in vivo* (180, 385, 386) and indicate that the mTTR promoter is capable of driving sustained and hepatospecific gene expression. Hence, the mTTR promoter sequence is a suitable alternative to other Pol II promoters and can be utilised to accomplish controllable and liver-specific expression of therapeutic RNAi effecters.

Efficient transduction of the liver was accomplished following LV delivery to young adult (via tail vein injection) and neonatal (via superficial temporal vein injection) HBV transgenic mice, evidenced by robust expression of Firefly luciferase in both animals cohorts. Importantly long-term sustained inhibition of markers of viral replication and circulating virion counts was observed in animal cohorts that received the HBV-silencing LV via temporal vein injection. In contrast, a sudden diminution in the level of reporter transgene expression accompanied by an increase in HBsAg serum levels as well as an increase in circulating VPEs was observed in transgenic mice subjected to systemic tail-vein LV delivery. The above observation was not unexpected and is consistent with reports of limited LV expression as a result of the development of transgene product-specific immune responses and clearance of transduced cells following systemic LV administration in immunocompetent mice(390). Nevertheless, even though the inclusion of *GFP* and

Firefly luciferase transgenes in the HBV-silencing LV facilitated convenient monitoring of LV transduction and expression, exclusion of the GFP-2A-Firelfy luciferase expression cassette will not have any consequences on the therapeutic efficacy of the approach. Importantly, the inclusion of liver-specific transcription regulatory elements in our therapeutic LV should preclude expression of the polycistronic pri-miR mimic in non-hepatic cells including APCs, largely improving the overall efficacy and safety of the HBV-silencing LV.

Data presented in this chapter demonstrate that the RNAi-based HBV-silencing LV is capable of mediating stable hepatic delivery and sustained expression of anti-HBV pri-miR sequences *in vivo*. Furthermore, the therapeutic sequences are processed according to the intended design and mediate powerful knockdown of markers of viral replication, circulating VPEs and intrahepatic viral RNA without inducing hepatotoxicity. To our knowledge this is the first demonstration of stable inhibition of viral replication in HBV transgenic mice using a LV that encodes an antiviral liver-specific polycistronic pri-miR sequence.

6 DISCUSSION

Over the past decade, the therapeutic potential of harnessing the RNAi pathway to achieve specific and potent silencing of pathology-causing genes has been widely explored (reviewed in (148, 391)). The field has been progressing with incredible speed and continual advancements have renewed enthusiasm for the development of novel and improved treatments for many diseases with limited treatment options, including chronic HBV infection. Persistent infection with HBV remains an important public health problem as the risk for development of serious life-threatening complications such as cirrhosis and HCC is significantly increased in chronic HBV carriers (40). HCC is the sixth most common cancer globally (35) and this aggressive malignancy has very poor prognosis (40). The efficacy of currently available therapies for chronic HBV infection is variable and long-term treatment using these agents is associated with the development of unwanted side effects as well as the emergence of resistance. Improved barrier to resistance and potent suppression of viral replication has been reported with some of the newer nucleoside/nucleotide analogues, such as entecavir and tenofovir (392, 393), nevertheless cessation of treatment results in recurrence of viral replication. This is as a result of the inability of this class of therapeutic agents to induce eradication of nuclear cccDNA from infected hepatocytes (394). Persistence of the episomal cccDNA minichromosome (17) allows for continual transcription of all HBV mRNA transcripts as well as pgRNA which serves as a template for reverse transcription perpetuating viral replication (341). Another shortcoming of nucleoside/nucleotide inhibitors is their limited ability to lessen the high HBV antigen load typically seen in chronic HBV infection (395). High antigenaemia in chronically infected individuals is likely to contribute to the development immunotolerance as well as chronic inflammation allowing for persistence of infected hepatocytes and

stimulating the development of a microenvironment that favours malignant transformation (34, 43). Nevertheless, the risk of developing HCC remains unchanged in the majority of chronic carriers receiving treatment and this is perhaps the most significant limitation of currently licensed therapies for HBV. Translation of the success observed using anti-HBV RNAi-based gene therapy in preclinical studies (153, 156, 157, 159, 160) could potentially provide the long-awaited novel and improved therapy for the approximately 350 million individuals worldwide that have been infected with HBV and progressed to chronicity (23)

HBV is amenable to direct RNAi-mediated silencing as the viral replication cycle involves the use of an RNA replication intermediate. The compact arrangement of the HBV genome limits the ability of the virus to mutate. In addition, all major HBV viral transcripts terminate at a common 3' end, allowing for simultaneous silencing of multiple HBV transcripts with a single RNAi effecter. The availability of a novel and potent silencing technology that could potentially be effective in overcoming some of the major limitations of currently licensed treatments for chronic HBV infection, spurred renewed enthusiasm for the development of anti-HBV RNAi-based gene therapy.

An abundance of preclinical studies has demonstrated the utility of highly effective expressed RNAi-based effecters to accomplish impressive silencing of HBV gene expression *in vitro* and *in vivo* (153, 156, 157, 159, 160). Nevertheless, rigorous preclinical testing of the efficacy of anti-HBV RNAi-based expression systems, revealed that several hurdles remain to be overcome before this novel technology can live up to its potential in a clinical setting. These include, optimisation of RNAi effecters to avoid unwanted toxicity (arising from immunostimulation, off-target silencing of cellular genes as well as saturation of the endogenous miR biogenesis pathway), accomplishing efficient delivery of RNAi effecters to the liver as well as the development of improved, suitable disease models for comprehensive testing of the efficacy of RNAi-based strategies.

