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RECOMBINANT PEGYLATED FIRST AND THIRD GENERATION ADENOVIRUS VECTORS FOR DELIVERY OF ANTI-HEPATITIS B VIRUS RNA INTERFERENCE EFFECTORS

Carol Crowther

A thesis submitted to the faculty of Health Sciences, University of the Witwatersrand, in fulfillment of the requirements for the degree of Doctor of Philosophy

Johannesburg 2013

DECLARATION

I, Carol Crowther 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 was not submitted before for any degree or examination at this or any other University.

.....

3rd day of June, 2013

DEDICATION

"If we knew what we were doing it would not be called research"-Albert Einstein

To my family:

My devoted husband, Nigel

My darling daughters, Emma and Kate

My mother, Blanche and brother, Kevin

PUBLICATION LIST

Journal publications related to this thesis

1. Crowther C, M Mowa, D Palmer, P Ng, A Ely and P Arbuthnot. *Sustained Hepatitis B virus inhibition in vivo using helper-dependent adenovirus vectors to deliver antiviral RNA interference expression cassettes*. Hum Gen Ther (under review, included in this thesis as Chapter 3).

2. Mowa, M, C Crowther, A Ely and P Arbuthnot. *Efficient silencing of Hepatitis B virus by helper-dependent adenovirus vector-mediated delivery of artificial antiviral primary micro RNAs*. MicroRNA, 2012. **1**: p. 1-7. (Printed in full in Appendix A2).

3. Crowther, C, A Ely, J Hornby, S Mufamadi, F Salazar, P Marion and P Arbuthnot. *Efficient inhibition of Hepatitis B virus replication in vivo using PEG-modified adenovirus vectors*. Hum Gene Ther, 2008. **19(**11): p. 1325-31. (Included in this thesis as Chapter 2 and printed in full in Appendix A2).

4. Carmona, S, A Ely, C Crowther, N Moolla, F Salazar, P Marion, N Ferry, M Weinberg and P Arbuthnot. *Effective inhibition of HBV replication in vivo by anti-HBx short hairpin RNAs*. Mol Ther, 2006. **13**(2): p. 411-21. (Printed in full in Appendix A2).

Journal publications indirectly related to thesis

5. Hean, J**,** C Crowther, A Ely, R Islam, S Barachievy, K Bloom, M Weinberg, W van Otterlo, C de Koning, F Salazar, P Marion, E Roesch, M LeMaitre , P Herdewijn and P Arbuthnot. *Inhibition of Hepatitis B virus in vivo replication using lipoplexes containing altrilol-modified antiviral siRNAs.* Artificial DNA: PNA and XNA, 2010. **1**(1): p 17-26.

6. Mevel, M, N Kamaly, S Carmona, M Oliver, M Jorgensen, C Crowther, F Salazar, P Marion, M Fujino, Y Natori, M Thanou, P Arbuthnot, J Yaouanc, P Jaffres and A Miller. *DODAG; a versatile new cationic lipid that mediates efficient delivery of pDNA and siRNA.* J Control Release, 2010. **43**(2): p. 222-32.

7. Carmona, S, M Keller, M Jorgensen, S Kolli, C Crowther, F Salazar, P Marion, M Fujino, Y Natori, M Thanou, P Arbuthnot and A Miller. *Controlling HBV replication in vivo by intravenous administration of triggered PEGylated siRNA-nanoparticles.* Mol Pharm, 2009. **6**(3): p. 706-17.

8. Ely, A, T Naidoo, S Mufamadi, C Crowther and P Arbuthnot. *Expressed anti-HBV primary microRNA shuttles inhibit viral replication efficiently in vitro and in vivo*. Mol Ther, 2008. **16**(6): p. 1105-12.

9. Weinberg, M, A Ely, S Barichievy, C Crowther, S Mufamadi, S Carmona and P Arbuthnot. *Specific inhibition of HBV replication in vitro and in vivo with expressed long hairpin RNA*. Mol Ther, 2007. **15**(3): p. 534-41.

Book chapters

1. Crowther, C, M Mowa, A Ely and P Arbuthnot. *Approaches to delivering RNAi therapeutics that target Hepatitis B virus*. In: Advanced Delivery and Therapeutic Applications of RNAi. Edited by Kun Cheng and Ram Mahato (In press, used as part of Introduction in this thesis and printed in full in Appendix A2).

2. Mowa, M, C Crowther and P Arbuthnot. *Therapeutic potential of adenoviral vectors for delivery of expressed RNAi activators*. Expert Opinion in Drug Delivery, 2010. **7**: p. 1373-1385. (Printed in full in Appendix A2).

ABSTRACT

Hepatitis B virus (HBV) is hyperendemic to southern Africa and parts of Asia where it is a major cause of serious liver disease. Licensed antivirals for chronically infected individuals are only partially effective and approximately one million deaths occur annually as a result of persistent infection with the virus. Although RNA interference (RNAi) based gene silencing of HBV has been successfully demonstrated, difficulties with delivery of anti-HBV RNAi effectors remains an obstacle to their clinical use. Recombinant adenoviruses (Ads), amongst the most efficient hepatotropic gene vectors following systemic administration, have been successfully used to deliver expressed anti-HBV RNAi sequences. However, a drawback of Ad vectors is diminished efficacy and toxicity that results from stimulation of innate and adaptive immunity.

To attenuate these effects we used polyethylene glycol (PEG) to modify first generation recombinant Ad (FG Ad) vectors that express an anti-HBV short hairpin (shRNA) sequence. Efficient hepatocyte transduction occurred and expressed shRNAs were processed to generate intended HBV-targeting guides. Inhibition of HBV replication was achieved after intravenous administration of PEGylated or native recombinant first generation Ads (FG Ads) to HBV transgenic mice. Circulating HBV viral particle equivalents (VPEs) remained low for 3 weeks and began to increase after 5 weeks. A second dose of PEGylated anti-HBV Ad caused a less sustained decrease in circulating VPEs, but no silencing after a second dose was observed in animals treated with unmodified vector. Release of inflammatory cytokines was elevated in animals receiving unmodified vectors and only a modest increase in monocyte chemotactic protein-1 (MCP-1) was observed in mice that received a second dose of PEG

Ads. Also, polymer-conjugated vectors induced a weaker adaptive immune response and were less hepatotoxic than their unmodified counterparts.

To address concerns about the transient nature of transgene expression by FG Ads resulting from immunostimulation, third generation helper-dependent (HD Ad) were utilised to delivered anti-HBV RNAi effectors. Seven days after intravenous administration of infectious HD Ads to HBV transgenic mice, 80-90% of hepatocytes were transduced and markers of HBV replication were decreased by approximately 95% which was sustained for 8 weeks. HD Ad-induced release of proinflammatory cytokines was minimal in preparations that were enriched with infectious particles. PEGylated HD Ad vectors caused similar anti-HBV effects and may be useful to evade interaction with vector-sequestrating receptors and further attenuate immunostimulation. Collectively these observations indicate that PEG modification of Ads and the use of HD Ads may have utility for delivery of therapeutic HBV-silencing sequences. Future work will focus on improving strategies to avoid immune detection and utilisation of HD Ad vectors for other HBV targeting sequences.

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LIST OF ABBREVIATIONS

AAV	-	Adeno-associated virus
Ad	-	Adenovirus
Ago 2	-	argonaute protein 2
ALT	-	alanine transaminase
Apo	-	apolipoprotein
AST	-	aspartate transaminase
bp	-	base pair
BSA	-	bovine serum albumin
CAR	-	coxsackie-adenovirus receptor
CBA	-	cytometric bead array
cccDNA	-	covalently closed circular DNA
CMV	-	cytomegalovirus
CsCl	-	cesium chloride
DAB	-	3,3'-Diaminobenzidine
DGCR8	-	DiGeorge syndrome chromosomal region
dH_20	-	distilled water
DIG	-	digoxigenin
DMEM	-	Dulbecco's Modified Eagle's Medium
DODAG	-	*N',N'*-dioctadecyl-N-4,8-diaza-10-aminodecanoylglycine amide
DOPC	-	dioleoylphosphatidylcholine
DOPE	-	dioleoylphosphatidylethanolamine
dsAAV	-	double-stranded AAV
dsDNA	-	double-stranded DNA

dsRNA	-	double-stranded RNA
eGFP	-	enhanced green fluorescent protein
ELISA	-	enzyme-linked immunosorbent assays
ER	-	endoplasmic reticulum
FBS	-	foetal bovine serum
FG Ad	-	first-generation adenovirus
GAPDH	-	glyceraldehydes-3-phosphate dehydrogenase
HBcAg	-	hepatitis B virus core antigen
HBsAg	-	hepatitis B virus surface antigen
HBV	-	hepatitis B virus
HCC	-	hepatocellular carcinoma
HD	-	high dose
HD Ad	-	helper-dependent adenovirus
HEK 293	-	human embryonic cell line 293
HIV	-	human immunodeficiency virus
HRP	-	horseradish peroxidise
HSPG	-	heparin sulphate proteoglycan
HV	-	helper virus
HVR1	-	hypervariable region 1
IFN	-	interferon
ifu/ml	-	infectious Ad particles per ml
Ig	-	immunoglobulin
IL	-	interleukin
ITR	-	inverted terminal repeat
kb	-	kilobase
kDa	-	kilo Daltons
KPBS	-	potassium-buffered saline

LD	-	low dose
lnRNA	-	long hairpin RNA
LV	-	lentiviral vector
mA	-	milli amps
MAP	-	mitogen activated protein
MAPK	-	mitogen activated protein kinase
MCP-1	-	monocyte chemotactic protein-1
MD	-	medium dose
MHC	-	major histocompatibility complex
miRNA	-	microRNA
moi	-	multiplicity of infection
mPEG-SPA	-	methoxypolyethylene glycol propionic acid N-succinimidyl ester
MW	-	molecular weight
Nab	-	neutralizing antibody
nm	-	nanometre
nt	-	nucleotide
NVV	-	non-viral vector
OD	-	optical density
ORF	-	open reading frame
OTC	-	ornithine transcarbamylase
PE	-	phycoerythrin
PEG	-	polyethylene glycol
pgRNA	-	pregenomic RNA
Pol	-	RNA polymerase
pre-miRNA	-	precursor miRNA
pri-miRNA	-	primary miRNA

PTGS	-	post transcriptional gene silencing
Q-PCR	-	quantitative polymerase chain reaction
RBC	-	red blood cells
RCA	-	replication–competent adenovirus
rcDNA	-	relaxed circular DNA
RISC	-	RNA induced silencing complex
RNAi	-	RNA interference
RPMI	-	Roswell Park Memorial Institue medium
SAPK/JNK	-	stress-activated protein kinase/ Jun N-terminal kinase
scAAV	-	self-complementary AAV
SEM	-	standard error of the mean
shRNA	-	short hairpin RNA
siRNA	-	small interfering RNA
SNALPS	-	stable nucleic acid-lipid particles
SREBP-1c	-	sterol regulatory element-binding protein-1c
TALEN	-	transcription activator-like effector nuclease
TBE	-	Tris-Borate EDTA
TMB	-	tetramethylbenzidine
TLR	-	toll-like receptor
TNF–α	–	tumour necrosis factor-α
tRNA	-	transfer RNA
TRBP	-	HIV transactivating response RNA-binding protein
vp	-	viral particles
VPE	-	viral particle equivalent
VV	-	viral vector
WHO	-	world health organization
X-gal	-	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

ZFP	-	zinc finger protein

LIST OF SYMBOLS

α	-	alpha
β	-	beta
Δ	-	delta
ε	-	epsilon
γ	-	gamma
Ψ	-	psi

1 INTRODUCTION

1.1 Hepatitis B virus

1.1.1 HBV prevalence

According to the World Health Organisation (WHO) 2 billion people have been infected by Hepatitis B virus (HBV) and there are an estimated 387 million chronic carriers of the virus world-wide (reviewed in [1]). Carrier rate varies in different regions of the world and is classified as low (0.1-2%), intermediate (2-8%) or high (8-20%) (Figure 1.1). HBV is highly prevalent in sub-Saharan Africa, east and south east Asia and the western Pacific islands where most infections occur perinatally or in early childhood [2]. The age at which HBV is acquired has a major impact on the development of chronic HBV [3]. Approximately 90% of infected infants or young children will remain chronically infected as compared to only 5% of patients infected as adults. [4]. A patient is chronically infected if they have persistence of HBsAg in their serum for six months or longer. There are ten known HBV genotypes (A-J) with particular genotypes being more common in certain geographical areas [5, 6]. It has been approximately 30 years since the first immunization programmes were implemented which has resulted in a progressive decrease in the number of new cases, yet vaccination has no benefit to already infected individuals. Persistently infected individuals have an increased risk of developing cirrhosis and hepatocellular carcinoma (HCC) and more than one million people are estimated to die annually as a result of HBV associated liver disease [7-9].

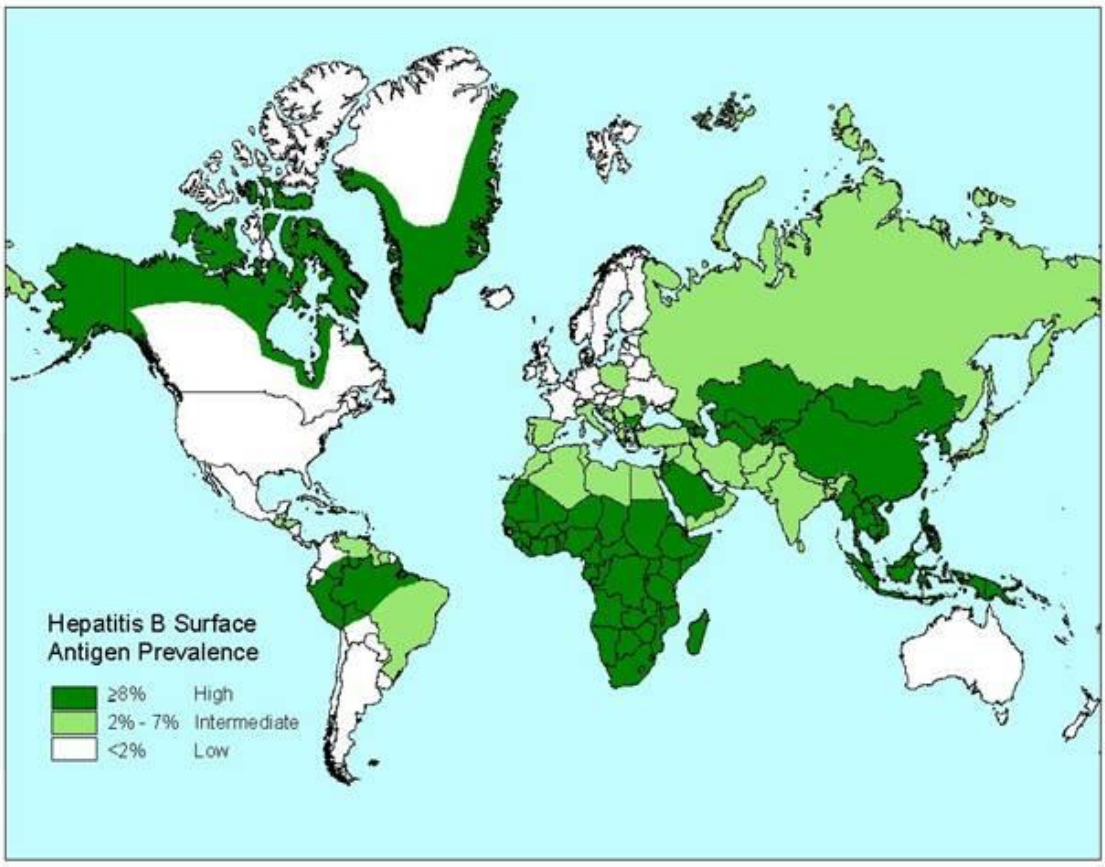


Figure 1.1: Global prevalence of chronic HBV infection. Map showing the prevalence of chronic HBV infection in different regions of the world. HBV is highly prevalent in sub-Saharan Africa, east and south east Asia and the western Pacific islands. There is a high prevalence in the far northern regions of Northern America but the numbers of carriers are low in this part of the world. http://www.stranorlarhealthcentre.com/hepatitis.htm

1.1.2 HBV genome structure

HBV belongs to the Hepadnaviridae family of hepatotropic DNA viruses and has a circular, partially double-stranded DNA genome of approximately 3.2 kb in length (Figure 1.2) [10, 11]. The infectious Hepatitis B virion, also known as the Dane particle, is a 42-44 nm spherical double shelled structure. The nucleocapsid is made up of core protein (HBcAg) which is enveloped by the surface proteins pre-S1, pre-S2 and hepatitis B surface antigen (HBsAg) embedded in a lipid bilayer [3]. The encapsidated relaxed circular genome (rcDNA) maintains its circularity by 5′ cohesive ends and is compactly organised with 4 overlapping reading frames (ORFs) [12, 13]. The ORFs encode seven different viral proteins: the 3 viral surface glycoproteins (pre-S1, pre-S2 and HBsAg) are encoded for by preS/S ORF; core and the non-structural Pre C protein (secreted e-antigen, HBeAg) are encoded for by the precore/core region; the multifunctional viral polymerase is encoded for by the pol ORF and the X protein by the X ORF [14].

1.1.3 HBV replication

HBV is distinctly liver tropic and viral replication occurs within polarised hepatocytes [15]. The specificity HBV has for the liver can partially be explained by hepatocyte-specific transcription factors [16]. HB virions bind to a still unknown receptor on hepatocytes and following endocytosis, the rcDNA is translocated to the nucleus where gaps in the DNA are repaired by second strand DNA synthesis to yield cccDNA (Figure 1.3) [13]. The cccDNA serves as the template for the four viral RNAs and the greater than genome length pregenomic RNA (pgRNA) produced during infection. There are also four promoter and two enhancer transcription control elements within the cccDNA (Figure 1.2) which direct cellular RNA polymerase II (pol II) to synthesise *pregenomic/preC*, *preS2*, *PreS1* and *X* mRNA.

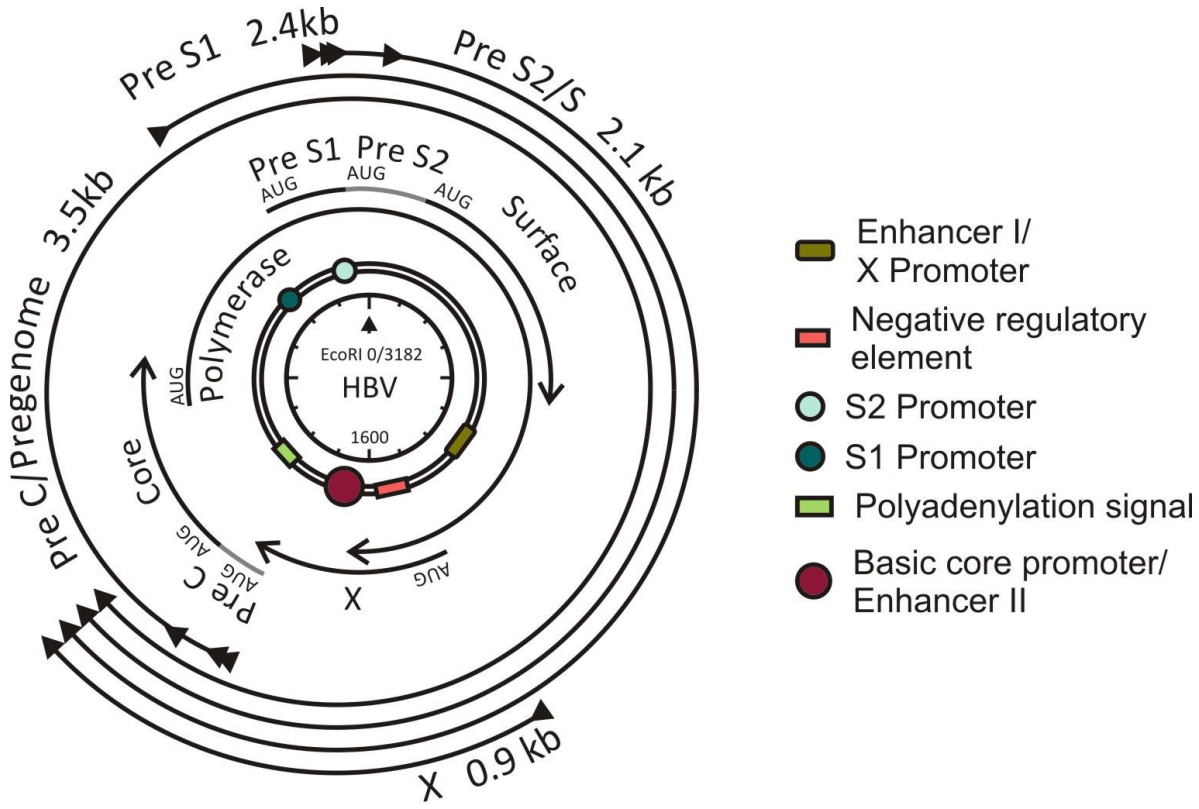


Figure 1.2: HBV genome organisation. Partially double-stranded HBV DNA consists of plus and minus strands with cohesive complementary 5′ ends. The circular and rectangular symbols represent the *cis*-acting regulatory elements that control transcription. Co-ordinates of the viral genome are given relative to the unique EcoRI restriction site. The immediately surrounding arrows indicate the *surface*, *core*, *polymerase* and *HBx* viral open reading frames (ORFs) (with initiation codons), encompassing the entire HBV genome. The four outer arrows indicate the major viral transcripts which have a common 3′ polyadenylation site.