Successful treatment of persistent HBV infection with RNAi-based therapy will necessitate long-term, sustained expression of active therapeutic effecters to counter chronic viral replication. Expressed RNAi effecters have the useful property of sustained production from a stable DNA template, making these silencing molecules better suited to treatment of chronic HBV infection than use of unmodified siRNAs. Initially, expression of shRNA sequences from U6 or H1 Pol III promoters (161, 396) was extensively applied as a means of activating RNAi to cause powerful silencing of HBV replication *in vitro* and *in vivo* (153, 156-158). Subsequently, Grimm and colleagues demonstrated that strong, U6-driven expression of these types of sequences, especially of shRNAs, resulted in severe toxicity *in vivo* and fatality as a consequence of saturating components of the RNAi pathway (165, 166). These studies highlighted the need to optimise the safety and efficacy of expressed anti-HBV RNAi effecters to allow for clinical advancement of this novel technology. Expressed RNAi-activating anti-HBV sequences should be capable of effecting potent knockdown of HBV gene expression when expressed at a low level. In addition, mature miR guide strands processed from these therapeutic effecter should be designed such that their incorporation into RISC is favoured to ensure specific and intended silencing. Concerted efforts to optimise RNAi expression cassettes led to the development of highly sophisticated and effective, Pol II-based expression systems encoding effecters that mimic naturally occurring pri- or pre- miRs and exhibit highly potent silencing activity (127, 159, 160, 182). Use of Pol II promoters to express silencing sequences allows for greater control over the dose of expressed effecters as well as for tissue-specific and/or inducible expression.

Expressed pri-miR mimics, derived from natural pri-miR-31 and engineered to generate anti-HBV RNAi guide sequences targeting single (monocistronic pri-miR sequences) or multiple (polycistronic pri-miR sequences) sites within the *HBx* ORF of the

viral genome have been developed in our laboratory (159, 160). Targeting of the highly conserved multifunctional *HBx* sequence (47, 190, 191) that shares sequence homology with all HBV viral transcripts enables simultaneous and potent knockdown of all HBV mRNAs. These silencing sequences have been demonstrated to be capable of mediating efficient, potent and safe knockdown of viral replication *in vitro* and *in vivo*. Nevertheless, accomplishing safe and efficient hepatic delivery of antiviral RNAi effecters remains the greatest challenge impeding the clinical translation of RNAi-based anti-HBV gene therapy.

Successful clinical application of RNAi-based anti-HBV therapy will depend on the availability of an efficient delivery system capable of mediating stable delivery to hepatocytes and sustained, long-term expression of the therapeutic effecters. Viral vector-mediated delivery allows for long-term expression of therapeutic genes, making this delivery method suited to countering persistent HBV infection. The use of viral vectors is a potentially effective and clinically relevant strategy (http://www.wiley.com//legacy/ wileychi/genmed/clinical/, accessed 12 March 12) for *in vivo* delivery of expressed RNAi activators. Adenoviral (157, 158, 397, 398) and adeno-associated viral (165, 167) vectors have been exploited to achieve efficient hepatic delivery and sustained expression of RNAi effecters following systemic administration. Nevertheless, to accomplish durable silencing of persistent HBV infection, long-term and perhaps indefinite expression of therapeutic RNAi effects, which is not attainable with the existing Ad and AAV vectors, is likely to be required. Recombinant adenoviruses are highly immunogenic and are known to induce potent innate and adaptive immune response after systemic administration (205-209). Sadly the highly immunogenic nature of adenoviruses led to the first reported death of a patient in a gene therapy clinical trial, following an intravenous, high-dose administration of recombinant adenovirus (210). A number of strategies has been employed since to improve the safety and efficacy of Ad vectors (reviewed in (399)). HDAd vectors have

been developed that are devoid of sequences encoding viral proteins and allow for sustained transgene expression (196, 214). Importantly, PEGylation of recombinant Ads as well as HDAds attenuates vector immunogenicity (219, 400, 401) and may further attenuate immunostimulation. Nevertheless technical considerations limit the large-scale propagation of pegylated Ads for clinical use. Although, recombinant AAV vectors have a considerably improved safety profile compared to Ad vectors (reviewed in (402)), accomplishing sustained expression of therapeutic genes using these vectors has proven to be a challenging task. Data from a recent clinical trial, using AAV vectors for the treatment of haemophilia, demonstrated that long-term transgene expression was precluded following the induction of an adaptive immune response and clearance of transduced cells (243, 244). Further improvement of AAV vectors (245, 246) can potentially overcome some of the existing challenges and enable long-term, durable transgene expression.

Recombinant lentiviral vectors derived from HIV-1 (251), have emerged as versatile and efficient transfer tools as a consequence of their unique ability to mediate stable transduction of dividing and non-dividing cells which allows for long-term and potentially indefinite gene expression. The transduction efficiency of recombinant LVs is enhanced when the virus is delivered to cells in the G1 phase of the cell cycle (320). While the majority of hepatocytes in a healthy adult liver are quiescent, lentivirus transduction is likely to be facilitated in the chronically infected liver where cell division is occurring as a result of HBV-induced chronic inflammation, cirrhosis and/or HCC (403, 404). These properties make recombinant LVs advantageous for gene transfer of expressed RNAi anti-HBV sequences to infected hepatocytes and for accomplishing the long-term sustained silencing of HBV gene expression necessary to counter chronic HBV.