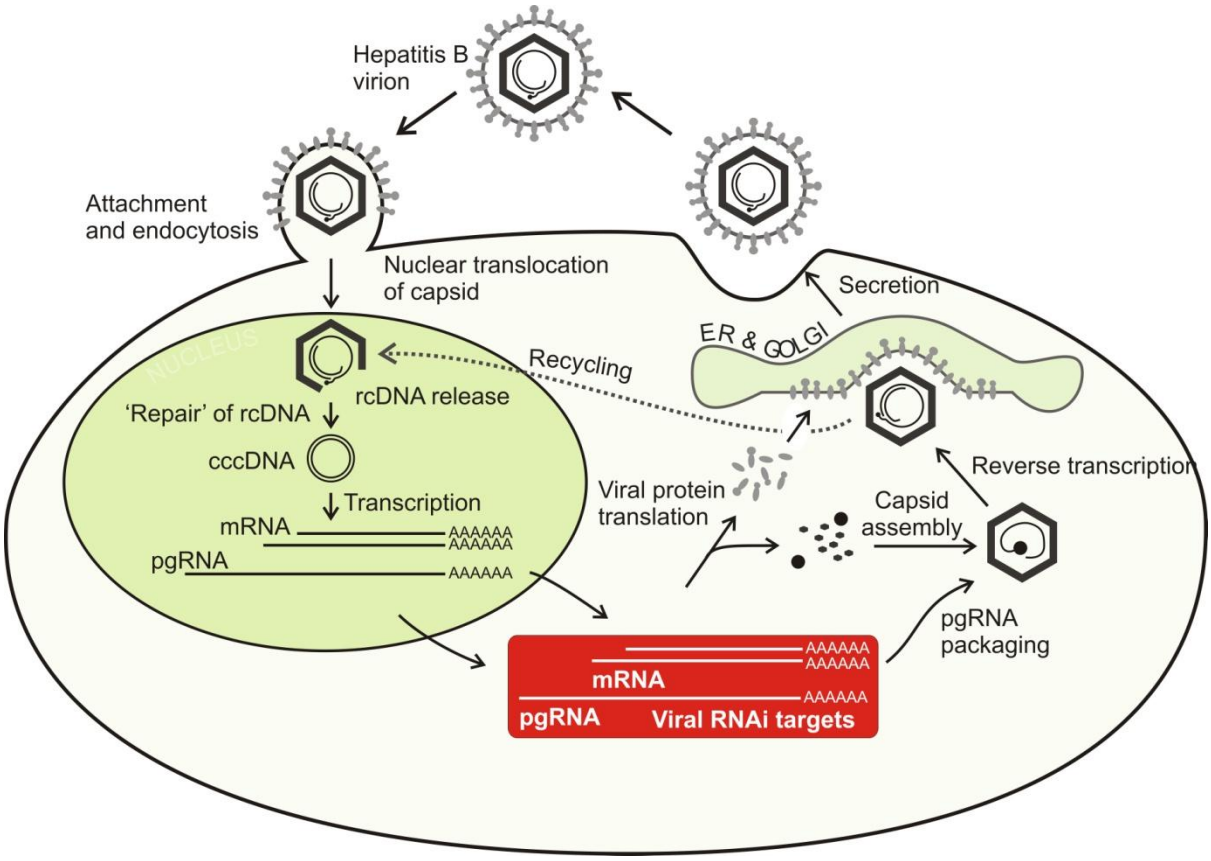


Figure 1.3: HBV replication cycle. Following attachment, endocytosis and nuclear release of the HB virion, rcDNA is repaired to form cccDNA. The cccDNA acts as a template for transcription of mRNA and pgRNA. Messenger RNA that codes for proteins and pgRNA which forms rcDNA are both potential RNA interference targets and are highlighted in red. The viral polymerase and pgRNA are packaged into capsid particles that comprise core protein. Within the capsid, pgRNA is reverse transcribed to form rcDNA which is either transported back into the nucleus and recycled to form more cccDNA, or incorporated into nascent virions which are secreted from the cell via the endoplasmic reticulum and Golgi apparatus. cccDNA, covalently closed circular DNA; rcDNA, relaxed circular DNA; pgRNA, pregenomic RNA; ER, endoplasmic reticulum.

Polyadenylated transcripts are then transported into the cytoplasm where they are translated into the seven viral proteins which are essential for virion assembly and replication. pgRNA has repeat sequences at the 5′ and 3′ ends that enables circularisation of reverse transcribed minus strand DNA which is followed by synthesis of the plus strand of rcDNA. The envelopment of HBV capsids occurs in the endoplasmic reticulum after which the virions are secreted and re-infect other hepatocytes. The stable circularised pgRNA and mRNA encoding the viral proteins are necessary for viral replication, making them an ideal therapeutic target and they are amenable to disruption by employing a RNA interference (RNAi) approach [17, 18].

1.1.4 Current treatment regimens for HBV

Effective anti-HBV therapy should achieve long term suppression of HBV replication and thereby avert the possible development of cirrhosis and HCC. An effective therapeutic response includes sustained suppression of serum HBV DNA levels, seroconversion of HBeAg to HBe antibody, loss of HBsAg and normalisation of liver pathology (reviewed in [18, 19]). Conventional treatments for chronic HBV infection include interferon-α (IFN−α), which functions as an immunomodulator, and nucleoside or nucleotide analogues (lamivudine, entecovir, adefovir and tenofovir) which inhibit HBV genome replication [20]. There is a variable response to treatment that has been attributed to different HBV genotypes (reviewed in [5, 6]).

1.1.4.1 Interferons

The development of chronic HBV has partially been attributed to a weak immune response to the virus [21]. Interferon-α has been used for treating HBV positive patients since the early

1980s as it has an immunomodulatory effect in addition to an antiviral effect. Interferon stimulates cytotoxic T cells and antibody secreting B cells which destroy HBV positive cells [20]. Presently there are two types of IFN–α approved for treating chronic HBV: IFN-α-2b and polyethylene glycol modified (PEGylated) IFN α-2a (PEG IFN α-2a) [22, 23]. The PEGylated form has a longer half-life, thus requiring lower doses to decrease the negative side effects of the drug. The more common side effects include flu-like symptoms, decreased white cell counts (leucopenia) and depression. Other drawbacks of this therapy are the necessity to administer IFN–α by subcutaneous injection, high cost, patient variable response to the treatment and the lack of suitability in patients with decompensated liver cirrhosis (reviewed in [8]).

1.1.4.2 Nucleotide or nucleoside analogues

Nucleotide and nucleoside analogues effectively suppress HBV replication without the side effects associated with IFN-α [24, 25]. They undergo phosphorylation in the cell whereby their structure changes and mimics naturally occurring nucleosides. During DNA synthesis these nucleoside analogues are incorporated into new DNA strands which results in termination of chain elongation and inhibition of DNA synthesis. Some nucleoside analogues competitively inhibit the reverse transcriptase activity of HBV polymerase (reviewed in [5]). The emergence of drug resistance to nucleotide and nucleoside analogues is a major concern and is commonly seen in lamivudine treated patients [24]. There are however some new generation drugs (entecavir and tenofovir) that hold promise as they do not drive the development of drug resistant HB virions [26].

It is apparent that the available repertoire of drugs for treating HBV has only limited success with none of the therapies targeting viral pgDNA or cccDNA. There is a need to develop alternative therapeutic strategies to inhibit HBV. In recent years there has been some notable success in preclinical trials employing RNAi strategies to target HBV which may prove to be a viable option for the management of chronic HBV infection.

1.2 RNA interference

The mechanism of RNAi was first described in 1998 by Fire and Mellow [27] which had such a profound impact in the field of gene regulation that they received the Nobel Prize for Physiology or Medicine in 2006. The Nobel citation stated they had "discovered a fundamental mechanism for controlling the flow of genetic information". RNAi is an evolutionary conserved biological process and is involved in a variety of essential cellular processes, including cell proliferation, apoptosis, and defense against viral infections [27-29]. The process involves the use of naturally occurring small double-stranded RNA molecules which direct homology-dependent genetic control of an organism. The pathway is activated by double-stranded RNA (dsRNA) molecules, which are processed to form small, 21–23 nucleotide (nt) guide strands that effect target silencing by pairing with complementary RNA sequences. Although transcriptional gene silencing has also been described, post transcriptional gene silencing (PTGS) is the best characterised RNAi-mediated gene inhibitory mechanism.

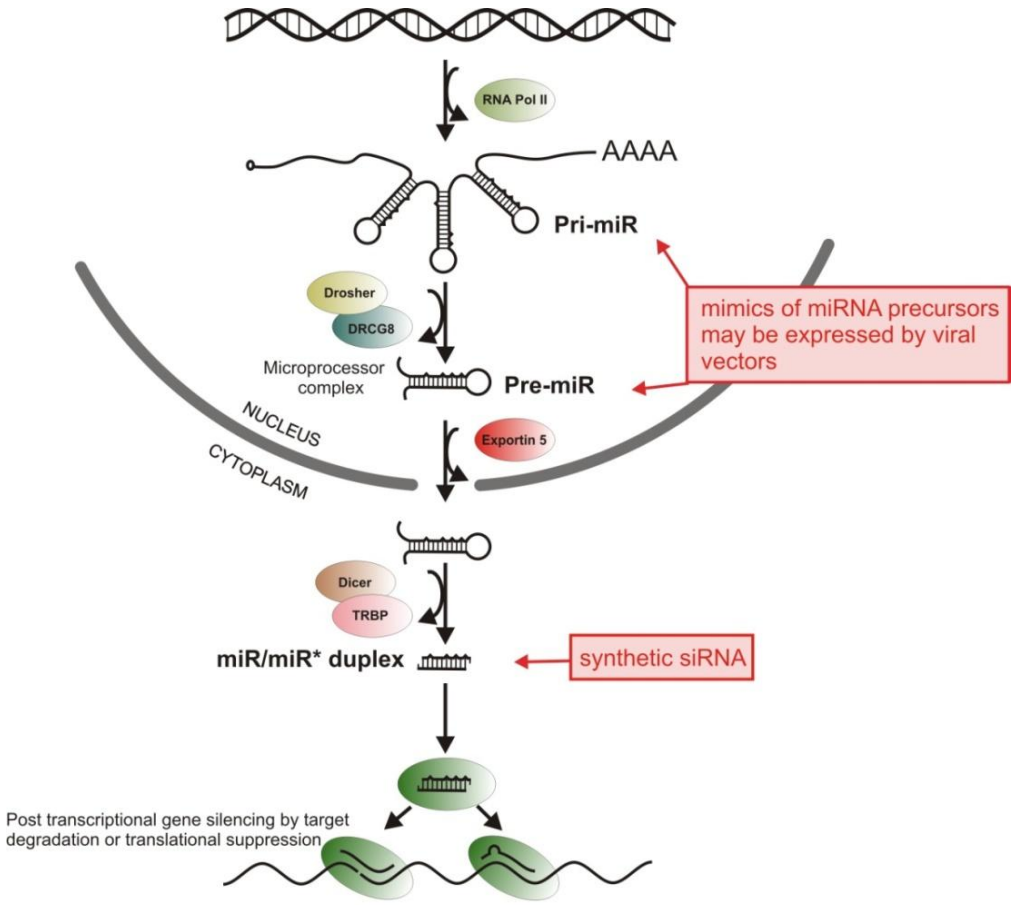


Figure 1.4: The RNA interference pathway. The pathway begins with an endogenously encoded primary microRNA transcript (Pri-miR) that is transcribed by RNA polymerase II (Pol II) which is then processed by Drosher/DGCR8 enzyme complex to yield precursor miRNA (Pre-miR). These precursors are exported into the cytoplasm by exportin 5 (Exp 5) and subsequently bind to the Dicer enzyme complex, which processes the pre-miR into a double-stranded RNA (dsRNA) for loading on the RISC complex. Within RISC the passenger (sense) strand is unwound leaving a mature miRNA which recognizes target sites in the 3′-UTR in the mRNA which mostly leads translational suppression. Exogenously added dsRNAs such as synthetic siRNAs lead to target degradation or translation suppression. Exogenous expression cassettes that transcribe miR intermediates, which may be pri-miR or pre-miR sequences, are typically incorporated into viral vectors. Non-viral vectors are normally used to deliver siRNA analogues of mature miR duplexes.

1.2.1 RNAi regulatory pathway

The mammalian endogenous microRNA (miRNA) pathway is well characterised and illustrated in Figure 1.4. The mechanism initially involves nuclear transcription of mono or polycistronic primary miRNAs (pri-miRNAs) by RNA polymerase II. These sequences, which contain hairpin-like RNA motifs, are then cleaved by the RNase III enzyme Drosha and its partner, DiGeorge critical region 8 (DGCR8), to yield precursor miRNAs (pre-miRNAs). The pre-miRNAs are transported to the cytoplasm for further processing by Dicer, another RNase III enzyme, to yield a mature duplex miRNA of 21-23 base pairs (bp). One strand from the miRNA duplex serves as a guide and is incorporated into the RNA inducing silencing complex (RISC) [30-33]. When the guide and target are completely complementary, Argonaute protein 2 (Ago2) within RISC inactivates the target RNA by cleaving it. When siRNA and target sequences are partially complementary, particularly at the 5′ end, translational suppression is induced without target cleavage. Complimentarity between the guide's seed region (nucleotides 2 to 8 from the 5′ end), is all that is required to effect translational suppression.

1.2.2 RNAi as a therapeutic strategy

There is compelling evidence that exogenous activators can be used to exploit the endogenous RNAi machinery and achieve specific and potent silencing of genes of interest. The mechanism provides the means of studying gene function and has potential application to silencing of pathology-causing genes. The RNAi pathway may be activated by synthetic or expressed exogenous RNAi activators. Chemically synthesized short interfering RNAs (siRNAs) typically resemble mature endogenous miRNAs, and DNA expression templates encode artificial mimics of upstream miRNA intermediates of the RNAi pathway. These

exogenous sequences reprogram the RNAi pathway to silence intended gene targets. The negative charge, sensitivity to nucleases, hydrophilicity and immunostimulatory effects of siRNAs has led to investigating use of chemical modifications to confer better therapeutic properties. These chemical modifications have aimed at improving delivery efficiency, reducing clearance, increasing stability, enhancing target specificity and minimising immunostimulatory effects [34-36]. Synthetic siRNAs have a smaller size than RNAi-activating expression cassettes. This feature, together with the cytoplasmic site of action, make siRNA delivery and dose control easier to achieve than with expressed RNAi activators.

The more sustained silencing that may be achieved with expression cassettes is useful for the treatment of chronic infection caused by HBV [37, 38]. These cassettes may be propagated conveniently using standard techniques of molecular biology and have been successfully used in our laboratory to target HBV. They are stable and compatible with incorporation into highly efficient viral vectors (VVs). Expressed short hairpin RNAs (shRNAs), which mimic pre-miRNAs, have been widely used to activate the RNAi pathway. Typically, their expression has been placed under control of RNA polymerase (Pol) III promoters and the powerful and constitutively active U6 small nuclear RNA promoter has been commonly used [39]. Although effective silencing is achieved, saturation of the endogenous RNAi pathway may occur which can lead to fatal toxicity [37]. To overcome these problems and improve transcriptional control of RNAi activators, pri-miRNA mimics that are compatible with expression from Pol II promoters have been developed [40-47]. These artificial expression cassettes are amenable to multimerisation to simulate natural polycistronic miRNAs. Using such a combinatorial RNAi approach enables simultaneous targeting of various regions of a viral sequence. This is a useful strategy to improve silencing efficacy and prevent viral

escape [40, 41, 46, 48]. Production of multiple antiviral siRNAs from long hairpin RNAs (lhRNAs) has also been described, but efficiency with which the individual siRNAs are generated from these templates is variable [49-51].

1.2.3 Hepatitis B virus as a target for RNAi-based gene silencing

Although evidence exists that viruses have evolved mechanisms to evade cellular RNAi silencing mechanisms [52], HBV-encoded factors that are capable of suppressing RNAi have not been described. In support of this, several investigations carried out *in vitro* and *in vivo* demonstrated that the virus is indeed susceptible to RNAi-based inhibition of replication [40, 53-57]. Synthetic and expressed RNAi activators have been used successfully to target different sites of the viral genome and features of the expressed and synthetic RNAi activators that have been used to counter HBV replication are described in Figure 1.5. In addition to typical Pol III expression cassettes, Pol II artificial mono- and polycistronic anti-HBV pri-miRNAs have been used successfully to knockdown HBV replication [40, 41, 57]. Whilst it is important to demonstrate the RNAi effects *in vitro*, it is success *in vivo* which remains critical, and the ultimate goal to successful gene therapy is the choice of a suitable vector for target delivery.

1.3 Suitable vectors for delivery of HBV gene silencers

Since HBV replicates and resides in the liver, efficient delivery of RNAi activators to hepatocytes is critically important to have therapeutic utility. This is challenging as vectors should ideally take antiviral sequences to their intended sites of action within hepatocytes after systemic administration. Optimally, only a single administration should be needed to achieve sustained inhibition of viral replication. If this is not possible vectors should then be

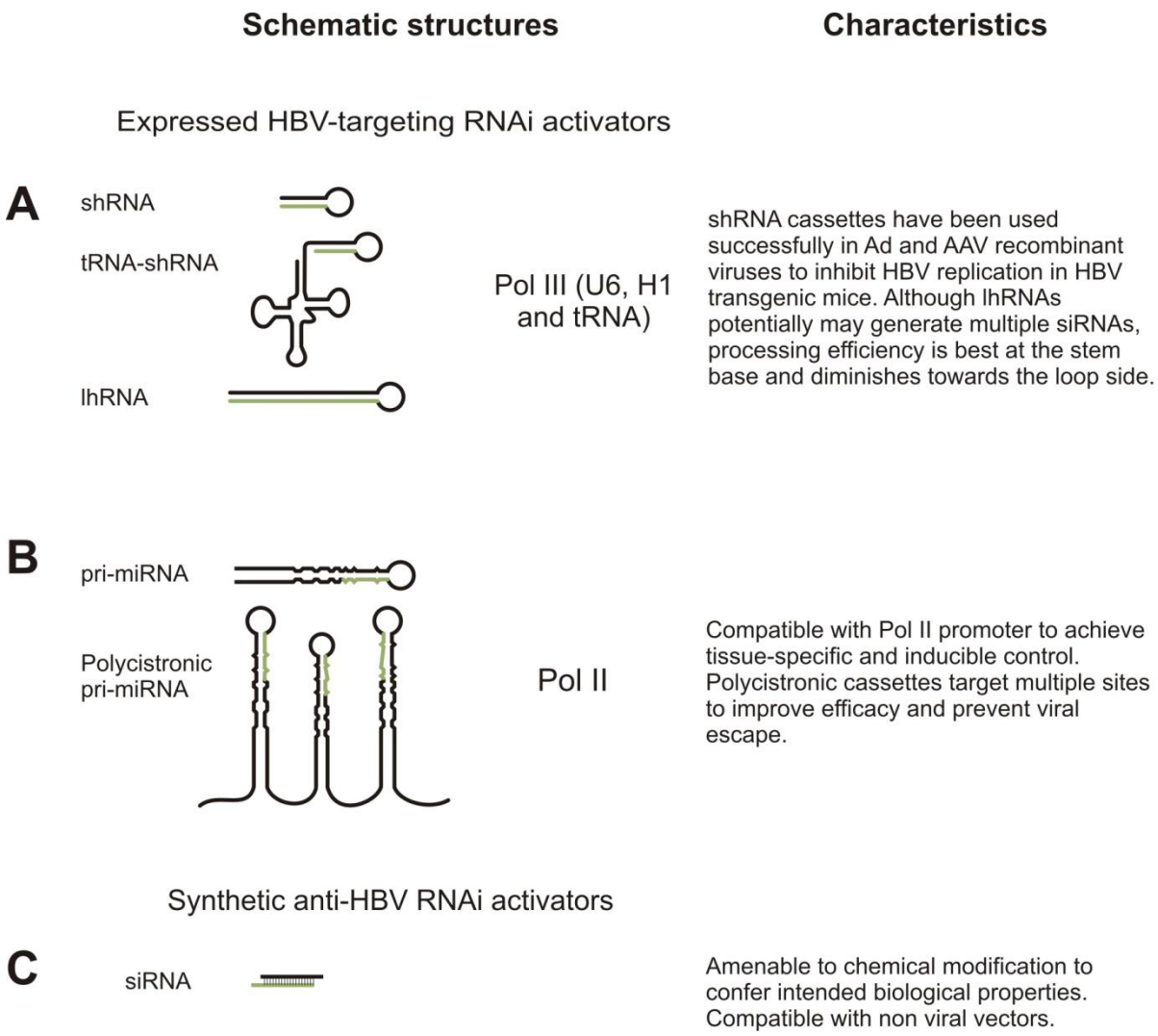


Figure 1.5: Types of RNAi activators that have been used to silence HBV replication. **A** and **B**. Expressed sequences, generated from either Pol III or Pol II promoters, are compatible with incorporation into viral vectors. C. Synthetic anti-HBV siRNAs are used in non-viral vector formulations. Characteristics of the different types of RNAi activator are briefly summarized. For each type of RNAi activator, the mature guide is illustrated in green.

amenable to re-administration and retain efficiency of inhibition of HBV replication. To gain access to hepatocytes, vectors need to traverse the endothelial fenestrated barrier between the blood and hepatocytes. Viral vectors (VVs) and non-viral vectors (NVVs) should therefore ideally have a uniform size of approximately 100 nm to enable them to cross the fenestrated barrier and come into contact with hepatocytes (reviewed in [41]). Sinusoidal endothelial cells range from 100-200 nm in size depending on the species, with the fenestrae in a healthy human liver measuring ±107 nm [58, 59]. NVVs have commonly been used to deliver synthetic siRNAs, and recombinant VVs engineered to deliver artificial RNAi expression cassettes. Characteristics of the types of vectors that have been used to silence HBV replication are summarised in Table 1.1.

1.3.1 Non-viral vectors

As gene delivery vehicles, NVVs offer a number of advantages over VVs. These include low immunogenicity, ability to accommodate large nucleic acids, modular assembly and the potential for large scale synthesis which is important for clinical application. Although NVVs can be complexed with both DNA and RNA, these delivery vehicles have primarily been used *in vivo* to deliver siRNAs to target cells. An important reason for this derives from the fact that delivery of siRNAs to their site of action (the cytoplasm) faces fewer hurdles than delivery of RNAi expression cassettes that comprise DNA (nuclear action). Nevertheless, delivery of siRNAs to the cytoplasm of target cells in sufficient quantities to have a desirable effect remains challenging.

A number of NVVs have been used successfully to deliver siRNA constructs targeting HBV [41]. These include cationic lipid-containing nucleic acid complexes (lipoplexes) and polymer-modified nucleic acid complexes (polyplexes) [60, 61]. Several

Table 1.1: Advantages, disadvantages and significant articles describing vectors used for delivery of nucleic acids to the liver.

	Ads	AAVs	Lentiviruses	NVVs
Advantages	Hepatotropic Efficient transduction of RNAi-activating RNA sequences Sustained therapeutic effect using HD Ads	Serotype-dependent liver tropism Efficient transduction of RNAi-activating RNA sequences Limited toxicity Sustained therapeutic effect for up to 8 weeks	Broad cell tropism Efficient transduction of RNAi-activating RNA sequences Limited immunogenicity Stable integration and long term therapeutic effect offset need for repeat administration	Targeting moieties facilitate liver tropism (e.g. galactose) Amenable to chemical modification to confer biological properties Feasible scale up of synthesis Suitable for delivery of synthetic RNAi sequences Repeat administration possible
Disadvantages	Large scale production is costly and labor intensive Immunostimulatory and potentially toxic Pre-existing immunity attenuates vector efficacy Repeat administration may be necessary	Labor intensive large scale production Immunostimulation limits repeat admninistration Repeated administration requires serotype switching	Labor intensive large scale production Poor transduction of liver cells following systemic administration Low hepatotropism	Poor delivery of expressed RNAi activators in vivo Variable immunostimulation Transient therapeutic effect
Significant publications relevant to HBV therapeutics	**Uprichard et al (2005)** Use of Ad to target HBV in transgenic mouse model **Carmona et al (2006)** Use of Ad to target HBV in transgenic mouse model **Crowther et al (2008)** Polymer modification to improve anti-HBV efficacy of Ads **Rauschhuber et al (2008)** Use of HD Ad to target HBV in transgenic mouse model	**Grimm et al (2006)** Utility of scAAV-8 vectors for targeting HBV in transgenic mouse model **McCaffrey et al (2008)** Highlighted the safety of AAVs for gene therapy **Chen et al (2009)** Repeated administration of AAVs possible with serotype switching	**Parks et al (2000)** Successful liver transduction of LVs using partial hepatectomy **Pichard et al (2011)** Pharmacological priming of hepatocytes to improve LV-mediated transduction	**Morrissey et al (2005)** Liposome delivery of siRNAs to HBV mouse model **Zimmerman et al (2006)** Using SNALPs for hepatotropic nucleic acid delivery in non-human promates

Footnote: Ads, adenoviruses; AAVs, adeno-associated viruses; NVVs, non-viral vectors.

other novel non-viral delivery strategies have been developed specifically for siRNA delivery but not utilised in targeting HBV. They include carbon nanotubes [62], lipidoids [63], membrane translocation peptides [64], universal base derivatives [65] and modified arginine peptides [66].

Cationic liposomes have the ability to neutralise the negatively charged siRNA nucleic acid sequences to facilitate transport across negatively charged lipid-rich membranes. These liposomes also condense the nucleic acids so that they are ideally contained in uniformly sized, small particles (presently still technically challenging) which can pass through liver fenestrations making these vectors suitable for *in vivo* application [67]. An important development in advancing lipoplex vectors was the incorporation of neutral lipids such as DOPE (dioleoylphosphatidylethanolamine) and DOPC (dioleoylphosphatidylcholine) into the formulations. DOPE improves lipofection by aiding release of NVVs from endosomes [68]. The modular way in which liposome formulations may be assembled has enabled evaluation of many combinations of cationic lipids, neutral lipids, targeting and 'stealth' components. This has been particularly useful to adjust the composition of lipoplexes to influence biological properties. Efficient liposome-mediated delivery of siRNAs in a mouse model of HBV replication achieved potent silencing of viral gene expression for 7 days, which could be maintained with repeated administrations [69]. The vectors used in this study, termed stable nucleic acid lipid nanoparticles or SNALPs, have since been used for other hepatic gene silencing applications [70, 71].

Cationic polymers comprise the second major class of NVVs. This group of compounds, as with cationic lipids, binds nucleic acids to neutralize negative charges through the formation of polyplexes. Condensation of nucleic acids enables generation of highly compact

nanoparticles, which may be taken up by cells through endocytosis (reviewed in [60]). Bioconjugation of the 5′ or 3′ end of the sense or antisense strands of siRNAs with lipids, proteins, peptides and inorganic molecules has also been explored as a means of targeted delivery (reviewed in [72]). More recently Zhu and Mahato successfully conjugated galactose-bound PEG (Gal-PEG) to the 3′ end of the sense strand of siRNAs and demonstrated silencing of target sequences in hepatocytes [73]. The silencing achieved with the siRNAs conjugated to Gal-PEG was however improved when encapsulated within a cationic liposome. Further characterisation of the siRNA conjugates *in vitro* and *in vivo* should provide insights into the therapeutic utility of the technology.

1.3.1.1 Using NVVs to deliver anti-HBV siRNAs

Generally, vectors carry their payloads to hepatocytes passively or by a receptor-mediated process. Examples of receptor and target pairings include interaction of the hepatocyte asialogycoprotein receptor [74] with galactose-containing NVVs, and binding of apolipoprotein A-1 (Apo A-1) [75] in NVVs with the hepatocyte high density lipoprotein (HDL) receptor. Hepatocytes exclusively and abundantly express the asialoglycoprotein receptor which interacts specifically to galactose moieties [74]. This fact has often been exploited to direct NVVs to the liver [76]. A novel galactose-modified DOPE derivative (1,2-dioleoyl-*sn*-glycerol-3-phosphatidyl-*N*-(1-deoxylactito-1-yl)etanolamine or GDOPE) has been used to prepare liposome formulations that achieve improved hepatocyte delivery of siRNAs [77].

Apo A-1 interaction with HDL receptors on liver cells has also been exploited to confer hepatotropism on siRNA-carrying lipoplex formulations [75]. Apo A-1 is a component of HDL and consequently is involved in the hepatocyte uptake of cholesteryl esters. Apo A-1-

conjugated liposomes were capable of delivering anti-HBV siRNAs to the livers of mice in a transient HBV replication model. Subsequent studies assessed efficacy of improved Apo A-1 conjugated liposomes carrying siRNAs targeting the hepatitis C virus [78]. These NVVs demonstrated better liver-specific targeting *in vivo*, more efficient target knockdown and minimal toxicity [78]. A novel cationic lipid DODAG (*N',N'*-dioctadecyl-N-4,8-diaza-10-aminodecanoylglycine amide) was recently shown to encapsulate anti-HBV siRNAs and mediate efficient hepatocyte delivery in a mouse model of virus replication [79]. DODAG-siRNAs, formulated without a neutral helper lipid, efficiently knocked down viral DNA and antigen markers of replication. Other lipoplex formulations have also been employed to deliver anti-HBV siRNAs *in vivo* [80-82].

Despite the need for repeated administrations of NVV-mediated delivery of siRNAs in chronic HBV, NVVs in general show potential as delivery vehicles of anti-HBV siRNAs. The field is developing rapidly and with positive data from clinical trials providing impetus (reviewed in [83]), NVVs are quickly gaining prominence as hepatotropic delivery vehicles for therapeutic siRNA sequences.

1.3.1.2 Off-target effects

Induction of an innate immune response by NVVs may reduce the duration of siRNA silencing [84]. Studies that comprehensively characterise potential toxic side effects of many of the reported NVVs are incomplete. Administration of dexamethasone prior to a lipoplex can effectively attenuate the innate immune response but does not have a significant effect on silencing activity of the siRNA [85]. This drug has similar utility for the reduction of immunostimulation by hepatotropic Adenoviral vectors (Ads) (see Section 1.3.2.3.8). Potential toxic side effects may also arise as a consequence of unintended NVV-mediated

delivery of siRNAs to untargeted cells and in general, there is a paucity of comprehensive analysis of the biodistribution of siRNAs delivered with NVVs [86-88]. Similarly, there is little information on the subcellular localisation of siRNAs after NVV-mediated delivery to target cells [88, 89].

1.3.2 Viral vectors

Adenoviruses and adeno-associated viruses (AAVs) are both capable of effective hepatocyte transduction and are able to achieve long term transgene expression in the liver. Lentiviral vectors (LVs) transduce hepatocytes stably, but efficiency of transgene delivery to these cells following systemic administration in adult animals is generally inadequate for treating chronic HBV infection. Nevertheless, LVs have potential therapeutic utility using *ex vivo* approaches.

1.3.2.1 Adeno-associated viral vectors

AAVs are non-enveloped viruses that belong to the *Parvoviridae* family. They are small (± 20 nm) and have a single-stranded DNA genome of 4.8 kilobases (kb). Recombinant AAVs can carry an insert of up to 4.6 kb which is adequate for accommodating typical RNAi expression cassettes [90]. An important advance in AAV vector design was the development of second generation double stranded or self-complementary AAV vectors (scAAVs). Transgene expression from these vectors is more efficient and allows for administration of lower vector doses [91]. There are 81 clinical trials in progress that utilize AAVs (http://www.abedia.com/wiley/vectors.php). These vectors are suitable for use in humans because they are non pathogenic, do not replicate without Ad co-infection, have low immunogenicity and high titres of the vectors may be produced conveniently [92]. Although

AAV safety is an advantage, a recent study showed that AAVs may cause liver inflammation by activating a Toll-like receptor-2 (TLR-2) mediated responses in hepatocytes [93]. An additional concern is that there is a high prevalence of neutralising antibodies (NAbs) to AAV-2 in human populations [94]. Some of the NAbs also cross-react with other AAV serotypes, which may limit their use as vectors in a clinical setting. Recombinant AAVs lack the viral Rep protein, which restricts integration into the host genome, and contributes further to vector safety [95]. The first AAV gene therapy vectors were based on AAV-2, which is capable of transducing many different cell types [96]. There are currently more than 100 known AAV serotypes and it is possible to package the AAV-2 genome with the capsid of any of these serotypes (pseudotyping). This feature is useful to change vector tropism and evade host immune responses. AAV-8 and AAV-9 have a high affinity for hepatocytes (3-4 times higher than AAV-2) and have consequently been used for hepatotropic delivery of RNAi effectors targeting HBV [97, 98]. Recently, DNA shuffling has been employed to generate libraries with variations in the exposed loops of AAV capsid proteins [99]. Subsequent positive or negative selection enables purification of vectors with defined properties, such as specific tissue tropism and attenuated NAb interaction. This method is likely to be very useful for future application in AAV vectorology.

Grimm *et al* were the first to demonstrate the utility of scAAV-8 vectors for RNAi-mediated HBV silencing [37]. However, although HBV replication was efficiently inhibited in the transgenic mice, there was an associated high mortality. This prompted subsequent studies which established that high concentrations of exogenous shRNAs compete with the natural miRNA machinery to prevent processing of essential endogenous miRNAs. Compromised function of hepatocyte miRNAs resulted in the death of the mice. Other studies have subsequently employed AAVs to deliver HBV-targeting RNAi expression cassettes. A

dsAAV-2/8 vector, a dsAAV-2 genome pseudotyped with an AAV-8 capsid, was successfully used to inhibit HBV replication in a transgenic mouse model [100, 101]. Significant HBV inhibition was maintained for 22 weeks. A reduction in appearance of complicating liver adenomas in HBV transgenic mice was also demonstrated after AAV delivery of anti-HBV expression cassettes [102, 103]. To overcome problems of neutralising antibodies to the AAV-8 capsid, a vector expressing the same anti-HBV sequence, but pseudotyped with AAV-9, was then administered. This dsAAV-2/9 vector silenced HBV effectively and evaded the AAV-8 NAbs. Thus, for successful repeated administration of anti-HBV AAVs, the serotype of the capsid protein may be changed for each administration to avoid neutralising effects of Abs [104].

An important recent advance in AAV vectorology has been the demonstration of clinical utility of AAVs that effect hepatic blood clotting factor IX gene expression in livers of haemophiliac patients [105]. This clinical study reported that peripheral vein infusion of the vectors resulted in improvement in the clotting profiles and 4 of the 6 treated patients did not require further factor IX prophylaxis. The authors suggest that concerns about toxicity and immune-mediated elimination of the vector may be countered by treatment with a short course of immunosuppressive glucocorticoids. This successful clinical study represents a significant milestone and paves the way for use of AAVs for other applications such as in RNAi-based HBV therapy.

1.3.2.2 Lentiviral vectors

LVs comprise a subgroup of retroviruses that transduce both dividing and non-dividing cells. An important feature of the vectors is that stable integration of their proviruses enables long term and potentially indefinite expression of transgenes, which may be up to 7.5 kb in length

[104, 106]. This is useful to achieve sustained expression of anti-HBV sequences and render infected cells resistant to HBV. Although provirus integration into host genomes is potentially mutagenic, targeting to heterochromatin should improve the vectors' safety profile [107]. Interestingly, it has recently been demonstrated that LVs were less likely to integrate into transcriptionally active sites in non-dividing cells than in dividing cells [108]. To date, LVs have been used in 40 clinical trials (http://www.abedia.com/wiley/vectors.php). Most trials involve *ex vivo* modification of hematopoietic stem cells and T-lymphocytes for the treatment of HIV-1 and monogenic diseases. Although *ex vivo* modification of hepatocytes to render them resistant to HBV infection offers interesting therapeutic possibilities, the methods required to employ this approach are yet to be established. Transduction of autologous hepatocytes derived from induced pluripotent stem cells followed by hepatic infusion may become a feasible method of populating the liver with HBV-resistant cells [109].

As for their utility for treating chronic HBV after systemic administration, a limitation is that LVs transduce only a small proportion of adult murine hepatocytes [110]. Liver cell transduction can be improved if cell proliferation is occurring at the time of LV administration. In support of this, injection of vectors into young and newborn animals [111] or following partial hepatectomy in adults [112] achieves greater hepatocyte transduction efficiency. Priming hepatocytes for LV infection by pretreating animals with cholic acid and phenobarbital has recently been investigated as a clinically relevant alternative to improving LV transduction of hepatocytes [113]. Interestingly phenobarbital has a weak stimulatory effect on cell proliferation, but cholic acid has no direct effect on the cell cycle. Without increasing markers of cell proliferation, both compounds were shown to improve transduction of hepatocytes *in vivo* following systemic administered LVs by a factor of 6 to 9 fold. This

priming strategy is easy to implement but may not enable transduction of adequate numbers of adult hepatocytes to be of use in RNAi-based HBV therapy with LV vectors. Adenoviral vectors have proven to be far more suitable for the efficient delivery of therapeutics to the liver.

1.3.2.3 Adenoviral vectors

1.3.2.3.1 Adenovirus structure

Adenoviruses belong to the *Adenoviridae* family and were discovered in the early 1950s. They naturally infect epithelial cells resulting in mild infections of the upper respiratory tract or gastroenteritis which are mostly asymptomatic in a healthy individual [114]. There are at least 57 known human Ad serotypes belonging to species A-G, with derivatives of human serotypes 5 (Ad5) and 2 (Ad2) from subgroup C, being most commonly used as gene therapy vectors, and are thus the best characterised [115, 116]. Adenoviral vectors are currently the most commonly used vector utilised in approximately 24% of clinical trials (Figure 1.6). They are relatively large non-enveloped icosohedral viruses and have a double-stranded linear DNA genome of approximately 36 kb. The capsid consists of 240 hexon capsomers which form the 20 triangular surfaces of the icosohedron. Three major proteins make up the capsid, i.e. hexon, penton base and fiber together with some minor proteins (reviewed in [115]) (Figure 1.7).

1.3.2.3.2 Adenovirus genome organisation

The 36kb linear genome of Ad5 is represented in Figure 1.8 It is flanked by *cis*-acting inverted terminal repeats (ITRs) which facilitate viral replication and a packaging signal (Ψ)

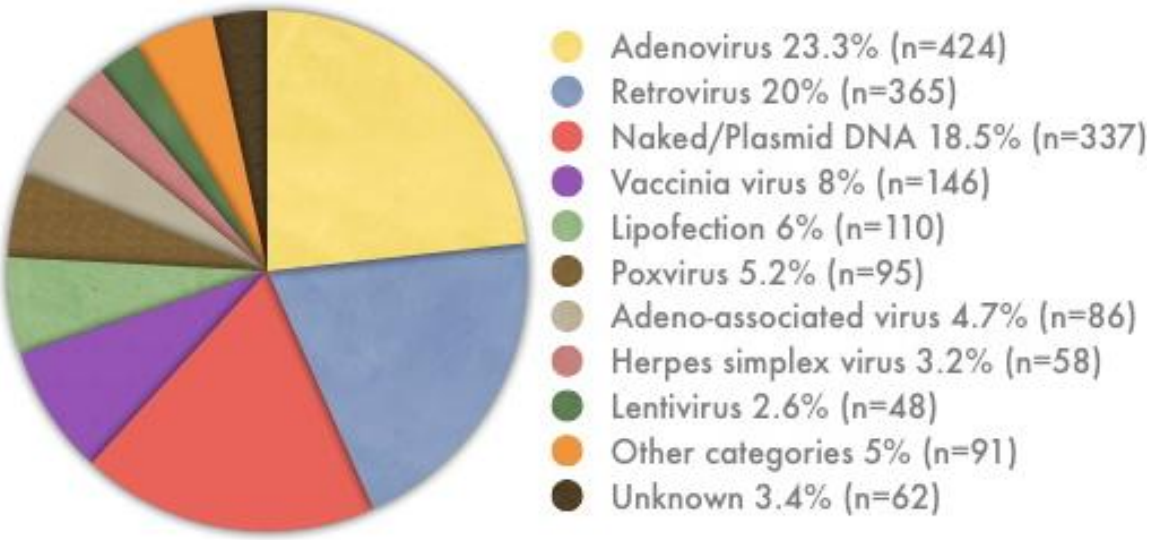


Figure 1.6: Vectors used in gene therapy clinical trials. Pie chart showing the proportion of gene therapy vectors being used in clinical studies as of June 2012. www.wiley.co.uk/genmed/clinical

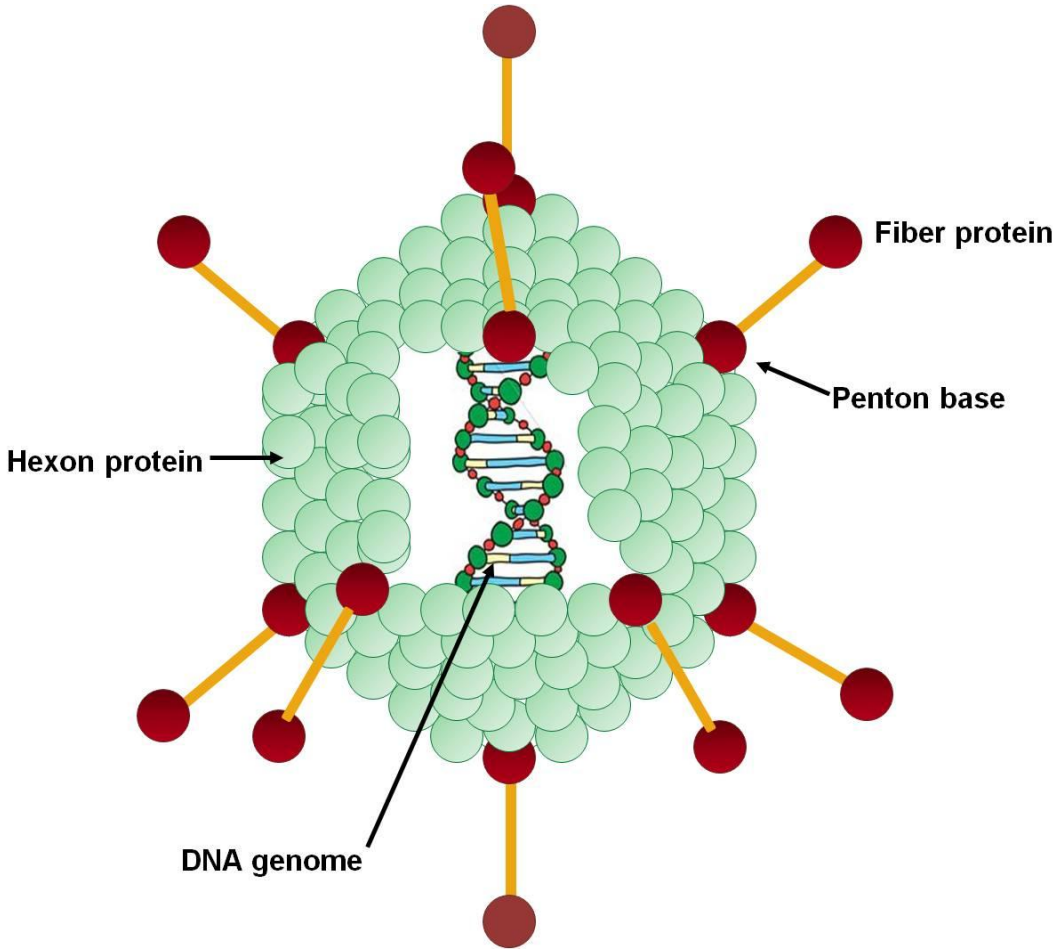


Figure 1.7: Structure of adenovirus. The icosohedral virion is 60-90 nm in diameter with the double-stranded DNA genome enclosed within the capsid made up of 240 hexon monomers and 12 pentons (152 capsomers).

which enables packaging of the genome into virion capsids. There are 2 sets of genes, the set expressed before DNA replication (E1A, E1B, E2, E3 and E4) and the late set (L1 to 5) which are expressed at high levels following initiation of DNA replication (reviewed in [115]).