Although accomplishing stable integration is useful to achieve durable silencing, lentiviral integration can result in genotoxicity. SIN lentiviral vectors have considerably

improved safety profiles in comparison to γ-retroviral vectors (257, 299, 300), however the ability of these vectors to cause insertional mutagenesis is not completely abolished and it is subject to ongoing investigations (268). Nevertheless, lentivirus-mediated insertional mutagenesis involving proto-oncogene activation has not been reported in ongoing clinical trials based on ex-vivo modification of HSC (308-311). Unlike hematopoietic systems where there is clonal expansion of individual LV-modified progenitor cells (250), liver regeneration involves generalised proliferation of residual hepatocytes (reviewed in (405)) thus clonal expansion of LV-modified hepatocytes is less likely to occur.

To advance potential clinical application of RNAi-based anti-HBV gene therapy, an original strategy was designed and evaluated *in vitro* and in an animal model of HBV replication. The novel strategy is based on the use of second generation, SIN lentiviral vectors with LoxP-containing LTRs to deliver potent and liver-specific HBV silencing pri-miR-31 mimics to infected hepatocytes while allowing for conditional ablation of transduced hepatocytes. This novel therapeutic approach aims to overcome concerns of LV-induced insertional mutagenesis and enable sustained and/or potentially indefinite knockdown of HBV gene expression to counter and possibly eradicate persistent viral infection.

Targeting expression of therapeutic transgenes to hepatocytes is an important method that can be exploited to achieve hepatospecificity of liver directed RNAi-based LV gene therapy. The incorporation of liver-specific promoters (179, 180) to limit expression of therapeutic genes to hepatocytes allows for efficient liver-specific expression while avoiding unintended off-target effects in non-targeted tissues and the induction of an immune repose by LV-transduced APCs (382). To generate liver-specific HBV silencing pri-miR mimics and optimise compatibility with the pLVTH lentiviral vector, the promiscuous CMV promoter, used to drive expression of previously characterised, mono-

and poly- cistronic pri-miR mimics (160, 406), was replaced with the mTTR enhancer-promoter sequence (180). Importantly, the constitutively active CMV promoter has been reported to affect viral titres negatively when used to control miR expression in the context of lentiviral vectors (329) and shutdown of CMV promoter activity in the murine liver has been reported (176, 177). Thus the use of tissue-specific Pol II promoters is a more suitable alternative.

Evaluation of the functionality and tissue-specificity of the mTTR enhancer-promoter sequence, revealed that the promoter is capable of driving robust and tissue-specific expression of the *Firefly luciferase* transgene in liver-derived Huh7 cells. Moreover, similarly to their CMV promoter counterparts, the monocistronic and polycistronic pri-miR mimics expressed from the liver-specific mTTR promoter were equally capable of mediating efficient knockdown of HBV gene expression. Furthermore, the mTTR promoter mediated stable and long-term (≥ 12 months) expression of the *Firefly luciferase* transgene in HBV transgenic mice that received Firefly luciferase-expressing LV via a neonatal superficial temporal vein injection. These data corroborate previous reports demonstrating capability of the mTTR promoter to drive sustained gene expression *in vivo* (180, 385, 386), and indicate that the mTTR promoter sequence can be exploited to accomplish controllable and liver-specific expression of therapeutic RNAi effecters. In addition, a recent study has demonstrated that moderate internal promoters in the context of SIN lentiviral vectors, are less likely to induce insertional mutagenesis by enhancer-mediated activation of oncogenes in comparison to strong enhancer-promoter sequences (268).

Following demonstration of functionality and antiviral efficacy of the liver-specific mono- and poly- cistronic pri-miR mimics, the HBV silencing expression cassettes were incorporated into second generation SIN pLVTH lentiviral vectors. Molecular engineering

of the HBV silencing lentiviral vectors involved meticulous design to ensure the generation of functional and effective therapeutic constructs that are suitable for potential clinical application. The mTTR pri-miR-encoding expression cassettes were inserted in the lentiviral vector in the sense orientation, with respect to the lentiviral genome, to ensure efficient expression of anti-HBV miR sequences. An internal polyadenylation signal, downstream of the liver-specific pri-miR expression cassettes was not included to prevent premature termination of the full length viral RNA genomic transcripts and the consequent reduction in viral titre (365). Assessment of antiviral efficacy of the liver-specific monocistronic and polycistronic pri-miR mimics in the context of the pLVTH lentiviral vector indicated that design of the HBV silencing lentiviral vectors was functional and efficient. It is likely that the polyadenylation signal in the viral 3' LTR contributes to improved stability of pri-miR transcript produced from the liver-specific anti-HBV pri-miR cassette, resulting in robust expression of mature miR sequences

The functionality of the novel anti-HBV RNAi-based lentiviral vectors was further validated following the propagation of VSV-G pseudotyped HBV silencing LVs. Previous reports indicated that Drosha-mediated recognition and processing of the pri-miR sequences present in the sense orientation of the genomic RNA transcript can negatively affect viral titre (329). However, a significant difference in viral titres of LVTH and LVTH-derived HBV silencing LVs was not observed. The above observations indicated that the design of the RNAi-based lentiviral vectors is functional and that these vectors are amenable to large-scale production. The absence of a negative effect on viral titre by Drosha-mediated processing of lentiviral vector-encoded miR sequences could be attributed to: 1) Drosha saturation, as numerous genomic RNA transcripts containing pri-miR sequences are being continually generated in producer cells and 2) effective nuclear export of unspliced and singly-spliced genomic lentiviral RNA, mediated by the Rev

protein interacting with the RRE sequence encoded in the genomic lentiviral transcript (183, 274). A nuclear export complex comprising RRE-containing lentiviral RNA, Rev and the CRM1 nuclear export factor is formed co-transcriptionaly and is likely to mediate evasion of Drosha recognition and processing of the pri-miR sequence (407).