Adenoviruses were one of the first vector systems to be developed as they can efficiently infect a wide range of both dividing and post-mitotic cells (reviewed in [117]). In 1977 the Human embryonic kidney (HEK 293) cell line was established which stably expressed the E1 genes so the gene product could be provided to the virus in *trans* thus enabling the mass production of replication deficient Ad vectors [118]. First generation (FG Ad) Ad5 vectors were rendered replication deficient by deletion of E1 genes (Figure 1.8). These vectors still have low level expression of viral antigens which activates a cytotoxic immune response resulting in limited transgene expression. The E3 gene was then deleted as it is dispensable for replication but involved with anti-host immunity (reviewed in [115]). However, it was subsequently discovered that the re-introduction of E3 could actually prolong transgene expression as it encodes functions involved in aiding viral escape from host immune responses [119]. The deletion of the E1 and E3 genes also allowed for approximately 6.5 kb of transgene to be inserted into the vector (reviewed in [115, 116]). It became apparent that some cells express E1-like proteins which allowed for E2 function at a low level. This resulted in the production of replication–competent adenovirus (RCA) which is not ideal for gene therapy applications [120]. Second generation Ad vectors were then developed where E2 was deleted to remove the risk of RCAs, but the vector's capsids still stimulated the host immune response resulting in transient transgene expression [121, 122]. To avoid these unwanted host immune responses and resulting liver toxicity, third generation, helper-dependent (HD Ad) or "gutless" vectors were developed which are devoid of all the viral

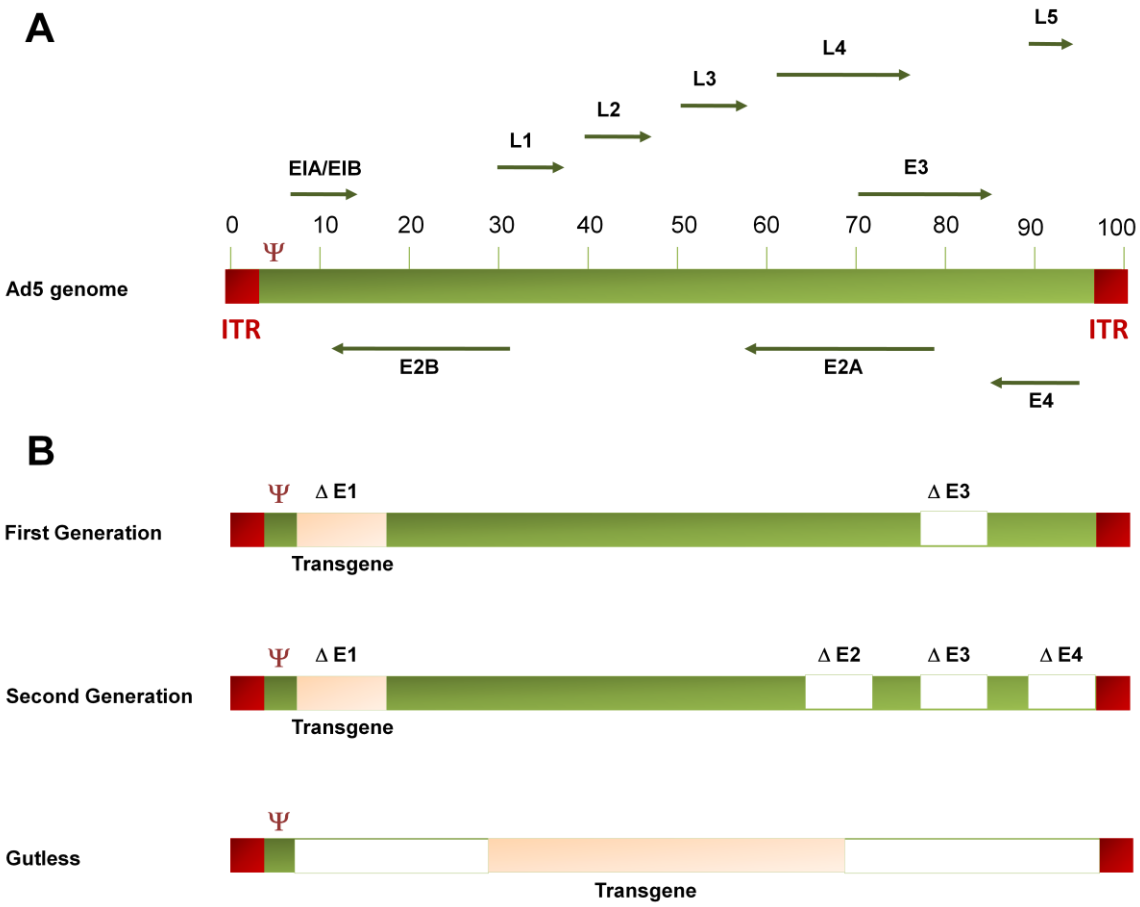


Figure 1.8: Genome organisation of the different generations of adenoviral vectors. Genome organisation and transcription map of Ad 5 and Ad 5-derived gene transfer vectors. The double-stranded ±36 kb Ad genome, which is divided into 100 map units, is schematically represented in **A**. Red boxes at the terminal ends represent inverted terminal repeats (ITR) and Ψ indicates the position of the packaging signal. Arrows show the direction of transcription for the major mRNAs. E1-E4 indicate early transcripts and L1-L5 represent late transcripts. **B**. Represents the first-, second-, and third generation (gutless) Ad vectors with the light boxes indicating which Ad genes have been deleted in the different vectors.

coding sequences (Figure 1.8). These vectors only contain the *cis*-elements required for genome replication and encapsulation so during the production process, a helper virus is used to provide all the necessary products in *trans* [117, 123-125]. The production and purification techniques initially used resulted in significant helper virus contamination but this was overcome by the development of the Cre/*loxP* helper-dependent system in 1996 [126-129].

1.3.2.3.3 The Cre-lox system of Ad production

HD Ad production using the Cre/*loxP* system initially entails inserting the gene of interest into a bacterial plasmid together with the 500 bp *cis*-acting viral sequences needed for replication (ITRs) and packaging (Ψ) (Figure 1.9). A genome that is too small leads to instability so "stuffer" DNA sequences are inserted to maintain an ideal size ranging from 27-38 kb [126, 130]. Following successful cloning, the plasmid form of the genome is linearised by restriction enzyme digestion with rare cutter *Pme* I (Figure 1.9) and the linear viral form is transfected into a HEK 293 cell line expressing Cre recombinase (116 cells). This cell line is then also infected with a helper virus, which is a FG Ad virus that has a packaging signal flanked by *loxP* sites. Following infection of the HEK 293 cells Cre-mediated *loxP* site-specific recombination excises the packaging signal (Ψ) in the helper virus genome to prevent packaging of the helper virus [126]. The genome size of the helper virus (HV) (36 kb) and HD Ad (30 kb) vary which enables them to be physically separated using cesium chloride (CsCl) density centrifugation thus reducing the chances of HV contamination. Modification of this system has enabled the mass production of high quality HD Ad with very low levels of contaminating helper virus [131].

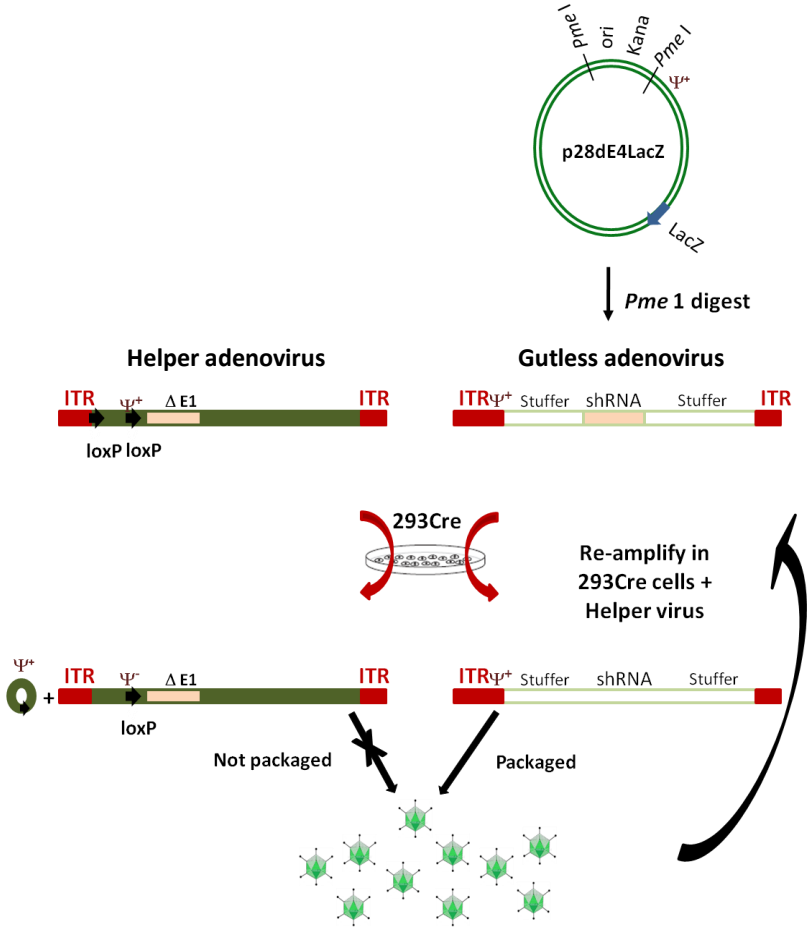


Figure 1.9: The Cre-lox system of helper dependent adenovirus production. The HD Ad genome is released from a bacterial plasmid (pΔ28LacZ) by restriction enzyme digestion with *Pme*I. The HD Ad genome consists of *cis*-acting sequences required for replication (ITRs) and packaging (Ψ) and occupies ± 500bp of the genome with the remainder consisting of "stuffer" sequences and the transgene of interest (shRNA). The liberated genome is transfected into Cre expressing HEK 293 cells (116 cells) and cells are infected with a helper virus with a packaging signal flanked by *loxP* sites. Cre mediated excision of Ψ renders the helper virus unpackagable yet still able to provide all the necessary factors for propagation of the HD Ad in *trans.* Serial coinfections of 116 cells with HD Ad and helper virus are performed until the desired viral titre is achieved. Adapted from [131]

1.3.2.3.4 Adenoviral vectors in gene therapy

Recombinant Ad vectors are used in 24% of current gene therapy clinical trials as they have several features which make them an attractive option, with the majority of the clinical applications being in cancer therapy (http://www.abedia.com/wiley/vectors.php). There are several advantages to using Ads for gene therapy: 1) they efficiently transduce a broad range of dividing and non-dividing cell types; 2) they can be produced in high titres (up to 10^{13} virus particles/ml; 3) they are capable of carrying large transgene inserts; 4) they do not integrate into the host genome which reduces the chances of insertional mutagenesis or carcinogenesis and 5) they are extremely hepatotropic which makes them well suited for use in HBV therapy [116]. HD Ad vectors have the added advantage of being able to carry large transgene inserts of up to 36 kb in length. Deletion of all of the viral genes in HD Ads limits induction of a cytotoxic T-cell mediated response and has an additional advantage of prolonging transgene expression [116, 126]. Therapeutic Ad vectors are most commonly administered systemically which unfortunately results in a number of adverse responses triggered by stimulation of host innate and adaptive immune responses and this remains a significant hurdle for clinical applications.

1.3.2.3.5 Adenoviral hepatotropism

A major advantage of using Ad vectors to target HBV is that they are naturally hepatotropic [132]. Ad particles have a diameter of 60-110 nm and are able to traverse fenestrations in liver. The level of transgene expression is often lower in humans than in pre-clinical animal models which may be attributed to the much smaller fenestrae in the human liver [133]. The fenestrations allow for access of the Ad to the microvillus surface of hepatocytes to enable receptor-mediated internalisation into these cells [134, 135]. The conventional understanding of Ad entry into cells is that there is initial binding of Ad fiber protein to coxsackie-

adenovirus receptors (CAR). This attachment receptor for coxsackie B viruses as well as Ad2 and Ad5 was identified 30 years ago, on non-polarised epithelial cell surfaces [136, 137]. It is becoming apparent that CAR is an important primary attachment receptor for Ads *in vitro* but *in vivo* other mechanisms play a role in hepatic Ad binding and CAR is of minor importance [138, 139]. A recently identified vitamin K-dependent pathway is responsible for effective delivery of systemically administered Ad5 whereby coagulation factors VII, IX, X and protein C bind to Ad hexon protein and transport the Ad5 to the liver. [140-142]. Coagulation factor X appears to be the most efficient at mediating binding of the hexon hypervariable region ofAd5. This interaction then promotes binding to cellular heparin sulphate proteoglycans (HSPGs) prior to hepatocyte internalisation [142].

The liver is made up of parenchymal hepatocytes (66%) and non-parenchymal/sinusoidal cells (33%). The non-parenchymal cells consist of endothelial cells (70%), Kupffer cells (20%), fat storing Stellate cells (10%) and pit cells (< 10%) [143]. The Kupffer and liver sinusoidal endothelial cells together with the spleen sinosoidal endothelial cells and macrophages, form part of the mononuclear phagocyte system, previously called the reticulo-endothelial system which plays an important role in defence [144]. The Kupffer cells are the most numerous macrophage population in the human body and play an integral role in Ad vector clearance after systemic delivery and also contribute to the innate immune response [143].

1.3.2.3.6 Innate immune response

The major challenge for effective Ad-mediated gene therapy targeting HBV is efficient delivery to parenchymal hepatocytes and the prevention of sequestration of the Ad and stimulation of the innate immune response by Kupffer cells. Once Ads reach hepatic tissue,

up to 98% of the virus particles are sequestrated by the mononuclear phagocyte system, and in particular the Kupffer cells [145]. These antigen presenting macrophages express a scavenger receptor A (SR-A) which binds negatively charged regions on the hypervariable region 1 (HVR1) of Ad5 hexon protein [146]. Ads are destroyed in the phagocytic Kupffer cells, which themselves undergo dose-dependent necrosis within 10 minutes of systemic delivery of the vector [145, 146]. Ad pattern recognition receptors on macrophages and dendritic cells trigger the innate immune response which results in an increase of chemokine and inflammatory cytokine levels [144, 147-149]. Activation of this response is characterised by a rapid release of inflammatory cytokines and may result in acute toxicity which can lead to systemic inflammatory response syndrome and possible organ failure [148]. The seriousness of such a response became evident by the unfortunate death of an 18-year old patient, Jesse Gelsinger, who died as a result of systemic inflammatory response syndrome, disseminated intravascular coagulation and multiorgan failure after receiving a high dose of Ad vector containing the ornithine transcarbamylase (OTC) gene [150]. His death highlighted the importance of understanding host-Ad interactions and the need for developing strategies to attenuate this response. This response is species-specific, with humans being acutely sensitive to the innate immune response (reviewed [143]).

The innate immune response is dose-dependent and occurs rapidly within 1 to 6 hours after intravenous injection of Ads [148, 151]. In mice the innate response is followed by a secondary release of pro-inflammatory cytokines and chemokines that occurs 5 to 7 days after infection. This effect is thought to result from an adaptive immune response to expressed viral proteins [115]. Receptor binding and endocytosis of Ads leads to activation of P13K and ERK/MAPK pathways causing NF-κβ dependent activation of the innate response [152]. There is a release of various chemokines and pro-inflammatory cytokines such as

tumour necrosis factor-α (TNF–α), interleukins (IL-6, IL-5, IL-12 and IL-1β), monocyte chemotactic protein-1 (MCP-1) and interferon-γ (IFN-γ). The cytokine surge is followed by leukocyte infiltration which results in liver tissue damage [148]. Toll-like receptors (TLRs) and MyD88, which is a TLR adapter gene, have been implicated in mediating the response *in vivo* [153, 154]. Toll-like receptors 2 and 9 are expressed on Kupffer, spleen and liver sinusoidal cells and exhibit species differences in expression which may account for differences seen in the innate response by different animals [154]. IFN-α and IFN-β production, which also contributes to the toxic effects of Ads [147, 151, 155, 156], occurs in splenic cells (myeloid dendritic cells) by a mechanism that is independent of TLRs and cytosolic receptors of RNA and DNA. The effect is however dependent on endosomal viral escape which activates MAP kinase and SAPK/JNK signaling pathways [156]. The tissue damage caused by the innate immune response eliminates the transgene expressing cells and hence reduces the duration of transgene expression.

1.3.2.3.7 Adaptive immune response

An adaptive immune response can be mounted after a single immunisation with Ads. Host B cell recognition of an epitope on a foreign antigen is followed by proliferation of T helper cells and the release of immunoglobulins targeting the Ads. Once an animal has been exposed to Ad it will trigger a humoral response and the production of Nabs within a week of exposure which limits the repeated administration of Ad vectors. Major histocompatibility complex (MHC) class I-restricted CD8+ T cells are directed against cells expressing the transgene [157]. MHC class II helper CD4+ T cells activate B cells to secrete NAbs directed at capsid proteins and inhibit transduction by the Ad vectors and decrease the amount of infectious virus [158-160]. A major obstacle in using Ad5 derived vectors is the high prevalence of human anti-Ad5 antibodies in humans resulting from previous exposure to Ad5

[161, 162]. The seroprevalence to Ad5 is 80% in sub-Saharan Africa but only 50% in the USA [19]. The immune response prevents the long-term expression of transgenes and inability to administer Ad5 vectors to pre-exposed individuals. Fortunately strategies have been developed to circumvent these potential problems. Overcoming the unintended effects resulting from immune-stimulation following Ad administration, as well as evading pre-existing immunity have been a priority of research involving use of Ads for gene therapy [163].

1.3.2.3.8 Strategies used to avoid immune stimulation

Helper dependent vectors versus first generation Ad vectors

The use of FG Ad generally results in transient gene expression in an Ad naïve animal and poor expression after repeated administrations resulting from activation of the host immune response. First generation Ad vectors express viral genes and display viral capsid peptides on transduced cell membranes resulting in immune clearance and memory T-cell killing of the transduced cell. It was found that reintroduction of the E3 region actually aids in viral escape resulting in prolonged transgene expression and decreased NAb generation [119, 164]. The development of third generation HD Ad vectors which have most of their genome deleted and do not express viral genes resulted in more sustained transgene expression and an improved safety profile [126, 165-167]. Although HD Ads and FG Ads induce the same inflammatory innate response, the inflammatory response does not last longer than 24 hours with HD Ads. Transgene expression from HD Ad vectors is not permanent, yet there have been reports of stable, long term-expression of transgenes for over 1 year following administration of a single dose of HD Ad vectors [168, 169].

Sequestration prevention

Various methods have been employed to avoid Ad sequestration by Kupffer cells. These include the intravenous administration of chemicals such as clodronate-encapsulated liposomes or gadolinium chloride which are specifically toxic to Kupffer cells and splenic macrophages [147, 170, 171]. Alternatively, an Ad5 'predosing' regimen has been employed where high doses of "null" Ad5 are injected to cause Kupffer cell death. Administration of the therapeutic viral vector soon thereafter results in greater efficiency of liver cell transduction as the Kupffer cells are unable to sequestrate the therapeutic Ad vector. [172-174]. Another strategy that has been employed in mice is preinjection of polyinosinic acid which is a scavenger receptor A ligand, resulting in decreased Kupffer cell numbers and increased circulating half-life of the Ad vector [175]. These strategies have been useful for studying Ad-host interactions in animal models but are not clinically viable. More clinically relevant approaches that have been employed are the administration of the anti-inflammatory glucocorticoid dexamethasone, transient pharmacological suppression of B and T cells and polymer modification of immunostimulatory epitopes [40, 176-181].

Another factor that diminishes efficiency of Ads in a clinical setting is vector sequestration by the CAR and Complement 1 receptor on human erythrocytes. These receptors are not present on mouse erythrocytes which emphasises limitations of murine models in predicting clinical utility of Ad vectors [182, 183]. To overcome this sequestration problem it may be possible to modify vectors with polymers or to isolate the liver circulation and deliver the Ad vector directly to hepatocytes by using an intravenous catheter [184, 185].

Serotype switching

With the high seroprevalence to Ad5 in humans, an alternative strategy to allow for repeated vector administration is serotype switching. There are more than 50 Ad serotypes that can infect humans. Neutralising Abs against one serotype usually do not cross reacting with another serotype [162]. Using the Cre/*loxP* system, it is possible to generate HD Ads with different serotypes by simply changing the serotype of the helper virus to that of a different Ad serotype. Replacement of capsid proteins with those from a rare, less seroprevalent Ad serotype, enables successful repeat administrations of Ad vectors *in vivo* [186, 187].

Capsid modification

Instead of changing the genetic makeup of Ad capsid proteins, the Ad capsid can be modified by conjugating polymers to "mask" the capsid from Kupffer cell, red blood cell, and NAb detection [180, 188, 189]189]. The shielding of the Ad capsid results in reduced innate and adaptive immune responses and decreased liver damage *in vivo* [188-190]. The main neutralizing antibody response is directed at the hexon proteins of the viral capsid but can also be directed at the fiber of penton base [19, 191]. Two types of synthetic polymer have been used to modify Ads, PEG and poly-N- (2-hydroxypropyl) methacrylamide. Polyethylene glycol is the most commonly used polymer and has been utilised since the 1970s to modify therapeutic peptides and proteins [192, 193]. Conjugation with PEG improves the stability, pharmacokinetic and toxicity profiles of compounds without decreasing their activity ([178], reviewed in [194]). Polyethylene glycol is an uncharged, monovalent, hydrophilic, non-immunogenic synthetic polymer which covalently attaches to hexon proteins on Ad caspids through free surface reactive amine groups [178]. Conjugation of viral capsids with PEG shields the negative charges on Ad5 hexon which diminishes vector interaction with Kupffer cells and subsequent sequestration, thus enabling efficient hepatocyte transduction [188, 190,

195-197]. PEGylation decreases immune detection, improves the toxicity profile and transduction efficiency of vectors is not compromised in non-primate animal models [198-201]. It has been noted however that transgene delivery to primate hepatocytes using PEG-modified vectors may be less efficient [180, 202, 203].

The focus of this thesis has been the employment of the above strategies to attenuate host immune responses, in particular the use of HD Ad vectors and PEGylated Ad vectors, to develop a viable delivery vehicle for a RNAi-based therapeutic option for the treatment of chronic disorders such as HBV.

1.3.2.3.9 Adenoviral therapy targeting HBV

In 2005 Uprichard and colleagues showed that HBV replication was inhibited by a recombinant first generation Ad vector that delivered a HBV-targeting shRNA expression cassette [204]. The effect lasted for at least 26 days in a HBV transgenic mouse model following systemic administration of the vector. In a similar study published by our group in 2006, first generation Ad vectors carrying an RNAi effector targeting the *X* ORF of HBV resulted in inhibition of HBV replication [56].

It became apparent that sustained hepatic transgene expression and attenuated immune stimulation may be achieved with HD Ad vectors [205, 206]. Assessing efficacy of HD Ads for RNAi-based treatment of other diseases has provided insights that are relevant to using these vectors for delivering HBV-silencing sequences. In a study aimed at inhibiting an endogenous hepatic gene, HD Ads successfully delivered an shRNA expression cassette targeting the gene encoding the sterol regulatory element-binding protein-1c (SREBP-1c) [207]. This transcription factor is an important mediator of insulin effects on lipid and

carbohydrate metabolism in the liver. Following systemic administration of 2×10^{11} HD Ad particles to mice that model type 2 diabetes, 90% knockdown of the target gene was observed in the liver after 1 week and the effect was sustained for 21 days. An interesting observation was that there appeared to be a limit to the level of gene silencing, and administration of higher vector doses did not augment knockdown but increased immunostimulatory effects [207, 208]. Usefulness of RNAi-activating anti-HBV HD Ad vectors has been assessed in one study reported to date [209]. Although potentially effective, specificity of the silencing effect could not be confirmed, which highlights the relevance of the work presented in this thesis.

There is a still a deficit in knowledge concerning Ad and host interactions and a better understanding of these interactions will enable the generation of safer HD Ad vectors that can be used in large animal models and ultimately humans. Although studies on use of HD Ads in large animal models of HBV infection have not yet been reported, results from investigations in other disease models are relevant. Successful long term expression of transgenes delivered with HD Ad vectors has been achieved in non-human primates [210-212]. However, when administered in high doses ($>1 \times 10^{13}$ viral particles) acute and sometimes fatal toxicity was reported to occur [39, 206]. The structure of the HD Ad virions is the same as that of the first generation vectors and thus HD Ads are capable of inducing an acute, dose-dependent innate immune response [39, 92]. This highlights the importance of developing strategies to attenuate these immune responses for the development of safe therapeutic Ad vectors.

1.4 Aims

An RNAi approach targeting HBV has proved to be successful, however the ultimate success of RNAi therapy in chronic HBV carriers will be defined by efficiency of the delivery vector.

In recent years there has been a growing interest in nanobiotechnology and the manipulation of viral vectors, in particular Ad vectors for therapeutic applications. Ads are highly efficient at transducing liver cells yet can be toxic as a result of the immunostimulatory effects in the host animal. The initial aim of the study was to investigate whether both the innate and adaptive immune responses could be attenuated by chemical modification of Ad vectors. FG Ad vectors carrying efficient anti-HBV RNAi sequences were PEGylated and tested in a transgenic HBV mouse model.

Chronic carriers of HBV require sustained inhibition of HBV for the therapy to be effective, so ideally an effective RNAi therapeutic for HBV should demonstrate long-term effects. FG Ad vectors expressing RNAi sequences have been shown to be efficient at inhibiting HBV but the effect is only transient. Third generation HD Ad vectors have an attenuated induction of a cytotoxic T-cell mediated response resulting in prolonged transgene expression. The principal objective of the second study was to clone anti-HBV RNAi effectors into HD Ad vectors, with the hope of obtaining sustained expression in the HBV transgenic mice. The combination of Ad capsid PEGylation and the use of HD Ads has the potential of generating an efficient delivery vector system for the treatment of HBV using an RNAi approach.

In summary the aims of the research presented in this thesis were to:

1. Chemically modify FG Ad vectors expressing ant-HBV shRNA effectors with the objective of attenuating immunostimulation and thereby increasing their efficacy *in vivo*.
2. Compare FG Ad and HD Ad vectors in the same *in vivo* model to access whether more sustained HBV inhibition can be achieved with HD Ad vectors.

2 EFFICIENT INHIBITION OF HEPATITIS B VIRUS REPLICATION IN VIVO USING PEG-MODIFIED ADENOVIRUS VECTORS

2.1 Introduction

Licensed anti-HBV therapeutics are only partially effective [213] and the demonstration that HBV replication is susceptible to RNAi-mediated silencing [53, 54, 204, 214-216] prompted investigations aimed at harnessing this pathway for therapeutic application. The HBV genome has a compact arrangement with overlapping open reading frames (ORFs) and *cis* elements embedded within protein-coding sequences (Figure 2.1). There is a single transcription termination signal, which results in all HBV transcripts having a common 3′ end that includes *HBx*. This arrangement limits sequence flexibility of the virus and makes *HBx* a particularly good target for RNAi based nucleic acid hybridisation gene silencing. Previous work from our laboratory demonstrated that Ads expressing hairpin sequences targeting *HBx* (HBV co-ordinates 1580–1604) are efficient silencers of HBV gene expression and replication [56].

The most promising viral vectors that have been used for *in vivo* delivery of anti-HBV sequences are recombinant Ads [56, 204] and AAVs [217]. Ads have the useful property of efficient targeting of hepatocytes *in vivo* after systemic administration (reviewed in [216]). However, widespread use of these vectors has been limited by pre-existing immunity and their powerful induction of innate and adaptive immune responses. Resulting damage to healthy tissues with decreased transgene expression is thus a significant concern for therapeutic use of recombinant Ads. Given the importance of Ad capsid proteins in mediating

immune responses, methods have been devised to attenuate immunostimulation by modifying Ad capsid proteins with polymers such as PEG [177]. Chemical modification of Ad capsid proteins is a well-established strategy and although decreased immunostimulation has been shown by this approach [178-180, 218, 219], there have been few studies that measured the efficiency of polymer-modified Ad vectors *in vivo* in disease models. In this study, we assessed PEG-modified first-generation vectors that express an RNAi activator that we have previously shown to silence HBV replication efficiently [56]. We observed that PEGylation improved silencing of HBV *in vivo* in a murine transgenic model of HBV replication. This chemical modification also significantly suppressed immunostimulation and hepatotoxicity.

2.2 Materials and methods

2.2.1 Adenoviral vectors

First-generation adenoviral vectors expressing enhanced green fluorescent protein (eGFP) together with anti-HBV short hairpin RNA 6 (shRNA 6) or shRNA 10 [56] were previously generated in our laboratory according to the AdEasy system (Stratagene, CA, USA) [220]. Purification of the recombinant Ad was carried out using a standard two-step CsCl centrifugation protocol and then the virus was dialysed and stored at -80 $^{\circ}$C.

2.2.2 PEGylation of first generation adenoviral vectors

The method used to modify the viral vectors with PEG was based on previously described protocols [178, 219]. Activated methoxypolyethylene glycol 5000 propionic acid N-succinimidyl ester (mPEG-SPA $_{5000}$) (Sigma Aldrich, MO, USA) was used to modifiy FG Ad vectors. Briefly, 1×10^{12} Ad particles were incubated with one ml mPEG-SPA $_{5000}$ (1 mg/ml)

for one hour at room temperature with constant mixing. Ten-fold excess of L-Lysine, Free Base, Monohydrate (Calbiochem, CA, USA) was then added to quench the reaction and incubated for one hour at room temperature with constant mixing. The FG Ad sample was then transferred to a Slide-A-Lyzer dialysis cassette (Pierce, Thermo Fisher Scientific, IL, USA) and dialysed overnight at 4 ºC in 10 mM potassium-buffered saline (KPBS), pH 7.4 to remove unreacted mPEG-SPA_{5000}. The PEG-conjugated Ad was concentrated to the original volume by placing the Slide-A-Lyzer containing the PEGylated Ad on solid PEG 20 000 for approximately two hours at room temperature. When the desired volume was reached, the PEGylated Ad virus was removed from the dialysis cassette and 10% glycerol was added before aliquoting and storing at -80 $^{\circ}$C. An aliquot was analysed to assess efficiency of PEGylation. A increase in molecular weight (MW) of the hexon protein results from PEGylation which was verified by polyacrylamide gel electrophoresis (PAGE) or microchip electrophoresis. The Experion microchip electrophoresis system (Biorad Laboratories, CA, USA) was used according to manufacturer's instructions. Briefly, it involves running the viral proteins under non-reducing conditions to resolve and quantify the PEG coupled hexon proteins accurately.

2.2.3 Titration of Ad vectors

The number of infectious particles in PEGylated or unPEGylated preparations was determined by titrating adenoviral stocks in HEK 293 cells and assaying the cells 48 hours later for Ad fiber protein expression using immunostaining. The protocol was based on the Adeno-X rapid titer kit (Clontech, Mountain View, CA, USA) with minor modifications. Briefly, HEK 293 cells were propagated as detailed in Appendix A1-1 and then plated at 5 × 10^5 cells/ml in a 24-well plate. The Ad was serial diluted (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} dilutions)

and 50 µl of diluent was added to triplicate wells at the time of plating. The cells were incubated at 37 °C, 5% CO_2 for 48 hours after which the medium was aspirated from the cells and the plate was allowed to air dry for five minutes. The cells were fixed to the plate by gently adding ice cold 100% methanol to each well and incubating at -20 °C for 10 minutes. The fixative was removed and three washes of phosphate-buffered saline (PBS) + 0.5% bovine serum albumin (BSA) were performed before adding two drops of diluted protein block (PBS + 1% BSA) per well which was incubated for five minutes at room temperature. The wells were then washed once with PBS + 0.5% BSA and the cells were permeabilised by adding 200 µl per well of PBS + 0.5% BSA + 0.25% Triton-X. The wells were again washed with PBS + 0.5% BSA before adding mouse anti-Ad fiber Ab (Biomeda, CA, USA) (diluted 1/1000 in PBS + 0.5% BSA) which was incubated for an hour at room temperature on an orbital shaker. The primary antibody was aspirated and the plate was washed once with PBS + 0.5% BSA. The buffer was removed and 2 drops of biotinylated secondary Ab (Biomeda, CA, USA) was added to the wells and incubated for 30 minutes at room temperature. The secondary Ab was aspirated and the plate washed, before adding 2 drops of diluted Detector Reagent (biotinylated horseradish peroxidise (HRP) complexed with avidin) (Biomeda, CA, USA) which was incubated for 30 minutes at room temperature. The wells were then washed with PBS + 0.5% BSA buffer followed by a distilled water wash before staining with the 3,3'-Diaminobenzidine (DAB) chromagen. The diluted metal enhanced DAB substrate (Roche Biochemicals, GmbH, Germany) was added to the wells and incubated at room temperature until a dark precipitate, which forms in the presence of HRP, was visible (5-15 minutes). The DAB positive cells were counted using a light microscope. The number of infectious Ad particles per ml (ifu/ml) of vector sample was determined using the following formula:

$$\text{Ifu/ml} = \frac{\text{(infected cells/field)} \times \text{(fields/well)}}{\text{volume virus (ml)} \times \text{dilution factor}}$$

2.2.4 Cell culture and Northern blot analysis

Culture and infection of Huh7 lines was carried out as detailed in Appendix A1-1. For Northern blot hybridisation, liver-derived Huh7 cells were infected with an approximate multiplicity of infection (moi) of 100 recombinant adenovirus vectors per cell and harvested 2 days thereafter, before extracting total RNA with TRI Reagent® (Sigma-Aldrich, MO, USA). Approximately 20 mg of RNA was separated on a 15% denaturing polyacrylamide gel. As a molecular marker a mixture of 200 *f*mol digoxigenin (DIG)-labeled probes (18 nucleotide shRNA6 and 24 nt U6 small nuclear RNA) was run alongside the samples. The gel was stained for 5 minutes in 0.5× Tris-Borate Ethylenediaminetetraacetic Acid (EDTA) containing 4 µg/ml ethidium bromide. The RNA was visualised under UV illumination to verify equal loading and quality of the RNA. The RNA was then transferred to Hybond-N^+ positively charged nylon membrane (Amersham, NJ, USA) at 4 $^{\circ}$C using the Semi-Dry Electroblotting Unit Z34,050-2 (Sigma-Aldrich, MO, USA) set at 3.3 mA/cm^2 for an hour The RNA was then cross linked to the membrane using a UV crosslinker (UVP Inc., CA, USA) set at 20 000 µJ/cm^2.

Probes against the putative 6 guide sequence (5′- TGC ACT TCG CTT CAC CTC -3′) and the U6 snRNA (5′-TAG TAT ATG TGC TGC CGA AGC GAG -3′) which was used as a control for equal loading of the cellular RNA [40], were prepared by labelling with the DIG Oligonucleotide 3′-End Labelling Kit (Roche Applied Science, Mannheim, Germany). The membrane was pre-hybridised in 10 ml/100 cm^2 of DIG Easy-Hyb solution (Roche Applied Science, Mannheim, Germany) at 42 $^{\circ}$C for 30 minutes. Following pre-hybridisation the labelled probes were denatured at 95 $^{\circ}$C for 5 minutes and added to the membrane at a concentration of 10 ng/ml in DIG Easy-Hyb solution and allowed to hybridise at 42 $^{\circ}$C overnight. Following hybridisation the membrane was subjected to a low stringency wash (5×

SSC, 1% SDS) at room temperature for 20 minutes and two high stringency washes (1× SSC, 1% SDS) at 42 °C for 15 minutes each. The hybridised probes were detected by equilibrating the membrane in 1× wash buffer (0.1 M malic acid; 0.15 M sodium chloride, pH 7.5; + 0.03% Tween-20) at room temperature for 2 minutes and then blocking in 5% milk powder in PBS at room temperature for 30 minutes. The Anti-Digoxigenin–Alkaline Phosphatase Fab Fragment Ab (Roche Diagnostics, IN, USA) (0.5 µl/10ml blocking solution) was then incubated with the membrane at room temperature for 30 minutes. Unbound Ab was removed by subjecting the membrane to four 15 minutes washes (1× wash buffer) at room temperature. The membrane was placed in detection buffer (0.1 M Tris.HCl; 0.1 M sodium chloride, pH 9.5) for 5 minutes before placing the membrane on a plastic sheet and then adding 20 drops of the chemiluminescent substrate for alkaline phosphatise, CDP-*Star* (Roche Biochemicals, GmbH, Germany). The membrane was covered with another piece of plastic and incubated at room temperature for 5 minutes before imaging the chemiluminescent signals using a G:Box Imaging and Analysis System (Syngene, Cambridge, UK). Membranes were stripped and reprobed with the DIG-labelled oligonucleotide to detect U6 snRNA.

2.2.5 Adenoviral vector administration to HBV transgenic mice

HBV transgenic mice which constitutively express HBV particles, have a greater than genome length HBV sequence stably integrated into their genome [221]. The mice were used to determine the effects of RNAi-activating adenoviral vectors on markers of HBV replication *in vivo*. These experiments were carried out in accordance with protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee. A dose of 5×10^9, 1×10^9, or 5×10^8 adenovirus infectious particles was injected as a bolus via

the tail vein and blood was collected by retroorbital puncture. In all experiments, groups of mice comprised eight animals each.

2.2.6 Serum HBV DNA and liver mRNA quantitation

Circulating viral particle equivalents (VPEs) and hepatic HBV mRNA were determined by real-time quantitative polymerase chain reaction (Q-PCR). An absolute Q-PCR method was used to measure serum HBV DNA. Total DNA was isolated from 50 µl serum samples using the MagNA Pure LC Total Nucleic Acid Isolation Kit and MagNA Pure LC system (Roche Diagnostics, GmbH, Germany). Real-time analysis was performed on the DNA using SYBR® Green Jumpstart *Taq* Ready mix (Sigma, MO, USA). The surface antigen region of HBV DNA was amplified using primer set: HBVs forward: 5′- TGC ACC TGT ATT CCC ATC -3′, and HBVs reverse: 5′- CTG AAA GCC AAA CAG TGG -3′ on the Roche Lightcycler v.2 (Roche Diagnostics, GmbH, Germany). Following a 95 $^{\circ}$C hotstart to activate the Taq polymerase, 50 cycles of the following parameters were used: annealing at 57 $^{\circ}$C for 10 seconds, extension at 72 $^{\circ}$C for 10 seconds and denaturation at 95 $^{\circ}$C for 10 seconds. Specificity of the amplicons was confirmed by melting curve analysis. The absolute value of viral particle equivalents was determined using a standard curve that was generated from a commercial HBV standard obtained from the National Institute of Biological Standards and Controls (NIBSC, Hertfordshire, UK) (Appendix A1-4).

A relative Q-PCR method was used to measure HBV mRNA in mice livers where HBV expression was quantified relative to the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). Total RNA was extracted from freshly harvested livers using TRI Reagent® according to procedure detailed in package insert. Approximately 30 ng of RNA

was reverse transcribed using Sensiscript® Reverse Transcription Kit (Qiagen, GmbH, Germany) and oligi-dT primer. The same HBVs primer set as used to measure HBV DNA was used and the GAPDH expression was determined using primer set: GAPDH forward: 5′-GAAGGTGAAGGTCGGAGTC -3′; GAPDH reverse: 5′- GAAGATGGTGATGGGATTTC-3′. HBV surface mRNA was quantified relative to GAPDH expression in the Roche Lightcycler v.2 using SYBR® Green as detailed above, except that a temperature of 58 $^{\circ}$C was used in the annealing step.

2.2.7 Liver sections

Adenovirus gene transduction was assessed by detecting eGFP microscopically in frozen liver sections done on mice sacrificed 48 hr after initial injection with adenovirus.

2.2.8 Cytokine assay

A cytometric bead array (CBA) mouse inflammation kit (BD Biosciences, CA, USA) was used to measure serum inflammatory cytokine levels. The assay quantitatively measured IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70 protein levels in the mouse serum samples. The protocol followed was according to manufacturer's instructions except that serum samples were diluted 2-fold. Briefly, the mouse inflammation standard was diluted in assay diluent and allowed to stand at room temperature for 15 minutes after which a dilution series was generated by serial dilutions of the standard. Each of the 6 capture beads (specific to each cytokine) was thoroughly mixed and 10 μl of each standard per test was added to make a stock solution. Fifty microlitres of bead mixture was then added to each assay tube. The standards and samples (50 μl) were then added to appropriate tubes followed by 50 μl of

Phycoerythrin (PE) detection reagent. The assay tubes were incubated at room temperature for 2 hours after which they were washed with 1 ml Wash Buffer. The assay tubes were centrifuged to pellet beads, Wash Buffer was removed and the beads were resuspended in 300 µl Wash Buffer. The contents of each tube were transferred to a microtiter plate and the inflammatory cytokine levels determined using the CBA machine and BD CBA Software (BD Bioscience, CA, USA).

2.2.9 Anti-adenovirus immunoglobulin assay

To measure mouse antibodies against complete Ad virions, a previously described protocol was followed [222]. Briefly, individual wells of a *MaxiSorp*™ ELISA plate (NUNC, Roskilde, Denmark) were coated with 100 µl of 0.05 M carbonate/bicarbonate buffer (pH 9.6) containing 1×10^{10} viral particles of FG Ad shRNA 6. The plate was left at room temperature overnight after which the coating solution was discarded and the plate washed three times with washing solution (0.9% NaCl; 0.05% Tween 20). The plate was dried by inverting on a paper towel. The remaining binding sites in wells were blocked with 150 µl dilution buffer (1 × PBS; 2% non-fat milk powder; 0.05% Tween-20; 0.02% sodium-azide) for 1 hour at room temperature. The solution was discarded and dried as before. Mouse serum samples were diluted 1:10 000 and 100 µl of the diluents were added to the ELISA plate before assaying for the amount of mouse antibodies to Ad hexon protein using the RIDASCREEN Adenovirus IgG kit (R-Biopharm, Pfungstadt, Germany). The protocol was followed according to the manufacturer's instructions except the kit secondary antibody was substituted with a peroxidase-conjugated secondary anti-mouse immunoglobulin (Dako, CA, USA) which was detected with *o*-phenylenediamine dihydrochloride as the chromogenic substrate. After 15 minutes the reaction was stopped by adding 100 µl 1N H_2SO_4 and the

optical density of the samples was measured at 496 nm on a Bio-Rad Microplate Reader, Model 680 (Bio-Rad, CA, USA).

2.2.10 Serum transaminase assay

Liver secreted aspartate transaminase (AST) and alanine transaminase (ALT) activities were measured in mouse serum samples by a kinetic assay with an automated photometric analyser (Roche Diagnostics, Rotkreuz, Switzerland) in a routine chemistry lab.

2.2.11 Statistical analysis

Means ± standard error of the mean (±SEM) were calculated and a statistical difference was considered significant when $p < 0.05$ and was determined according to the Student two-tailed *t* test. Calculations were performed with the GraphPad Prism software package (GraphPad Software, CA, USA).

2.3 Results

2.3.1 HBV target sequence

The previously described HBV targets of expressed RNAi activators are located in the *HBx* ORF of HBV (Figure 2.1). This viral sequence was selected as it is conserved in HBV genotypes and is also present in all of the viral transcripts. Previously optimised hairpins which were complementary to the target sites in HBV, were cloned downstream of a pol III (U6) promoter within FG Ad vectors and consisted of 25-bp stems with four CA and GU

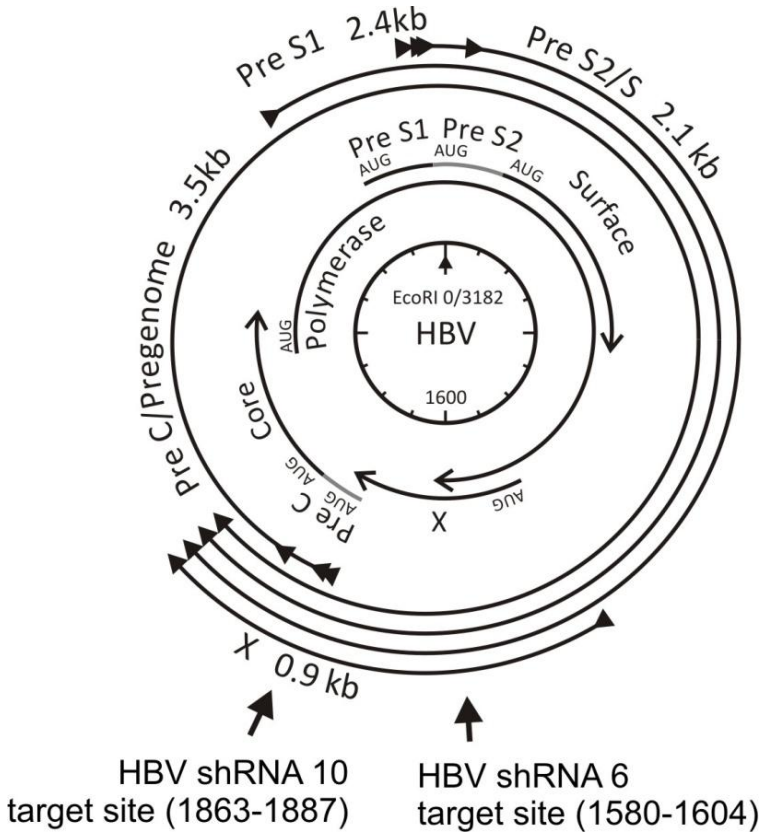


Figure 2.1: Schematic illustration of Hepatitis B virus genome showing sites targeted by shRNA sequences. Co-ordinates of the viral genome are given relative to the unique EcoRI restriction site. The *surface*, *core*, *polymerase* and *HBx* viral open reading frames (ORFs) (with initiation codons), encompassing the entire genome, are indicated by arrows. Four outer arrows represent the HBV transcripts with common 3′ ends. The sites targeted by HBV shRNA 6 and HBV shRNA 10 are located within the *HBx* ORF and are found in all of the viral transcripts.