The HBV silencing lentiviruses stably transduced liver-derived Huh7 and HepG2.2.15 cells. Northern blot analysis of RNA from stably transduced Huh7 cells verified that the integrated anti-HBV pri miR sequences were processed to form RNAi-activating guide strands according to the intended design. The pri-miR sequences were efficiently processed as unprocessed precursors (pri and pre- miR transcripts) were not detectable. This is in contrast to shRNA expression systems which often yield large amounts of unprocessed precursors and cause saturation of the endogenous miR biogenesis pathway, inducing severe toxicity *in vivo* (127, 165). Saturation assays revealed that there was limited disruption of endogenous miR function in cells which had been stably transduced with HBV silencing LVs, indicating that expression of integrated liver-specific pri-miR sequences is not likely to induce saturation of components of the endogenous miR biogenesis pathway and unintended toxic effects. PCR analysis of genomic DNA extracted from Huh7 cells, which had been stably transduced with the polycistronic HBV silencing LV, showed a lack of evidence for major recombination-mediated deletion of repeated sequences in the pri-miR-31 scaffold, in contrast to previous reports (183, 186). The extent of recombination occurring may be related to particular pri-miR scaffolds used as well as the number of miR effecters within the polycistron (185). It is likely that there is low frequency of recombination-mediated deletion when a polycistronic pri-miR-31 sequence encoding three miR effecters is used. Nevertheless, extrapolation of results obtained for a multi-targeted anti-HIV RNAi approach indicates that an effective anti-HBV RNAi-based

multi-targeted approach is not likely to necessitate simultaneous expression of more than three miR effecters (182).

Potent knockdown of HBsAg, a marker of HBV replication, was observed when a HBV-replication competent plasmid was transfected in LVTH-, LVTH miR-31/5- and LVTH miR-31/5-8-9- transduced Huh7 cells. Furthermore, a dual luciferase reporter assay demonstrated that effective knockdown of HBV gene expression was mediated by all three miR effecter sequences in Huh7 cells which had been stably transduced with LVTH- and LVTH-derived HBV silencing LVs. Nevertheless, knockdown potency corresponded to the quantity of guide sequence detected by Northern blot analysis, with the miR-31/5 guide sequence being most effective at silencing gene expression. Assessment of the ability of the LV-delivered liver-specific monocistronic and polycistronic pri-miR mimics to inhibit viral replication of a mutated HBV sequence revealed that only the polycistronic pri-miR-31/5-8-9 sequence was capable of silencing the expression of both a wild-type and mutated *HBx* sequence. These observations are likely to be the result of additive knockdown effects arising from simultaneous expression of several active anti-HBV pri-miR molecules and possibly enhanced processing of the polycistronic pri-miR transcript (408). The use of a multitargeted RNAi approach is likely to be beneficial in limiting the emergence of viral escape mutants and allow for improved barrier to the emergence of resistance in a clinical setting.

As the polycistronic anti-HBV, liver-specific pri-miR sequence was shown to have superior efficacy to the monocistronic pri-miR sequence, further evaluation of antiviral efficacy was carried out using LVs expresing the polycistronic pri-miR mimic. Powerful knockdown of markers of viral replication and circulating VPEs was demonstrated in LVTH miR-31/5-transduced HepG2.2.15 cells. Rigorous evaluation of the anti-HBV efficacy of the polycistronic silencing LV in the HepG2.2.15 HBV replication cell line

(378) and demonstration of potent antiviral activity augured well for further *in vivo* evaluation of the novel RNAi-based lentiviral vector HBV silencing strategy.

Preclinical analysis of the efficacy of the RNAi-based lentiviral vector system was assessed using the HBV transgenic mouse model which is well suited for rigorous evaluation of the antiviral efficacy of novel therapies (345, 379). LVTH- and LVTH-derived HBV silencing LVs encoding a liver-specific polycistronic pri-miR 31/5-8-9 sequence, were delivered to young adult animals (~3 weeks old) via a tail-vein injection as well as to neonatal mice via a superficial temporal vein injection. Both LVTH and LVTH miR-31/5-8-9 LV were modified to encode the bicistronic 2A-linked Firefly luciferase-eGFP expression cassettes placed under the control of an internal EF1-α promoter.

Lentiviral transduction following systemic delivery of recombinant lentivirus has been reported to be significantly higher in young and newborn rodents (180, 322-324). Thus, the aforementioned LV delivery methods were exploited to allow for optimal assessment of the antiviral efficacy of the HBV silencing RNAi-based lentiviral vector therapy. Robust expression of Firefly luciferase in both animal cohorts indicated that efficient liver transduction was accomplished following systemic LV delivery to young adult and neonatal HBV transgenic mice. Nevertheless, genomic qPCR analysis revealed that significantly higher transduction efficiency was accomplished when the LVs were delivered to neonatal mice. Reduction of serum HBsAg and HBV DNA was significant in animals that received neonatal temporal vein or adult tail vein injection of LV expressing the polycistronic pri-miR-31/5-8-9 molecule when compared to mice injected with control LVTH LV. In addition to knockdown of HBV gene expression at protein and DNA levels *in vivo*, a significant reduction in the concentration of intrahepatic HBV *surface* mRNA was mediated following LV-delivered polycistronic pri-miR sequences. While long-term, sustained inhibition of HBsAg secretion and circulating VPEs was observed over a 12

week period (study end point) in mice that received HBV silencing LV via temporal vein injection, only short-term attenuation of HBV replication (~4 weeks) was observed in mice subjected to systemic tail-vein LV delivery.