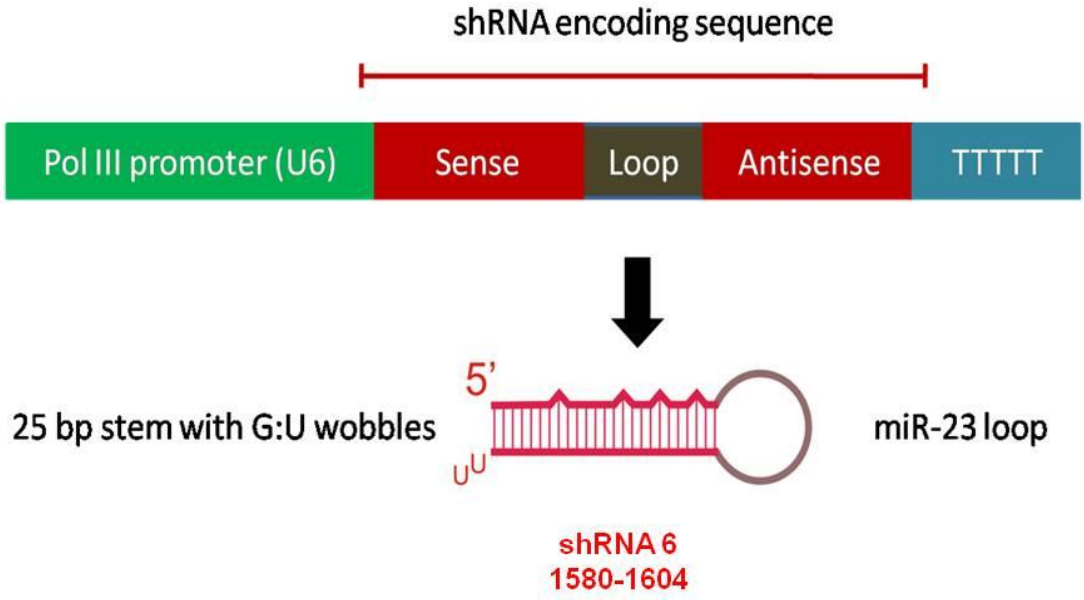


Figure 2.2: shRNA encoding vector targeting HBV. Schematic of the DNA cassette that was inserted into FG Ad vectors with pol III (U6) promoter, sense miRNA loop and antisense-encoding sequences is illustrated above. The expressed anti-HBV shRNA 6 indicating miRNA 23 loop and mismatches in the sense strand is shown below.

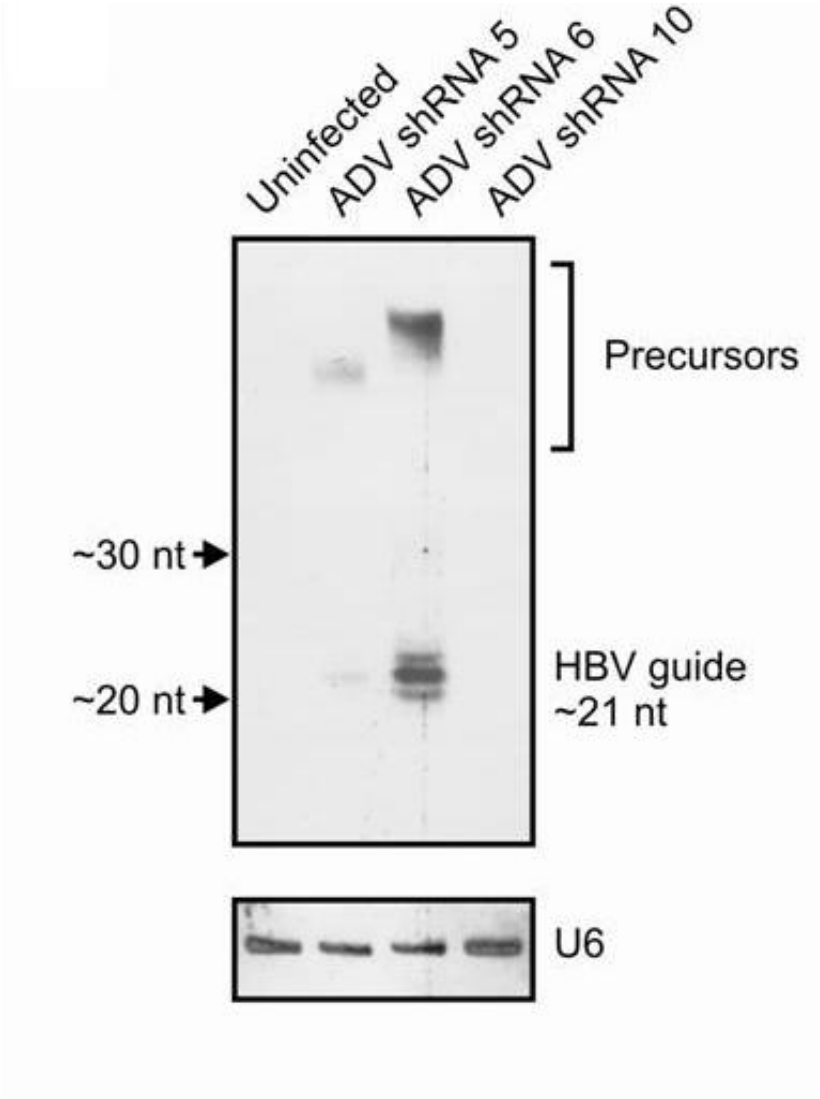


Figure 2.3: Northern hybridisation analysis of expressed anti-HBV sequences. RNA was extracted from Huh7 cells after infection with recombinant viruses expressing the indicated shRNA cassettes (shRNA 5, shRNA 6 and control shRNA 10) or from uninfected cells. Hybridisation of a ^{32}P labeled probe complementary to the intended shRNA 6 guide strand, indicates the presence of an efficiently processed 21nt guide sequence. The probe was then stripped and rehybridised to an endogenous U6 snRNA probe to confirm equal loading of cellular RNA (*bottom*).

mismatches (Figure 2.2). U6 shRNA 6 is highly effective against HBV, but U6 shRNA 10 does not silence HBV replication and has been used as a control in this study. To assess potential improvement by polymer modification, we selected the recombinant adenovirus vector that expresses anti-HBV shRNA 6 (Ad HBV shRNA 6) and subjected this to analysis after PEG conjugation. After infection of Huh7 cells in culture with Ad HBV shRNA 6, the intended anti-HBV guide sequence was detected (Figure 2.3), which verified that transcripts of the expression cassette were indeed processed by the RNAi related machinery.

2.3.2 Anti-HBV efficacy of PEG-modified Ad vector

Ad HBV shRNA 6 vectors were modified with mPEG-SPA, and efficient addition of PEG to the Ad hexon protein was verified by microchip electrophoretic analysis. An estimated 93.3% of the hexon protein present in the sample was PEGylated (Appendix A1-2). Titration of PEG-modified vectors did not result in an appreciable decrease in infectivity, and suitable concentrations for *in vivo* application were routinely achieved. Hepatic delivery of transgenes was assessed after injection of mice with 5×10^9 infectious recombinant virus particles. Fluorescence microscopy of frozen liver sections showed that approximately 80% of liver cells were transduced 48 hr after injection, and that this was achieved with both native and modified vectors (Figure 2.4). Enhanced GFP was also not detectable in any significant amounts in non-hepatic tissue (data not shown). Our PEG modification procedure therefore did not compromise delivery efficiency and liver targeting of these vectors after systemic administration. HBV transgenic mice were then injected with PEGylated and native Ads expressing HBV shRNA 6 and anti-HBV efficacy was measured by determining the number of circulating VPEs (Figure 2.5). Initial effects of PEGylated and unmodified vectors on circulating VPEs were similar in mice treated with both vectors. There was a significant drop in the circulating VPEs in each of the groups of animals 1 week after administration of the

virus. This effect persisted for approximately 4 weeks, and at week 5 the VPE titre had increased to approach baseline levels. At week 5, a second dose of Ad vectors was administered to the mice via the tail vein. At week 7, the PEG-modified vectors effected a decrease in VPEs, which was not observed after treatment with the unmodified Ads. By 3 weeks after the second PEG Ad vector injection the number of circulating VPEs had returned to baseline concentrations, which indicates that silencing efficacy after the second Ad injection is less sustained than after the initial vector administration. There appeared to be an increase in the VPEs measured in mice receiving the PEG-modified vector following the second administration. However the increase in VPEs was not significantly greater than baseline levels and is in keeping with the natural variation of HBV levels seen over time, in the HBV transgenic mice used in this study.

Knockdown of intrahepatic HBV mRNA was also measured 1 week after administration of various doses of Ad HBV shRNA 6 or control Ad HBV shRNA 10 (Figure 2.6). Quantitative reverse transcription PCR, using primers that target the HBV *surface* ORF relative to eGFP expression (indicative of the amount of vector expressed in the liver), showed that intrahepatic HBV mRNA was decreased in animals that had been treated with 5×10^9 Ad HBV shRNA 6 particles. This effect was significant at a high dose but diminished in mice receiving lower doses of the PEG-modified recombinant vector. Moreover, PEGylated Ad HBV shRNA 10 did not demonstrate decreased HBV mRNA concentration, which is in keeping with the previously reported observations showing that this unmodified Ad vector had no effect against HBV replication [56].

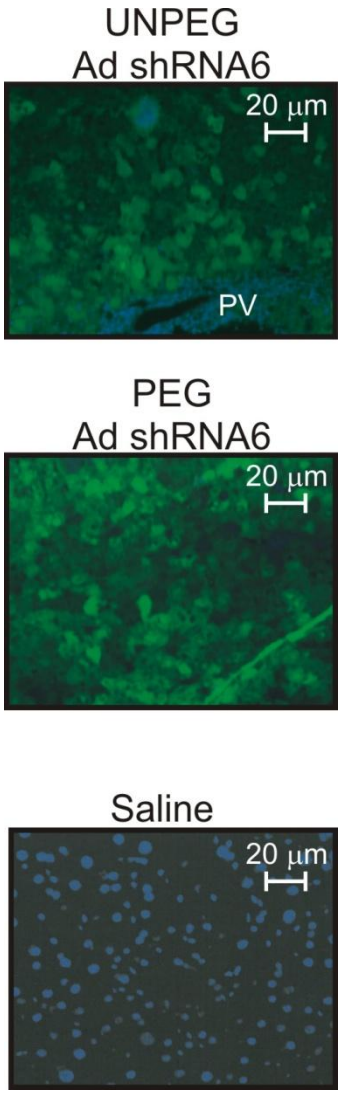


Figure 2.4: Adenoviral vectors efficiently transduce hepatocytes. Representative fluorescence microscopy analysis of frozen sections from mouse liver that was processed 48 hrs after administration of 5×10^9 native (top) and PEG-modified (middle) Ad vectors. Transduced cells expressing enhanced GFP and the location of the portal vein (PV) is indicated. Saline control mice livers showed no eGFP expression.

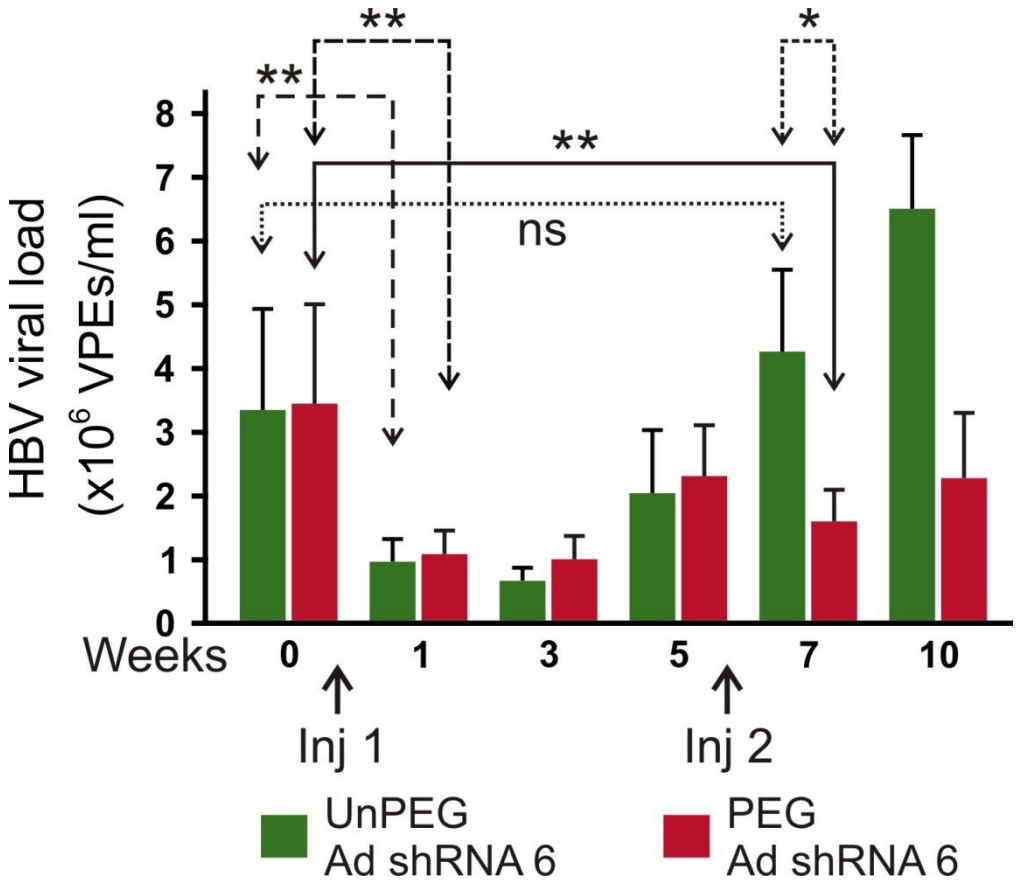


Figure 2.5: **Circulating VPEs in HBV transgenic mice after administration of PEG-modified and native Ads**. Animals received two injections of 5×10^9 infectious Ad particles, which were administered at commencement of the experiment and 5 weeks after the initial injection. Titers of VPEs were determined by real-time quantitative PCR. Results are expressed as the mean (±SEM) from 8 mice. Statistically significant differences (* $p<0.05$; ** $p<0.01$) between PEG-modified and unmodified shRNA 6-expressing vectors are indicated and were determined according to the Student's 2 tailed paired t-test.

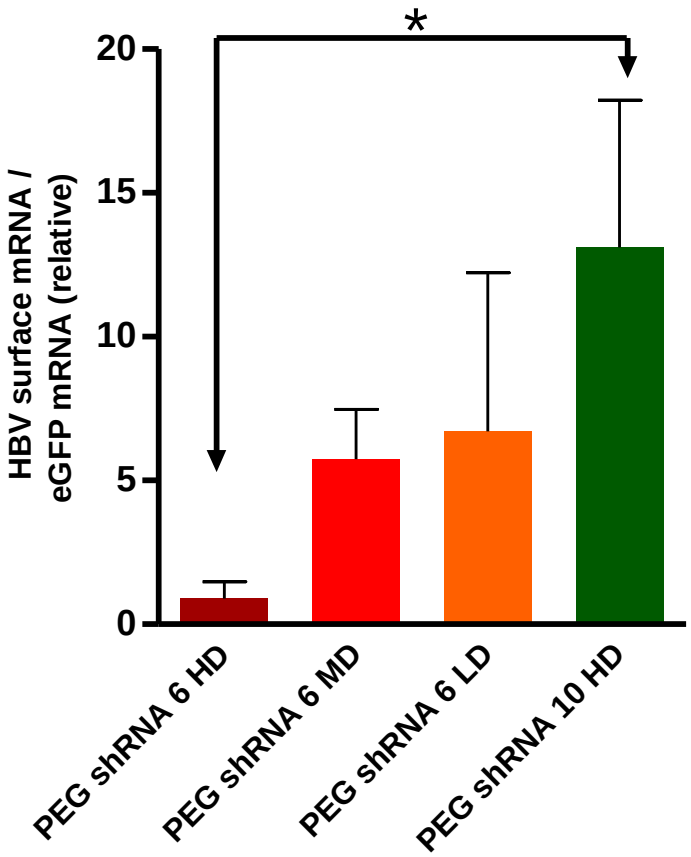


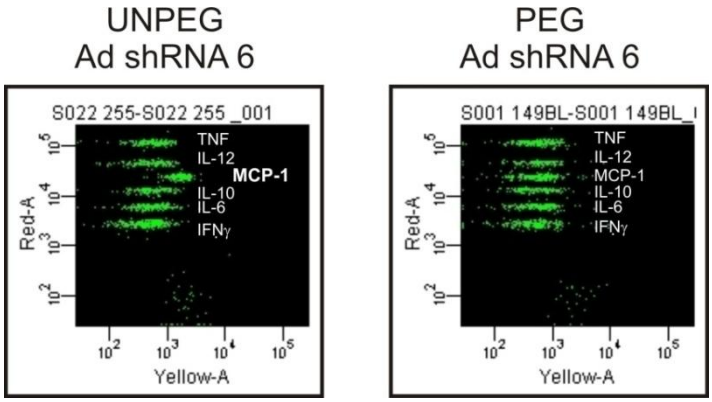
Figure 2.6: **Effects of Ad dose on hepatic HBV mRNA**. Animals were killed 1 week after intravenous administration of PEG-modified vectors at doses of 5×10^8 (LD), 1×10^9 (MD) and 5×10^9 (HD), vector particles. Mean ratios of HBV surface mRNA (±SEM) were measured relative to eGFP mRNA, using Q-PCR. Statistically significant differences (* $p<0.05$) between vectors is indicated and were determined according to the Student's 2 tailed paired t-test.

2.3.3 Serum cytokine concentrations after injection of PEGylated and unmodified Ad vectors

The improved anti-HBV efficacy of PEGylated vectors is likely to result from an attenuated immune response to the polymer-coated Ads [180, 218, 219]. Innate and adaptive immune responses against recombinant Ads are the major contributors to toxicity. The initial response caused by Ad capsid interaction with macrophages, Kupffer and dendritic cells occurs within one hour of vector administration and peaks at approximately 6 hours and may persist for 4 days depending on vector dose [203]. This response is characterised by a release of pro-inflammatory cytokines and chemokines to recruit effector cells resulting in neutrophil-dependent liver injury. A secondary but self-limiting inflammatory process of the liver occurs 5 to 7 days after vector administration where activated lymphocytes remove Ad infected cells. The humoral immune response develops after a week and is characterised by the production of NAbs which target Ad vectors upon re-administration. To assess the effects of PEGylated and unmodified Ad HBV shRNA 6 on the release of inflammatory cytokines, serum concentrations of a selection of cytokines and chemokines were determined. Initially, blood was collected 6 and 24 hr after the first injection of mice with recombinant vectors and the cytokines were measured by a CBA assay. The panel of cytokines, comprising TNF-α, IL-12, MCP-1, IL-10, IL-6, and IFN-γ, included markers that give a broad indication of innate and adaptive immune response activation.

At the time point of 6 hr after injection of the first dose of Ad HBV shRNA 6, MCP-1 was elevated in mice receiving the unPEGylated vector but not in the serum of animals receiving PEG-modified recombinant virus (Figure 2.7). By 24 hr after injection, the serum MCP-1 concentration reverted to baseline control levels (data not shown). MCP-1 is produced from a

A



B

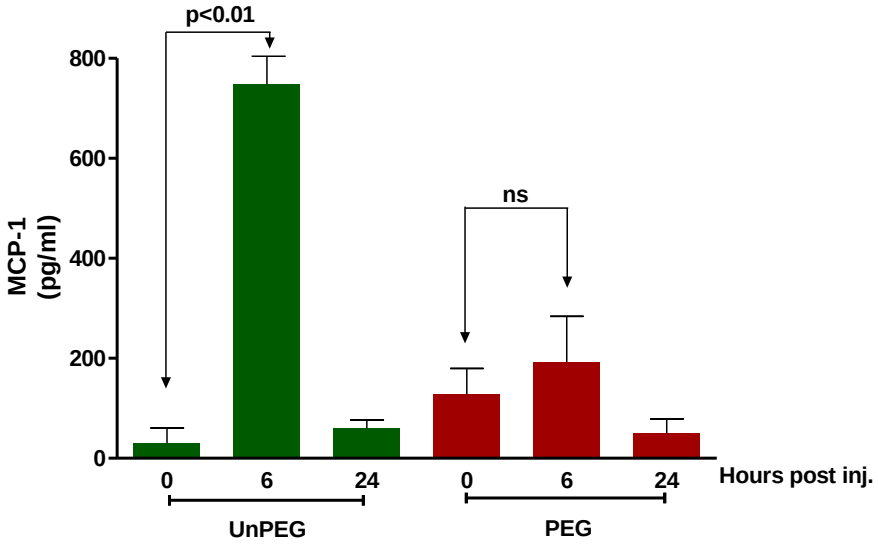


Figure 2.7: Serum cytokine concentrations after first administration of Ads to HBV transgenic mice. A. Representative flow cytometry data from CBA assay for TNF-γ, IL-12, MCP-1, IL-10, IL-6, and IFN-α at baseline and 6 hrs after adenovirus injection. Elevated levels of MCP-1 were shown in mice receiving unPEGylated Ad shRNA 6. **B**. Mean concentrations of MCP-1 levels are shown in mice at baseline, 6 and 24 hours. Levels returned to normal 24 hours after administration of PEG-modified and native adenoviral vectors. Statistically significant differences ($p<0.001$) between time points is indicated and were determined according to the Student's 2 tailed paired t-test.

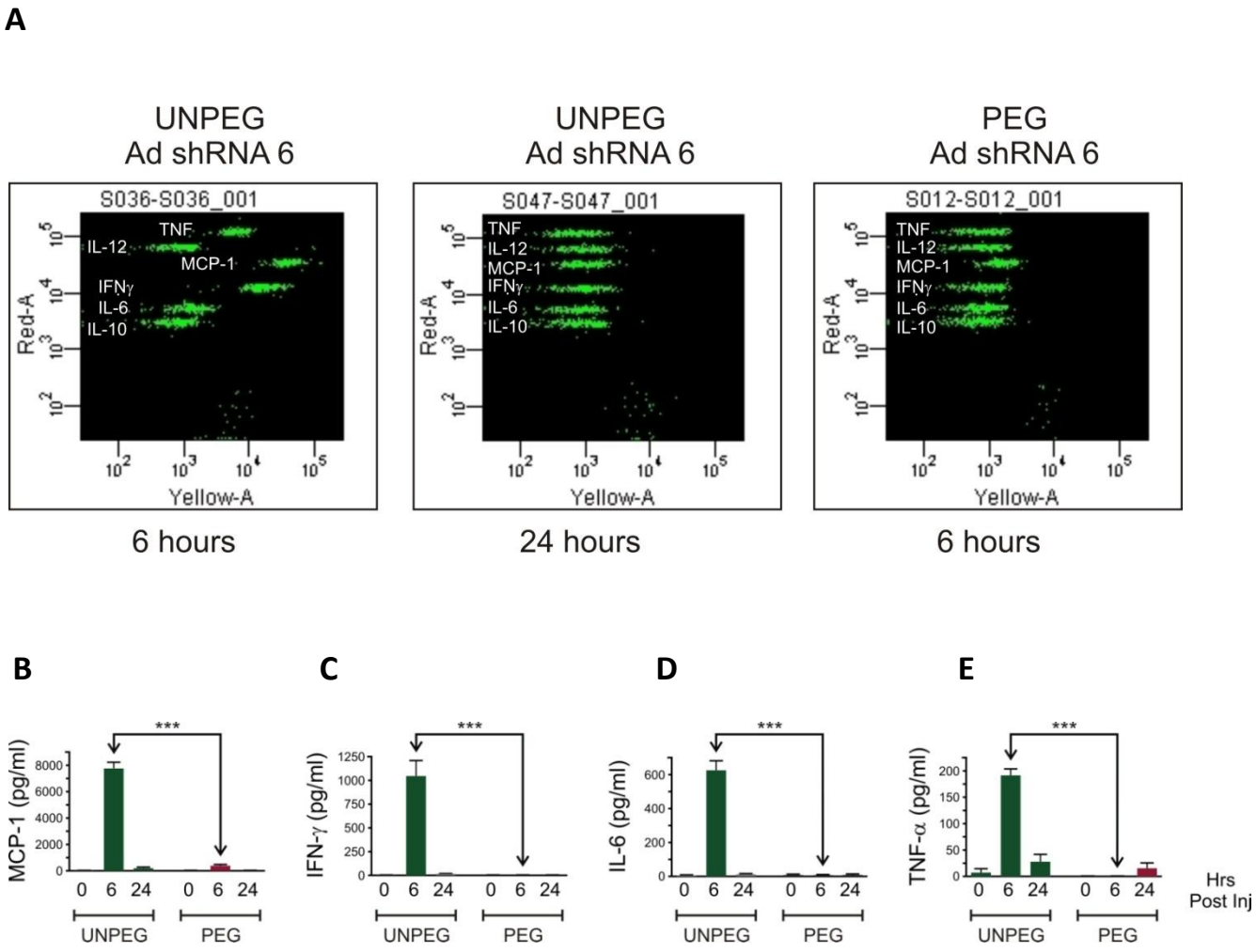


Figure 2.8: **Serum cytokine concentrations after second administration of Ads to HBV transgenic mice**. **A**. Representative flow cytometry data from CBA assay for TNF-α, IL-12, MCP-1, IL-10, IL-6, and IFN-γ 6 and 24 hr after adenovirus injection. Shown are mean concentrations (±SEM) of MCP-1 (**B**), IFN-γ (**C**), IL-6 (**D**), and TNF-α (**E**) in mice at baseline and 6 and 24 hr after administration of PEG-modified and native adenoviral vectors. Statistically significant differences (*** $p<0.005$) between PEG-modified and unmodified shRNA6-expressing vectors are indicated and were determined according to the Student's 2 tailed paired t-test.

variety of cells and functions as a monocyte chemoattractant [223, 224] and mediator of inflammation [225, 226]. Although other studies have reported stimulation of secretion of additional proinflammatory cytokines after Ad administration, our observations may be specific to the line of HBV transgenic mice studied here and also the low dose of Ad that these animals received.

Serum concentrations of cytokines were again measured in animals that received a second dose of native and PEG-modified Ad. PEG-modified vectors caused modest elevation of only MCP-1 at 6 hr, and the concentration of this chemokine reverted to baseline 24 hr after injection (Figure 2.8). Because a raised serum MCP-1 concentration was also the only marker of immunostimulation after initial administration of native Ad, secretion of this chemokine may be the most sensitive indicator of Ad-induced immunostimulation under the experimental conditions described here. CBA analysis revealed that concentrations of TNF-α, IFN-γ, MCP-1, and IL-6 were markedly elevated in mice 6 hr after receiving the unmodified vector. By 24 hr, the concentrations had returned to normal baseline levels. This observation contrasts with the modest acute elevation in serum MCP-1 concentration after injection of PEGylated Ads and indicates that this modification diminishes immunostimulatory properties of the anti-HBV vectors.

2.3.4 Assay of anti-Ad vector immunoglobulin titres and markers of hepatocyte toxicity

To measure the concentrations of humoral immune response to Ad vectors, enzyme-linked immunosorbent assays (ELISAs) were performed to detect antibodies interacting with Ad hexon proteins and also complete Ad particles. Comparison of the relative optical density (OD) readings indicated that there was a significant difference in immunoglobulin titres in

animals 5 weeks after receiving either unmodified and PEGylated vectors (Figure 2.9). Lower values observed after administration of PEGylated vectors confirm that the humoral immune response is attenuated and correlates with diminished cytokine release, as well as data reported by others [180, 218, 219]. Hepatotoxic effects of the Ad administration were determined by measurement of ALT and AST activities in mouse serum 1 day and 1 week after receiving a second dose of the PEG-modified or unmodified Ad vectors (Figure 2.10). Compared with mice receiving the PEGylated Ads, the serum concentrations of both transaminases were significantly increased at 24 hr in animals receiving unmodified vectors. Taken together, our data support the interpretation that PEG modification improves anti-HBV efficacy, attenuates immunostimulatory properties, and improves the safety profile of recombinant Ad vectors in a stringent model of HBV infection.

2.4 Discussion

Recombinant adenovirus vectors have several properties that make them useful for therapeutic transfer of anti-HBV sequences [216]. These include efficient infection of non-dividing cells, hepatocyte targeting after systemic administration *in vivo*, and transient transgene expression that is sustained for weeks without integrating into host DNA. However, immunostimulatory effects of Ads may be toxic and also diminish the efficiency of transgene expression. In addition to modification with synthetic polymers, as was used here, a number of approaches has been employed to diminish activation of host immune responses. These include immunosuppression [227, 228], silencing mediators of hepatocyte injury [229], serotype switching [162], genetic manipulation of capsid-encoding sequences [191], and use of high-dose vectors [126]. Although these strategies have had success, there are limitations

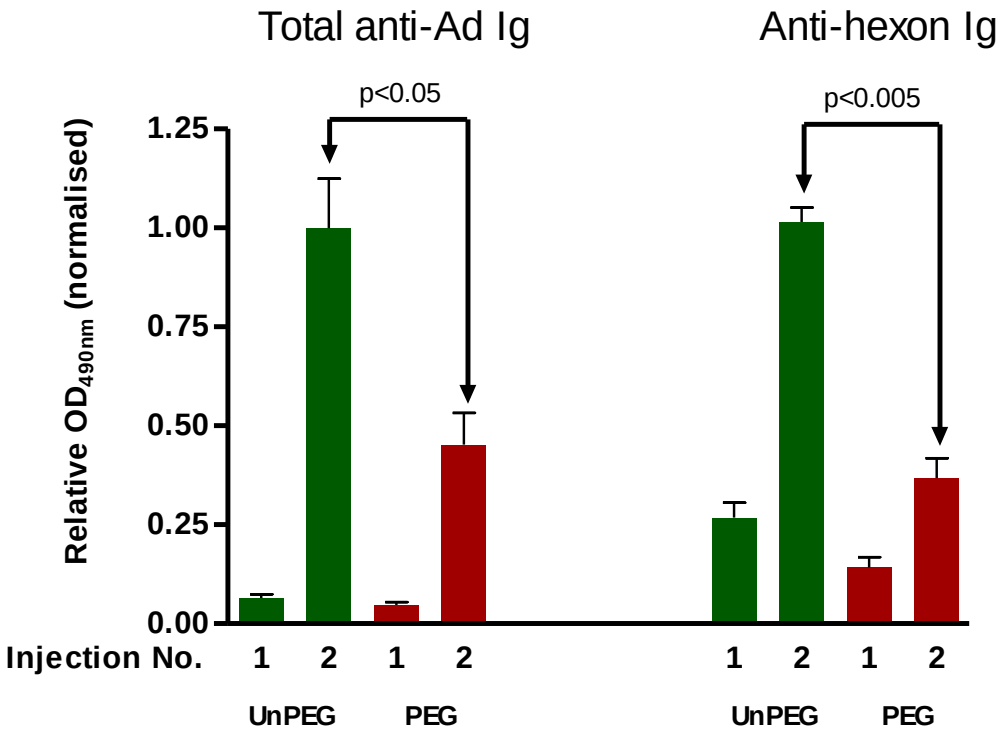


Figure 2.9: Relative serum anti-Ad immunoglobulin concentrations after vector administration to mice. Total anti-Ad and anti-hexon protein-specific antibodies were measured by ELISA. Mean relative optical density readings (±SEM) are given. Statistically significant differences are indicated and were determined according to the Student's 2 tailed paired t-test.

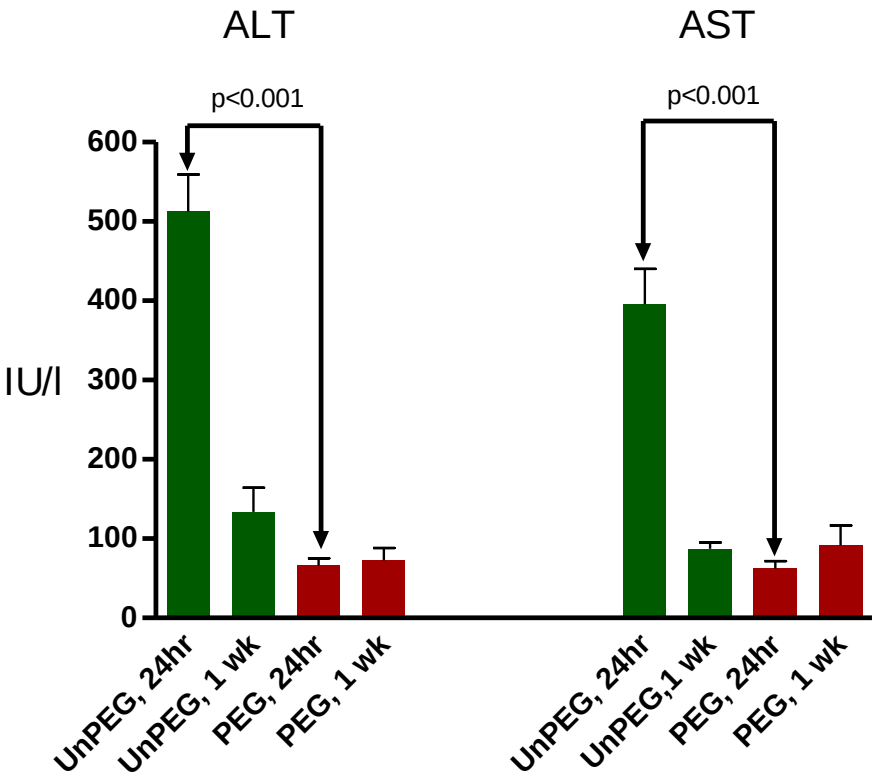


Figure 2.10: **Serum transaminase activities after vector administration to mice**. Transaminase (ALT and AST) activities were measured 24 hrs and 1 week after the second injection of PEGylated or unmodified adenoviral vectors. Mean (±SEM) enzyme activities are shown. Statistically significant differences are indicated and were determined according to the Student's 2 tailed paired t-test.

to their general applicability. For example, immunosuppression has side effects that would not be desirable in a clinical setting of HBV treatment. Importantly, Ad immunostimulation is largely mediated by viral capsid proteins, and is not dependent on viral gene expression or transduction [149]. Modification of viral capsid proteins with PEG is therefore an attractive method to control Ad immunostimulatory properties. PEG has been widely used in various therapeutic applications and has well-characterised pharmacologic properties. The polymer has low immunogenicity, is nontoxic, and improves the water solubility of PEG complexes (reviewed in [177]). In addition, PEG has other advantageous properties such as simultaneous modification of many surface proteins without the need for genetic manipulation, improvement of vector stability, and diminishment of nonspecific interactions. PEGylation of Ads can be carried out under mild reaction conditions to preserve vector bioactivity. The modification employed in this study entailed use of the covalent attachment of PEG to the Ad surface. This occurs when monomethoxypolyethylene glycol activated by succinimidyl propionate (MPEG-SPA), reacts preferentially with the ε-amino terminal of lysine residues, the most abundant functional group on the Ad hexon, fiber, and penton proteins [188]. Although we were able to demonstrate decreased immunostimmulation with our PEGylated vectors the attachment of mPEG-SPA moieties is random, which may lead to a heterogenous sample of PEGylated Ad [203].

There has recently been a shift to use second generation PEG derivatives which have been developed to preferentially bind thiol groups on cysteine residues. The immunostimulatory hexon protein on the Ad capsid (720 monomers per virion) has hypervariable regions (HVRs) protruding from the capsid which are the main target of NAbs and Kupffer cell receptors [230]. It is thought that the scavenger receptors of the Kupffer cells interact with the HVRs thus making it an ideal site for PEG modification [145]. The genetic modification of this

HVR by the introduction of a single point-mutation converts an alanine residue into a cysteine residue which is then efficiently targeted by maleimide PEG in a site specific manner [173, 190]. This site directed PEGylation results in improved stability of the modified Ad and more reproducible experimental results.

The bolus of 5×10^9 Ad infectious particles administered systemically to mice in this study represents a low dose compared with that used in other studies. Ad activation of dendritic cells and macrophages with resultant release of IL-6 and IL-12 was observed in mice treated with $0.3–5 \times 10^{11}$ particles per animal [144]. In primates, intraportal administration of up to 5×10^{12} particles/kg led to a self-limiting hepatitis, but higher doses caused massive hepatic necrosis and coagulopathy within days of vector administration [149]. This dose-dependent effect has also been reported on re-administration of Ad vectors [231]. The significantly lower dose that was used in this study (approximately 2×10^{10} particles/kg) is likely to contribute to our observation of attenuated immunostimulation and improved safety. However, it appears that efficacy of RNAi-activating Ads is dose dependent, as administration of lower amounts of Ads did not cause significant HBV gene silencing. Although complete silencing of HBV replication was not observed in this study, a higher Ad dose may well silence HBV replication more effectively. However, higher doses have lethal toxic effects (data not shown), which are likely to result from Ad immunostimulation and saturation of the endogenous miR pathway [232]. The low dose of vector that was required to be effective as well as the favourable effects of PEG modification of Ad vectors are important means of improving the safety profile of RNAi-activating recombinant Ads for potential therapeutic application. The therapeutic effects of our RNAi effectors targeting HBV are likely to be further enhanced by the use of HD Ads as they have more sustained transgene expression and are less immunogenic.

3 SUSTAINED HEPATITIS B VIRUS INHIBITION *IN VIVO* USING HELPER-DEPENDENT ADENOVIRUS VECTORS TO DELIVER ANTIVIRAL RNA INTERFERENCE EXPRESSION CASSETTES

3.1 Introduction

Safe and efficient introduction of RNAi activators into target hepatocytes to achieve sustained inhibition of HBV replication is important before therapeutic application of gene silencing technology is realised. Recombinant Ads have valuable properties as vectors for HBV-silencing (reviewed in [233]). We and others previously demonstrated that FG Ads that express HBV-silencing sequences were successful in inhibiting viral replication in HBV transgenic mice [56, 204, 234]. Nevertheless, the transient nature of the silencing and potentially toxic immunostimulation, are problematic. Modification of anti-HBV FG Ads with PEG improved their efficacy by diminishing immunostimulation, toxicity and augmenting target silencing after repeat administration [234]. Use of HD Ads, which have all of the Ad protein coding sequences removed from the vectors, may present an added advantage for the delivery of HBV-silencing expression cassettes. Attenuated adaptive immunity improves long term expression of transgenes delivered with these vectors. However, induction of the innate immune response which does not depend on viral gene expression and is caused by Ad particles in a dose-dependent manner [133, 149, 235, 236],

may occur following administration of HD Ads. The one study to date that reported on use of HD Ads against HBV demonstrated modest silencing [209]. Poor antiviral action of the RNAi expression cassettes may be the reason for the inadequate anti-HBV efficacy. To investigate the utility of HD Ads more comprehensively, we generated recombinant HD Ads that express a Pol III U6 anti-HBV cassette that has previously been shown to be highly effective against HBV [56]. Testing was carried out using HBV transgenic mice [221], which stringently simulate the human condition of chronic HBV infection. Our results show that anti-HBV U6 shRNA expression cassettes, delivered with native or PEGylated vectors, are processed according to intended design and safely suppress HBV replication.

3.2 Methods and materials

3.2.1 First generation adenoviral vectors

Preparation of the FG Ad HBV shRNA 6 vector expressing anti-HBV sequences from a U6 Pol III promoter [56] and the eGFP reporter from a cytomegalovirus immediate early promoter/enhancer (CMV) were previously propagated in our laboratory according to the AdEasy system from Stratagene as described in Section 2.2.1 [220].

3.2.2 Helper-dependent adenoviral vectors

Vectors used for experimentation were HD Ad Δ28, which lacked an RNAi expression construct, HD Ad HBV shRNA 6 and HD Ad HBV shRNA 10 (control, ineffective at HBV knockdown). To generate shuttle plasmids required to form anti-HBV HD Ads, U6 shRNA 6 and U6 shRNA 10 cassettes were initially excised from pG-U6 shRNA 6 and pG-U6 shRNA

10 [56] using AscI and MluI restriction enzymes. Thereafter, the fragments were inserted into the AscI site of a cytomegalovirus (CMV) promoter driven plasmid, pΔ28E4LacZ (Appendix A1-3) [131] to generate pHD Ad HBV shRNA 6 and pHD Ad HBV shRNA 10. HD Ad genomic DNA template required to initiate recombinant virus propagation was prepared and then amplified using published protocols [131] . Briefly, the plasmid form of the HD Ad was digested with PmeI restriction endonuclease to release the HD Ad genome. A HEK 293 producer cell line (116 cells) that expresses bacteriophage P1 site specific Cre recombinase was propagated as detailed in Appendix A1-1. The producer cells were transfected with the HD Ad together the helper virus AdNG163. The helper virus has a packaging signal (Ψ) flanked by *lox*P sites which is excised in the presence of Cre recombinase rendering it unpackageable but still able to replicate and provide the necessary viral proteins *in trans* for the successful packaging of HD Ads. The HD Ads were amplified by performing serial coinfections of 116 cells until a maximum HD Ad titre was obtained. The lysate from the passage having the highest titre was then added to a 150 mm dish of 116 cells and 48 hours later these cells were harvested and used for large-scale production of the HD Ad vector. The mass produced HD Ad vectors were purified from the cell lysate using CsCl gradient centrifugation and dialysis and then stored in a potassium buffer (KPBS) (10 mM K_2PO_4; 150 mM NaCl; 1 mM $MgCl_2$; pH 7.8) supplemented with 5% sucrose.

3.2.3 Titration of adenoviral vectors

Infectious viral particle titre of the FG Ad vector was determined using a method based on the Adeno-X rapid titer kit (Clontech, Mountain View, CA) as detailed in Section 2.2.3. As HD Ads have a LacZ expression cassette it was possible to determine infectious titre by performing staining for β-galactosidase (X-gal) on titrated virus. HEK 293 cells were plated

at 5×10^5 cells/ml with 0.5 ml per well in a 24-well plate. The HD Ad was serial diluted (10^{-2} 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} dilutions) and 50 µl of diluent was added to triplicate wells at the time of plating. The cells were incubated at 37 °C, 5% CO_2 for 48 hours after which the medium was aspirated from the cells and the plate was allowed to air dry for 5 minutes. The cells were fixed to the plate by gently adding 500 µl fixative (1% formaldehyde; 0.5% glutaraldehyde in PBS) to each well and incubated at room temperature for 5 minutes. The fixative was removed and fixed cells washed twice with PBS. The staining solution (4 mM potassium ferricyanide; 4 mM potassium ferrocyanide; 2 mM $MgCl_{2;}$ 0.4 mg/ml X-gal) was added to the wells and incubated at 37 °C overnight. The stain was then washed off with dH_2O and the cells observed under a microscope. The X-gal positive cells stained blue and were counted using a light microscope and the ifu/ml calculated using the formula detailed in Section 2.2.3.

3.2.4 Physical titre

The physical titre is the concentration of viral particles in a vector preparation and was calculated by measuring absorbance at 260 nm (OD_{260}) following lysis of the virions, and correcting for vector genome size. Virion lysis buffer (TE; 0.1% SDS) was added to an aliquot of purified vector to a total volume of 100 µl. A blank sample was prepared by adding the same volume of lysis buffer to the buffer. The samples were vortexed briefly and incubated at 56 °C for 10 minutes. Samples were vortexed vigorously for 1 minute before measuring absorbance on a Nanodrop® ND-1000 (Thermo Fisher Scientific, USA). The physical titre was calculated from absorbance reading at 260 nm (OD_{260}), where vp/ml = (OD_{260}) (dilution factor) (1.1×10^{12}) (36/size of vector in kb).

3.2.5 Quantitative PCR to determine helper virus contamination

Quantitative PCR was used to determine the level of HV contamination in the HD Ad preparations. Absolute quantitation of HD Ad and HV levels was necessary so standards were generated for both Ad types. Using conventional PCR, HV sequences were amplified using primer set: AdNg163 forward 5′ - TGGGCGTGGTGCCTAAAA - 3′ and AdNg163 reverse: 5′ - GCCTGCCCCTGGCAAT- 3′; and HD Ad sequences were amplified with primer set: pΔ28LacZ forward: 5′ - GAAAAAACACACTGGCTTGAAACA - 3′ and pΔ28LacZ reverse: 5′ - TGCCACCTCGTATTTCACCTCTA - 3′. The PCR amplicons were run on a 2% agarose gel, excised, gel purified and ligated into the TA cloning vector pTZ57R/T (Fermentas, USA). The plasmids were prepared using the High Pure Plasmid Isolation kit (Roche, GmbH, Germany) according to manufacturer's instructions and subjected to restriction enzyme digestion with PvuII to screen for correct sized inserts. Plasmids containing the correct sequence were prepared in bulk using the Plasmid Midi Kit (Qiagen, GmbH, Germany) and a serial dilution of the linearised plasmid DNA was used to generate a standard curve (Appendix A1-5 and A1-6).