The transient inhibition of HBV replication observed in transgenic mice to which LV was delivered via the tail vein is likely to have resulted from the induction of a specific immune response to *GFP* and/or *Firefly luciferase* transgenes expressed by the internal EF1-α promoter (380, 381). Development of transgene product-specific immune responses and clearance of transduced cells following systemic LV administration in immunocompetent mice has been previously reported (390). Induction of a transgene-specific immune response was not observed in neonatal mice that received LV via a superficial temporal vein injection as the immune-system of these newborn animals was not fully functional at the time of LV administration and the response to foreign antigens was likely to have been decreased. Antigen-specific adaptive immune responses to LV-encoded GFP can be attenuated by targeting transgene expression to hepatocytes (382) or incorporation of haematopoietic lineage-specific miR targets in the 3' UTR of the transgene to negatively regulate transgene expression in APCs (318, 387). Although the above approaches are clinically relevant to reduce immunogenicity of the HBV silencing LVs, full exclusion of the marker transgene-encoding cassette will not have any consequences on the therapeutic efficacy of the approach. Finally, analysis of serum ALT levels in both neonatal (superficial temporal vein LV injection) and young adult (tail-vein LV injection) cohorts did not reveal significant hepatotoxicity associated with the administration (young adult cohort) or LV persistence (neonatal cohort). Processing of the integrated pri-miR mimics was according to their intended design as mature miR-31/5 guide sequence could be detected at necropsy in the livers of HBV transgenic mice that received the LVTH-LUC miR-31/5-8-9 LV via a temporal or tail vein injection.

Data presented in this thesis demonstrate that the RNAi-based HBV-silencing LVs encoding a liver-specific polycistronic pri-miR sequence are capable of mediating stable hepatic delivery and sustained expression of anti-HBV pri-miR sequences *in vivo*. Furthermore, the therapeutic sequences are processed according to the intended design and mediate powerful knockdown of markers of viral replication, circulating VPEs and intrahepatic viral RNA without inducing hepatotoxicity. To our knowledge this is the first study to evaluate the efficacy of RNAi-activating anti-HBV lentiviral vectors in the HBV transgenic mouse model. Although this constitutes a preliminary step towards clinical application of HBV silencing RNAi-based lentiviral vectors, it has provided proof of principle for a therapeutic strategy that can be potentially useful for the treatment of chronic HBV infection.

RNAi-activating HBV silencing lentiviruses could be used in combination with currently existing treatments. Combining the potent inhibition of viral replication that could be achieved with nucleoside/nucleotide analogues with effective silencing of HBV gene expression mediated by highly efficacious LV-delivered polycistronic anti-HBV RNAi sequences has the potential to enable simultaneous inhibition of persistent viral replication and high load of HBV antigen while providing a high barrier to emergence of escape mutants. Sustained reduction of viraemia and antigenaemia, accomplished by RNAi-activating LVs or in conjunction with available therapies, should provide a relief from immune exhaustion (30), stimulating an adaptive immune system response and potentially eradicating the infection. Importantly, activation of the adaptive immune system will fuel clearance of infected cells stimulating cell division which will in turn lead to dilution of the cccDNA pool and formation of a population of hepatocytes free of cccDNA (reviewed in (409)). Studies have shown that following recovery of chronic infection the population of hepatocytes that are not infected is in fact derived from

parentally infected cells (410, 411). Ultimately, the treated liver might be repopulated by healthy hepatocytes as they may have a growth advantage.

Furthermore, the lentiviral vector platform that has been developed and characterised as part of the current set of investigations may be beneficial for advancing potential clinical application of RNAi-based gene therapy for other persistent hepatic viral infections, such as HCV.

Although the long-term antiviral efficacy of novel therapies can be rigorously assessed in the HBV transgenic model, cccDNA is not formed in the hepatocytes of HBV transgenic mice (344). Further development of the RNAi activating HBV silencing lentiviral vectors into a clinically relevant anti-HBV therapeutic will necessitate evaluation in an animal model that supports the formation of cccDNA. Analysing the effects of RNAi activating HBV silencing LVs on this persistent HBV replication template would provide deeper insight into their efficacy and ability to eradicate persistent viral infection. The uPA-SCID mouse model (350) may be exploited to assess the potential clinical utility of the anti-HBV RNAi lentiviral vector hybrid technology. Human hepatocytes can be transduced *ex vivo* with the anti-HBV RNAi-activating LVs and subsequently transplanted into uPA/SCID mice. Following successful grafting, animals may be infected with HBV (351) to determine whether HBV-targeting lentiviruses confer viral resistance. Successful methods for *ex vivo* LV-modification of human hepatocytes and subset transplantation, corroborate the feasibility of using the uPA/SCID mouse model (412) to provide insights into the potential utility of *ex vivo* gene therapy for serious chronic viral infections of the liver.

Ex vivo gene therapy has been the method of choice for lentiviral vector-based therapies currently being assessed in clinical trials (http://www.abedia.com/wiley/vectors.php, accessed 15 March 2014). Most trials are based on *ex vivo* lentiviral

gene modification of peripheral T lymphocytes or HSC, and these studies highlight the feasibility of *ex vivo* therapy with RNAi-activating HBV silencing LVs. Similar to *ex vivo* HSC modification, hepatocytes may be efficiently transduced with viral vectors in suspension and transfused back into the patient. A recently developed protocol has described efficient hepatocyte transduction with lentiviral vectors and immediate transplantation. (314-317). The SLIT protocol is clinically applicable and it results in engraftment and proliferation of transduced hepatocytes while preserving their functionality. The feasibility and biosafety of this approach has been demonstrated in non human primates and the protocol was shown to be effective using human hepatocytes (315-317), corroborating potential feasibility of lentiviral vector-based *ex vivo* therapy for hepatic diseases and/or viral infections

An *ex vivo* gene therapy approach to transfer therapeutic RNAi activators to the liver offers several advantages when compared to *in vivo* liver gene transfer. Although recombinant viruses can readily reach the liver via the blood circulation, *in vitro* hepatocyte manipulation allows for selective modification of hepatocytes, eliminating the possibility of immune stimulation as a result of antigen-presenting cell transduction, avoiding VSV-G inactivation in human serum as well as general toxicity or inflammatory responses following systemic administration (reviewed in (413)).

Ex vivo modification of hepatocytes with a lentiviral vector carrying a therapeutic anti-HBV RNAi molecule, prior transplantation would inhibit viral replication and decrease antigen expression. As a result, these engineered cells will be invisible to circulating immune cells, gaining growth advantage over endogenous hepatocytes and possibly repopulating the liver. Preclinical studies have demonstrated that hepatocytes transplanted in the liver or spleen can engraft, survive and function in the native liver and also in the setting of a cirrhotic rat liver (414-416). However, obtaining successful

hepatocyte engraftment and survival in humans has proven to be a challenging undertaking (417-419). Recently described technological advances allowing for the generation of induced pluripotent stem cells (iPSCs) by reprogramming and differentiating somatic cells have renewed enthusiasm in the field (reviewed in (420)). New developments in the field (421-423) can potentially allow for the generation of an unlimited source of patient-specific autologous hepatocytes, available for *ex vivo* modification and transplantation. Nevertheless, expansion of hepatocytes following transplantation has been one of the major hurdles impeding clinical translation of this approach (424, 425). A recent study has demonstrated the generation of hepatocyte-like cells differentiated from multipotent progenitor cell (iMPC) capable of proliferating following transplantation and repopulating the liver of immunodeficient mice (426). Translation of promising preclinical data may lead to potential clinical success of *ex vivo* RNAi-based lentiviral vector therapy for liver-related disease.

APPENDIX

A1 GENERAL LABORATORY METHODS AND PROTOCOLS

A1-1 Plasmid DNA purification

Plasmid DNA preparation

Reagents

Luria Bertani medium

Luria Bertani medium was prepared by dissolving 10 g of tryptone (Oxoid, England, UK), 10 g NaCl and 5 g of yeast extract (Oxoid, England, UK) in one litre of deionised water. The solution was sterilised by autoclaving at 121°C for 15 minutes and stored at room temperature.

1000× Ampicillin

Five grams of ampicillin (Sigma-Aldrich, MO, USA) was dissolved in 50 ml of 50% ethanol and stored at -20°C.

1000× Kanamycin

Five hundred milligrams of kanamycin (Sigma-Aldrich, MO, USA) was dissolved in 10 ml of deionised water and stored at -20°C.

Procedure

Plasmid DNA was prepared using the Qiagen Plasmid Maxi or Giga Kit (Qiagen, GmbH, Germany). The steps involved in preparing transfection-grade plasmid DNA are

summarised in the table below (Table 6.1).

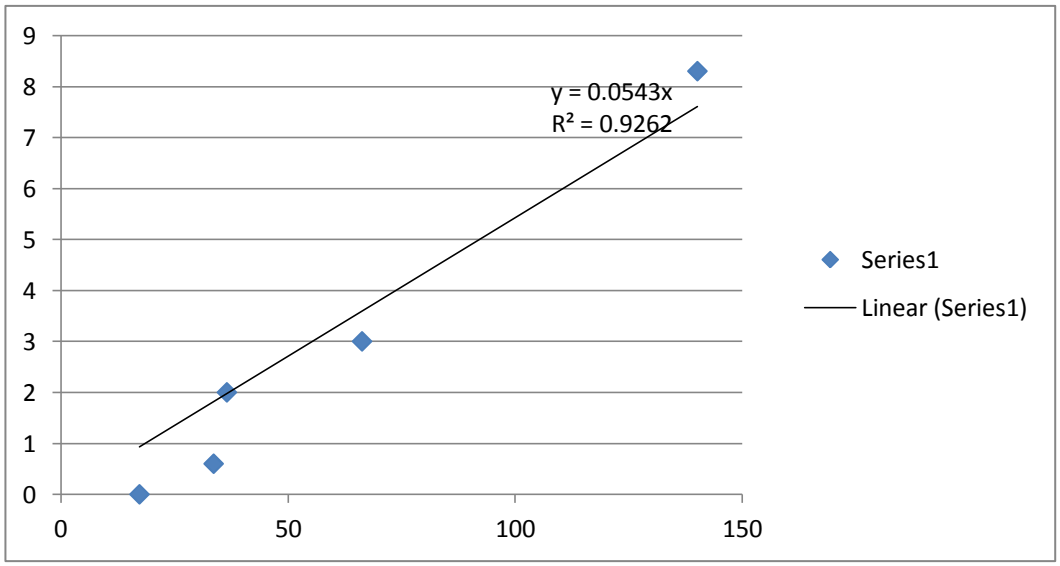
Table 6.1: Preparation of transfection grade plasmid DNA using Qiagen Plasmid Maxi and Giga Kits.

Step	Description	Maxi Kit	Giga Kit
I	Luria Bertani medium was inoculated with a single colony containing the desired plasmid. The cultures were incubated at 37°C with shaking (200 rpm) for 16 hours.	100 ml Luria Bertani (100 µg/ml ampicillin)	2.5 litres Luria Bertani (100 µg/ml ampicillin)
II	Bacterial cells were harvested by centrifugation at 6000 × g for 20 minutes at 4°C and the pellet was resuspended in chilled buffer P1 (50 mM Tris-HCl, pH 8.0; 10 mM EDTA, 100 µg/ml RNase A).	10 ml Buffer P1	125 ml Buffer P1
III	Buffer P2 (200 mM NaOH; 1% SDS) was added and the cell suspension was mixed thoroughly followed by a 5 minute incubation at room temperature.	10 ml Buffer P2	125 ml Buffer P2
IV	Chilled Buffer P3 (3 M Potassium acetate, pH 5.5) was added to the cell suspension which was then incubated on ice for 30 minutes.	10 ml Buffer P3	125 ml Buffer P3

	To clarify the lysate, the suspension was centrifuged at 20,000 × g for 30 minutes at 4°C.		
V	Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% Isopropanol; 0.15% Triton® X-100) was applied to the column to equilibrate the resin. The clarified lysate was added to the column and allowed to drain by gravity flow.	10 ml Buffer QBT	75 ml Buffer QBT
VI	Two washes were performed using buffer QC (1 M NaCl; 50 mM MOPS, pH 7.0; 15% Isopropanol)	60 ml Buffer QC	600 ml Buffer QC
VII	To elute plasmid DNA bound to the resin, buffer QF (1.6 M NaCl; 50 mM MOPS, pH 7.0; 15% Isopropanol) was dispensed into the column. Isopropanol was added to the eluted solution followed by centrifugation at 15,000 × g for 60 minutes at 4°C.	15 ml Buffer QF 10.5 ml Isopropanol	100 ml Buffer QF 70ml Isopropanol
VIII	The supernatant was decanted and the DNA pellet was washed with 70% ethanol, air-dried and resuspended in a suitable volume of deionised water.	5 ml 70% Ethanol	10 ml 70% Ethanol

A1-2 qPCR vector copy number quantification data

	WPRE Ct	UCR1 Ct	Average WPRE	Average UCR1	dCt	$2^{-\Delta Ct}$	VC
C0			22.92	27.03	-4.11	17.26765	
C0							
C1			22.91	27.98	-5.07	33.59093	
C1							
C3			22.25	27.44	-5.19	36.50444	
C3							
C4			21.69	27.74	-6.05	66.25696	
C4							
C5			21.62	28.75	-7.13	140.0696	
C5							



	x Average WPRE	Average UCR1	dCt	$2^{-\Delta Ct}$	y vector CN
LVTH	21.82	26.85	-5.03	32.67238802	1.7741107
LVTH miR-31/5	22.28	27.32	-5.04	32.89964245	1.7864506
LVTH miR-31/5-8-9	22.47	27.03	-4.56	23.58830748	1.2808451

A1-2 Cell culture

Reagents and Solutions

Gibco TrypLE™ Express Trypsin (Life Technologies Inc., CA, USA)

Gibco Opti-MEM®I (Life Technologies Inc., CA, USA)

Linear 25 kDa PEI (Polysciences, PA, USA)

Saline (Dismed CritiCare, South Africa)

0.4% Trypan blue stain (Lonza, MD, USA)

DMSO (Sigma-Aldrich, Germany)

Saline-EDTA: saline containing 1 mM ethylenediamine tetraacetic acid (EDTA)

Freezing medium (DMEM, 40% FCS and 10% DMSO)

PEI stock solution (1 mg/ml PEI in sterile 150 mM NaCl (pH 7.0)

Maintenance of cultured cells

Cells were incubated at 37°C in a humidified incubator with atmosphere of 5% CO_2. HEK293T, HeLa and Huh7 cells were maintained in complete DMEM (Section 2.1.1.2). HepG2.2.15 cells were maintained in complete William's E culture medium (Section 2.1.1.2). Cells were passaged before reaching confluence by enzymatic dissociation. Briefly, a thorough saline wash was carried out before the cells were incubated in saline-EDTA solution for 5 minutes at 37°C. Following removal of the saline-EDTA solution, an appropriate volume of TrypLE™ Express Trypsin was added and an additional 5 minute incubation step at 37°C was carried out. Cells were detached by gently tapping the culture dish, followed by addition of pre-warmed, complete culture medium. An appropriate volume of the cell suspension was aliquoted into sterile culture dish containing complete growth medium and the cells were returned to the incubator. Typically a 1:10 subculturing ratio was used when passaging HEK293T cells, while Huh7

and HepG2.2.15 cells were passaged at a 1:3 ratio. The growth medium was replenished when a drop in pH was evident until further cell passaging was required. Before seeding cells for an experiment that involved transfection or lentivirus infection, the number of viable cells was determined using trypan blue exclusion and a Neubauer hemacytometer.

Cryopreserving cultured cells and thawing cryopreserved cells

To prepare stocks of cultured cells for freezing, cells were detached as described above and the number of viable cells was determined using trypan blue exclusion and a Neubauer hemacytometer. The cells were centrifuged at 200 × g for 4 minutes and resuspended in the appropriate volume of freezing medium (1×10^7 cells/vial). Finally, the cell suspension was aliquoted in cryogenic vials (Corning Inc., NY, USA). The cell-containing cryogenic vials were slowly frozen at -80°C overnight in a freezing container and transferred to liquid nitrogen for long-term storage.

To thaw cells stocks, cryogenic vials containing frozen cells were taken out of liquid nitrogen storage and immediately placed into a 37°C water bath for less than 5 minutes. The tube was sterilised with 70% ethanol and the cell suspension was added drop wise into a 25 cm^2 tissue culture flask containing 10 ml of pre-warmed complete culture medium appropriate for the respective cell line.

Transfection of cells

Lipofectamine-mediated transfection

The day before transfection was carried out cells were trypsinised and seeded in 24 well culture plates at a density of 80 000 cells/well (Huh7 cells) or 50 000 cell/well (HEK293T). Cells were plated in complete growth medium and allowed to grow overnight at 37°C and 5% CO_2. The growth medium was removed the following day and replaced

with complete culture medium. To prepare a transfection mixture, 1 µg of DNA was diluted into 50 µl of OPTI-MEM and 1 µl of Lipofectamine was diluted into 50 µl of Opti-MEM® for each well of cells to be transfected. The mixes were incubated at room temperature for 5 minutes before the DNA:Opti-MEM® mix was added to the Lipofectamine:Opti-MEM® mix and the final transfection mix was incubated at room temperature for an additional 20 minutes. One hundred microlitres of the final transfection mix was added to each well and the cells were incubated at 37°C in a CO_2 incubator for 5 hours. After 5 hours the growth medium was removed and fresh, complete growth medium was added to the each well.

PEI-mediated transient transfection of HEK293T cells for lentivirus production

Six million HEK293T cells were seeded in a 15 cm^2 culture dish in a final volume of 18 ml of complete DMEM without phenol red on the day before transfection. The following day a plasmid DNA mix containing 30 µg of transfer lentiviral vector, 22 µg of psPAX2 or pCMVΔR8.74 and 15 µg of pMD2.G was prepared and diluted in 1 ml of sterile 150 mM NaCl solution (pH 7.0). One millilitre of PEI stock solution was added to 1 ml of the plasmid DNA mix. The DNA:PEI transfection mixture was briefly vortexed and incubated at room temperature for 10 minutes. Finally, the transfection mixture was added to each 15 cm^2 dish in a circular drop wise manner. The plates were incubated for 12 hours at 37°C in the presence of 5% CO_2. After 12 hours the growth medium was removed and 20 millilitres of fresh, complete growth medium was added to the dish. Transfection mixture volumes and the corresponding amount of plasmid DNA were scaled up accordingly for large scale lentivirus production.

A3 ANIMAL ETHICS CLEARANCE CERTIFICATES

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2013/38/04

APPLICANT: Mr D Ivacik

SCHOOL: Medical School
DEPARTMENT: Molecular Medicine & Haematology
LOCATION: Faculty of Health Sciences

PROJECT TITLE: Assessing the efficiency of lentiviral vectors for delivery of anti-HBV RNA interference based gene therapy to the mouse liver

Number and Species

204 Mice

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20/08/2013. This approval remains valid until 19/08/2015.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None.

Signed: _____ (Chairperson, AESC) Date: 20/8/2013

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 20/08/2013

cc: Supervisor: Prof P Arbuthnot
Director: CAS

Works 2000/lain0015/AESCCert.wps

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Dejana Ivacik

b. Department: Antiviral Gene Therapy Research Unit, Department of Molecular medicine and Haematology

c. Experiment to be modified / extended

	AESC NO	
Original AESC number	**2013/38/04**	
Other M&Es :		X

d. Project Title: Assessing the efficiency of lentiviral vectors for delivery of anti-HBV RNA interference based gene therapy to the mouse liver.

		No.	Species
e.	Number and species of animals originally approved:	205	HBV transgenic mice (72) and NMRI mice (72)
f.	Number of additional animals previously allocated on M&Es:		
g.	Total number of animals allocated to the experiment to date:	20	HBV transgenic mice
h.	Number of animals used to date:	20	HBV transgenic mice

i. Specific modification / extension requested:
Permission to perform tail vein injection to deliver therapeutic recombinant lentiviruses.

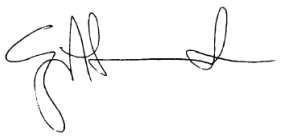
j. Motivation for modification / extension:
To gain a full insight into the efficacy of the antiviral strategy we have developed we have considered expanding our study to include gene transfer to the liver of adult HBV transgenic mice. This application is to request permission to perform tail vein injections (TVIs) in adult HBV transgenic mice to deliver recombinant lentivirus using the animals that have been originally approved for use in the on-going study. No new animals are required for the study. The TVI procedure has been approved in previous AESC protocols and is well established in our hands. We are intending to perform TVIs to reduce the number of neonatal animals that would receive the recombinant viruses via an IV injection into the superficial temporal vein. The TVI procedure is brief, less complex to perform and is likely to cause minimal or no distress and/or harm to the animals. The recombinant lentiviral vectors we are intending to administer have been described in the original ethics application.

Date: 24/9/2013

Signature: 

RECOMMENDATIONS
Approve: tail vein injections as a route of deliver.

Date: 3 October 2013

Signature: 

Chairman, AESC

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