Viral DNA was isolated from 50 µl of Ad vector preparations using the QIAamp DNA Mini Kit (Qiagen, GmbH, Germany) according to manufacturer's protocol. Sensimix capillary kit SYBR® green (Bioline, London, UK) was used to perform Q-PCR on 5 µl of viral DNA using the relevant primer set. Dilutions of known copy number of the previously generated standards were also included in the run which was done using the following cycling parameters: a hotstart at 95 $^{\circ}$C for 10 minutes and 50 cycles of annealing at 59 $^{\circ}$C for 10 seconds, extension at 72 $^{\circ}$C for 10 seconds and denaturation at 95 ºC for 10 seconds on the Roche Lightcycler v.2. Absolute copy numbers of HD Ad and HV were calculated and the

relative ratio of contaminating HV within a sample was determined. A representative result is shown in (Appendix A1-5 and A1-6).

3.2.6 PEGylation of helper dependent adenoviral vectors

Helper dependent Ad vectors were PEGylated according to a previously published protocol (Mok et al, 2005). The HD Ad was re-suspended in 0.1 mM KPBS (pH 8.2) following dialysis to remove TRIS contained in the buffers used to produce the HD Ads. Approximately 1×10^{12} Ad particles were incubated with 1 ml mPEG-SPA $_{5000}$ at a concentration of 2 mg/ml for 1 hour at room temperature with constant mixing. Ten fold excess L-Lysine, Free Base, Monohydrate (Calbiochem, CA, USA) was then added to quench the reaction, for one hour at room temperature with constant mixing. The PEGylated HD Ad sample was transferred to a Slide-A-Lyzer dialysis cassette (Pierce, Thermo Fisher Scientific, IL, USA) and dialysed overnight in 0.1 mM KPBS (pH 8.2) to remove unreacted mPEG-SPA $_{5000.}$ The PEG-conjugated HD Ad was then concentrated to the original volume by placing the Slide-A-Lyzer containing the PEGylated HD Ad on solid 20 000 Da PEG (PEG $_{20\,000}$) for approx- imately two hours. When the desired volume was reached the Ad PEGylated virus was removed from the dialysis cassette, sucrose was added to a final concentration of 0.5 M and then aliquoted and stored at -80 $^{\circ}$C. Microchip electrophoresis as previously described in Section 2.2.2, was used to show that polymer modification of PEG HD Ad HBV shRNA 6 occurred on approximately 40% of vector hexon proteins.

3.2.7 Quantitative PCR to detect HD Ad genomes in liver

To determine intrahepatic HD Ad Δ28 genome copy numbers, freshly resected liver sections were homogenized with equal volumes of saline. DNA was extracted from 200 μl of the homogenate using the QIAMP Mini extraction kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions. SYBR green-based quantitative PCR was performed as detailed in section 3.2.5 using 50 ng of DNA and HDAdΔ28 specific primers on the Roche Lightcycler v.2. Absolute copy numbers of HDAdΔ28 were calculated from the standard curve generated in Section 3.2.5.

3.2.8 Cell culture and northern blot analysis

Culture, transfection and infection of Huh7 and HEK 293 lines were carried out as has been described in Section 2.2.4.

3.2.9 Adenoviral vector administration and measurement of HBV silencing

HBV transgenic mice [221] were used as a model to determine the effects of RNAi-activating Ads on markers of HBV replication *in vivo*. These experiments were carried out in accordance with protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee. A dose of 5×10^9 infectious Ad particles was injected as a bolus via the tail vein and groups of mice comprised 8 animals each. Circulating VPEs were measured using Q-PCR as detailed in Section 2.2.6.

Mouse serum HBsAg levels were quantified using the MONOLISA® HBs Ag Assay kit (Bio-Rad, CA, USA). The mouse serum was diluted 20-fold with saline and 100 µl of diluent was added per well of an ELISA plate coated with mouse monoclonal anti-HBs antibodies. Conjugate solution which contains monoclonal and polyclonal anti-HBV antibodies bound to peroxidase, was added to each well (50 µl) in the plate, the plate was sealed and then incubated at 37 $^{\circ}$C for 90 minutes. Unbound antibodies were then removed by performing five washes with Washing Solution (Tris NaCl buffer, pH 7.4; 0.004% ProClin™ 300). Development Solution containing the chromagenic substrate tetramethylbenzidine (TMB) was added to each well (100 µl) and the plate was incubated in the dark at room temperature for 30 minutes. The enzymatic reaction was stopped by adding 100 µl of Stopping Solution (1 N H_2SO_4) to each well and the optical density of the samples was measured at 420/690 nm on a Bio-Rad Microplate Reader, Model 680 (Bio-Rad, CA, USA).

Similarly HBcAg was detected in formalin fixed paraffin-embedded liver sections according to previously described standard immunohistochemical techniques [56]. Briefly, sections were rehydrated by the following series of washes: two xylene washes of 5 minutes each, followed by two 100% ethanol washes (5 minutes each), followed by 95% ethanol, 70% ethanol, 50% ethanol, 30% ethanol and finally dH_2O washes (5 minutes each). Endogenous peroxidase activity was quenched by incubating the sections in 0.3% hydrogen peroxide (H_2O_2) for 30 minutes. The sections were then washed in PBS for 20 minutes and then processed further using the Ultra-Sensitive ABC Peroxidase Staining Kit (Pierce, Thermo Scientific, IL, USA). All incubations were performed flat with the sections covered by a glass coverslip. Three drops of diluted protein Blocker Buffer were added onto the sections and covered with glass coverslips and incubated for 20 minutes at room temperature. The glass coverslips were carefully removed and the sections washed once with PBS and the cells were

then permeabilised by adding 200 μl of PBS + 0.5% BSA + 0.25% Triton-X onto each section and incubated at room temperature for 10 minutes. The sections were then washed with PBS before applying rabbit polyclonal Ab against HBcAg (Signet Laboratories Inc., MA, USA) (diluted 1/1000 in Blocking Buffer) which was incubated with the sections at room temperature for 1 hour. The primary Ab was aspirated and the sections washed once with PBS. The buffer was removed and 3 drops of Biotinylated Secondary Antibody was added to each section and incubated for 30 minutes at room temperature. The secondary Ab was aspirated and the sections washed, before adding 3 drops of ABC Reagent (biotinylated horseradish peroxidise (HRP) complexed with avidin) per section which was incubated for 30 minutes at room temperature. The sections were then washed with PBS followed by a dH_2O wash before staining tissue sections with DAB substrate at room temperature until suitable staining developed (2-5 minutes). The sections were rinsed with water, counterstained with haematoxylin, rinsed in water and finally dehydrated by using two rinses of 100% ethanol followed by 2 rinses of xylene before mounting coverslips using Permount (Thermo Fisher Scientific, USA).

3.2.10 β-Galactosidase staining of liver sections

To determine efficiency of HD Ad delivery to hepatocytes, livers were harvested from separate groups of animals 7 days after Ad administration and frozen tissue sections were stained for β-galactosidase activity. The frozen sections were fixed to the glass slides by incubating them in fixative (1% formaldehyde; 0.5% glutaraldehyde in PBS) at room temperature for 5 minutes. The fixative was removed and fixed sections washed twice with PBS at room temperature for 5 minutes each. The sections were then incubated in the staining solution (4 mM potassium ferricyanide; 4 mM potassium ferrocyanide; 2 mM $MgCl_{2;}$ 0.4

mg/ml X-gal) at 37 °C overnight. The stain was washed off with dH_20 and the sections analysed using a light microscope.

3.2.11 *In vivo* shRNA processing

To detect processed anti-HBV sequences, total hepatic RNA was extracted using Tri Reagent® at day 7 after Ad administration. Thereafter, 25 μg RNA was resolved on a 15% denaturing polyacrylamide gel. The gel was stained in 0.5× Tris-Borate EDTA (TBE) containing ethidium bromide at a final concentration of 4 μg/ml for 5 minutes and visualised on a UV transilluminator to confirm equal loading and RNA integrity. Decade RNA molecular weight markers (Ambion, TX, USA) were labelled and run alongside the cellular RNA. The RNA was then transferred and blotted onto positively charged nylon membranes (Hybond-N+, Amersham, NJ, USA). Electroblotting was done at 3.3 mA/cm^2 for an hour at 4 °C in 0.5× TBE using the Semi-Dry Electroblotting Unit Z34.050-2 (Sigma-Aldrich, MO, USA). RNA was immobilised on the nylon membrane by UV crosslinking at 200 000 μJ/cm^2 of energy using a crosslinker (UVP Inc., CA, USA).

The membranes were prehybridised in Rapid-Hyb (Amersham, NJ, USA) at 42 °C for 15 minutes. Probes specific to shRNA 6 and shRNA 10 guide sequences were labelled at their 5′ ends with 20 μCi of [γ-^{32}P] ATP and T4 polynucleotide kinase (Fermenras, MD, USA) and the probes were purified using standard protocols. The relevant probe was hybridised at a final concentration of 10 ng/ml overnight at 42 °C and then subjected to a low stringency wash with 5× SSC, 0.1% SDS at room temperature. This was followed by 2 high stringency washes with 1× SSC, 0.1% SDS solution at 42 °C. The probed membrane was exposed to X-

ray film and then stripped and reprobed with ^{32}P labeled oligonucleotide to detect U6 small nuclear RNA which was used as a control to verify equal loading of the cellular RNA. The probe oligonucleotide sequences were as follows: probe shRNA 6: 5′- TGC ACT TCG CTT CAC CTC - 3′; probe sh10: 5′- GTT CAA GCC TCC AAG CTG - 3′and U6 small nuclear RNA probe: 5′- TAG TAT ATG TGC TGC CGA AGC GAG - 3′.

3.2.12 Cytometric bead array assay

To measure murine TNF-α, IL-6, IL-12p70, MCP-1, IL-10, and IFN-γ, the CBA Mouse Inflammation Kit (BD Biosciences, CA, USA) was employed as detailed in Section 2.2.9.

3.2.13 Serum transaminase assay

Serum enzyme activities of AST and ALT activities were measured using a kinetic assay with an automated photometric analyzer (Roche Diagnostics, Rotkreuz, Switzerland) and were carried out by the South African National Health Laboratory Services.

3.2.14 Statistical Analysis

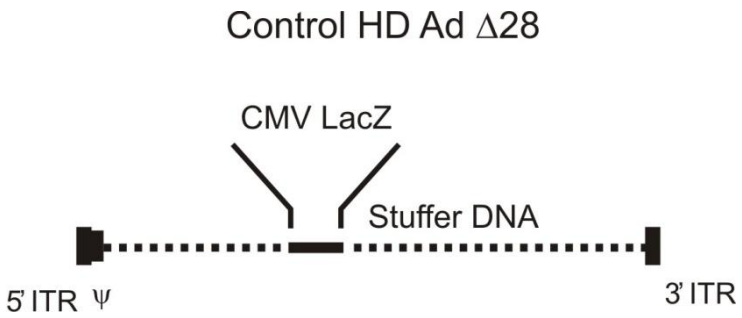
Data are expressed as the mean ± SEM. Statistical differences were considered significant when $P<0.05$ and were determined according to the Mann Whitney or Student 2 tailed t-tests. Calculations were performed with the GraphPad Prism software package (GraphPad Software Inc., CA, USA).

3.3 Results

3.3.1 Structure of HD Ads containing anti-HBV RNAi expression cassettes

Previously described HBV targets of expressed RNAi activators were used in this study as detailed in Section 2.3.1. Expression cassettes were incorporated into the HD Ads according to the schematic illustrations shown in Figure 3.1. All vectors, including the HD Ad Δ28 control, contained ITRs, packaging sequence (Ψ) and a *LacZ* expression cassette. Inclusion of the β-galactosidase reporter was useful for convenient tracking of cells infected with the vectors. In our hands, after CsCl gradient purification, helper virus contamination was routinely less than 0.1%. Expression cassettes comprised a typical arrangement with U6 Pol III promoter, downstream shRNA-encoding DNA and transcription termination signal. To verify that the HD Ad-delivered HBV shRNA6 expression cassette was capable of reprogramming the RNAi pathway according to intended design, Huh7 cells were infected with the recombinant viruses. Extracted RNA was subjected to Northern blot hybridisation using a probe that was complementary to the anticipated anti-HBV guide (Figure 3.2). Results show presence of a band of approximately 21 nt in length, which is also the size of a mature guide, and confirms that shRNAs expressed from HD Ads are processed by the RNAi pathway in cultured liver-derived cells.

A



B

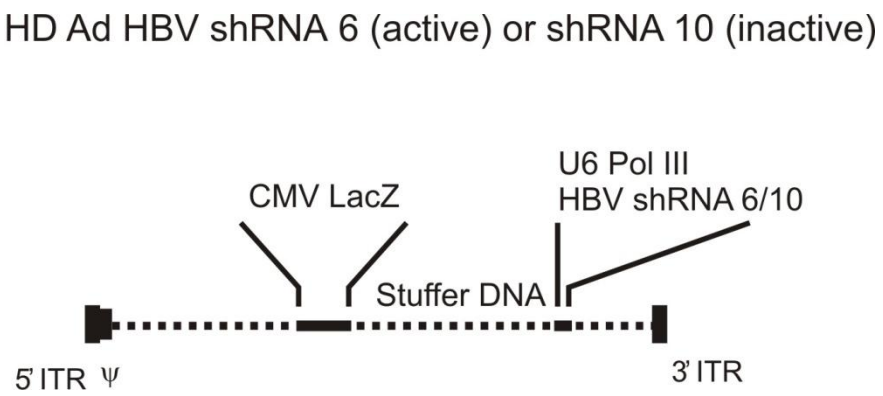


Figure 3.1: Schematic illustration of anti-HBV Ad vectors. **A**. HD Ad Δ28 contains the CMV LacZ-expressing reporter cassette, but no HBV-targeting cassettes. Apart from ITRs and viral packaging signal (Ψ) the remainder of the vector DNA comprises stuffer sequence with all Ad ORFs removed. **B**. Anti HBV HD Ad shRNA 6 and HD Ad shRNA 10 contain active and inactive Pol III U6 shRNA sequences respectively, as well as the CMV LacZ-expressing reporter.

3.3.2 HD Ad-mediated inhibition of markers of HBV replication *in vivo*

To assess efficiency of liver cell transduction by HD Ads, HBV transgenic mice were treated by intravenous administration of a single bolus dose of 5×10^9 infectious vector particles. Approximately 80-95% of hepatocytes were positive for β-galactosidase activity (HD Ads) or eGFP expression (FG Ads) when livers were subjected to analysis at day 7 (Figure 3.3). To measure efficacy of HD Ad HBV shRNA6 and PEG HD Ad HBV shRNA6, groups of HBV transgenic mice received 5×10^9 infectious vector particles. Control groups were either injected with FG Ad HBV shRNA 6, Ads lacking antiviral sequences (HD Ad Δ28) or containing the ineffective U6 Pol III HBV shRNA 10 expression cassette [56]. Blood samples were collected at regular time intervals to measure serum HBsAg (Figure 3.4) and circulating VPEs (Figure 3.5). We and others have reported that these measurements correlate with other markers of HBV replication, such as the intrahepatic concentrations of the viral nucleic acid [56, 204, 234]. Ad vectors that incorporated HBV shRNA 6 expression cassettes (FG Ad HBV shRNA 6, HD Ad HBV shRNA 6 and PEG HD Ad HBV shRNA 6) achieved suppression of HBsAg concentrations (Figure 3.4). The concentration of circulating Hepatitis B VPEs followed a similar trend after mice received the HD Ad HBV shRNA 6 vector (Figure 3.5). Effects on markers of HBV replication were initially observed at 1 week after vector injection when knockdown efficiency of approximately 95% was observed. This markedly decreased HBsAg concentration was sustained for a period of 5 weeks after which the concentration of HBsAg gradually increased to reach a level at 12 weeks that was not statistically significant from the baseline. Results from measurement of serum markers of HBV replication were corroborated by immunohistochemical detection of HBV core antigen (HBcAg) in liver sections of mice that had been treated with the Ad vectors (Figure 3.6.A).

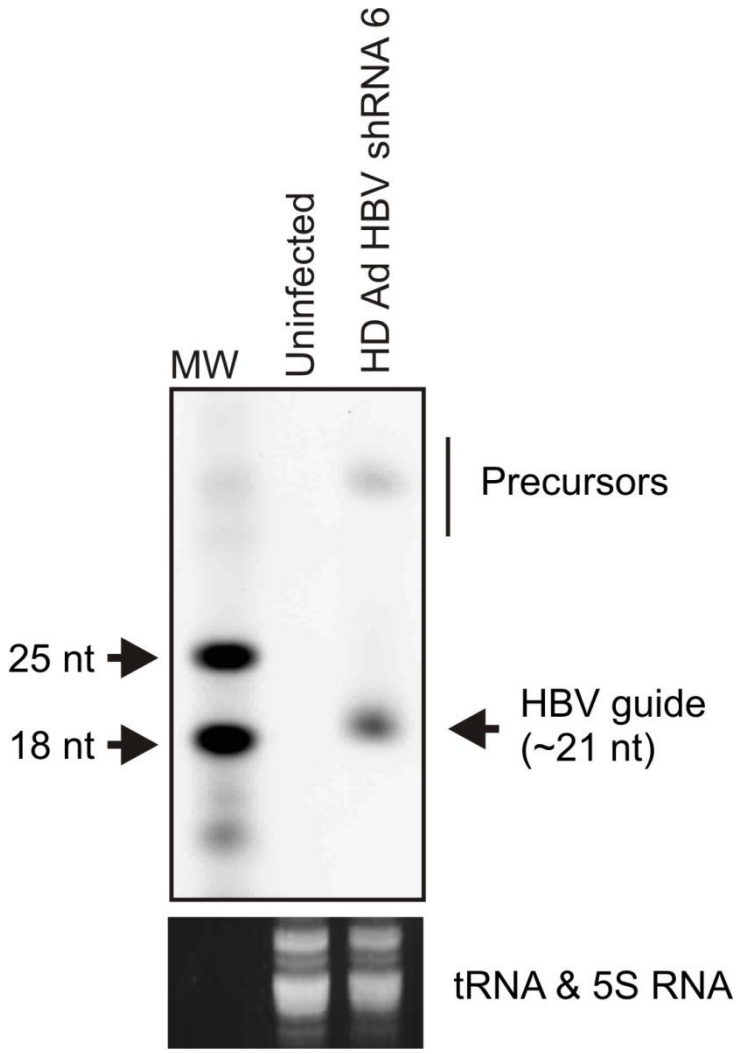


Figure 3.2: Processing of anti-HBV shRNA in infected cultured hepatic cells. RNA extracted from HD Ad-transduced liver-derived Huh7 cells was subjected to Northern blot hybridisation using a probe that was complementary to the intended shRNA 6-derived guide strand. DNA oligonucleotides were used as markers to determine approximate molecular weights of guide strands. Prior to blotting, the gel was stained with ethidium bromide to confirm equal loading of the lanes.

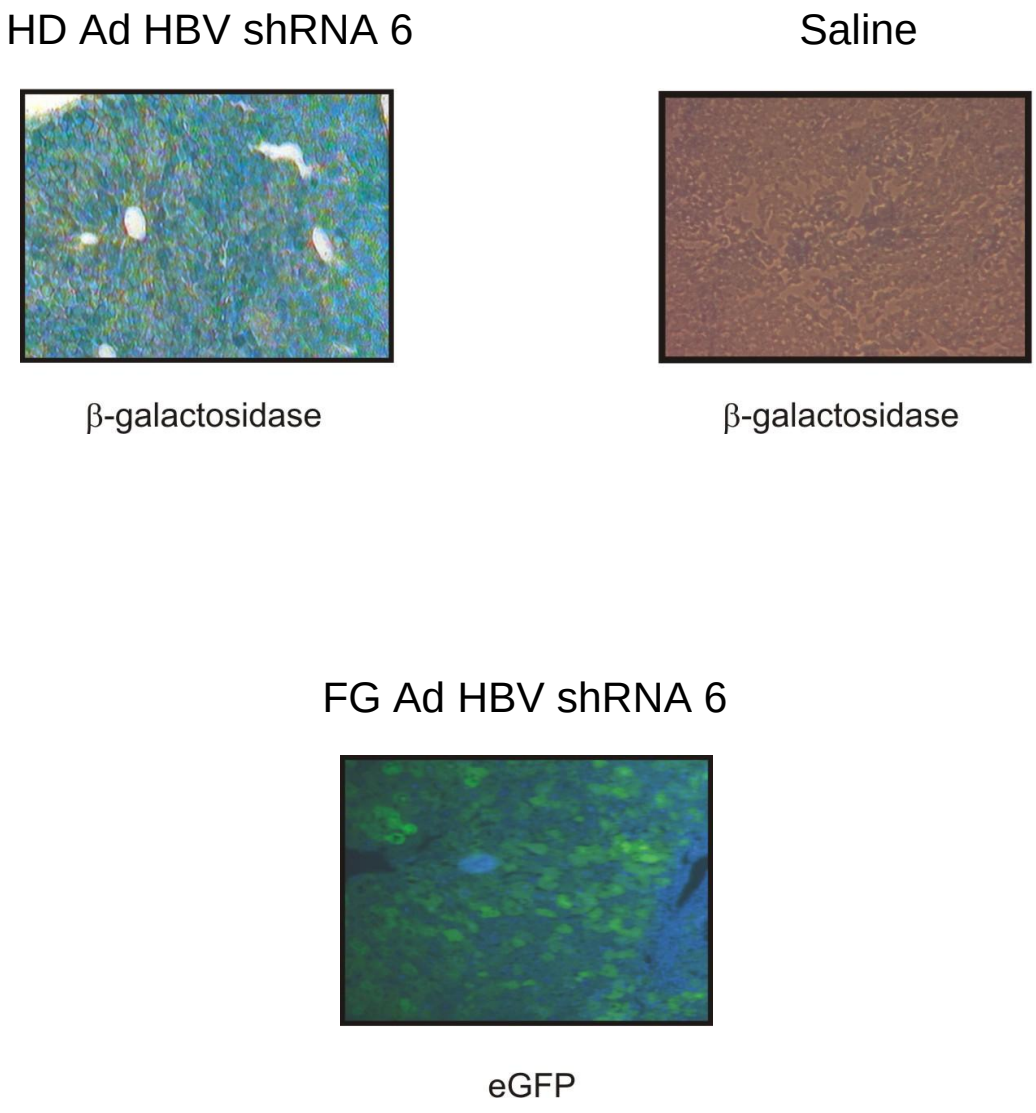


Figure 3.3: HD Ad transduction efficiency in mice. β-galactosidase (positively stained blue cells in the top panel) and eGFP (positively stained green cells in the bottom panel) reporter gene expression was detected in frozen sections of livers 7 days after vector administration.

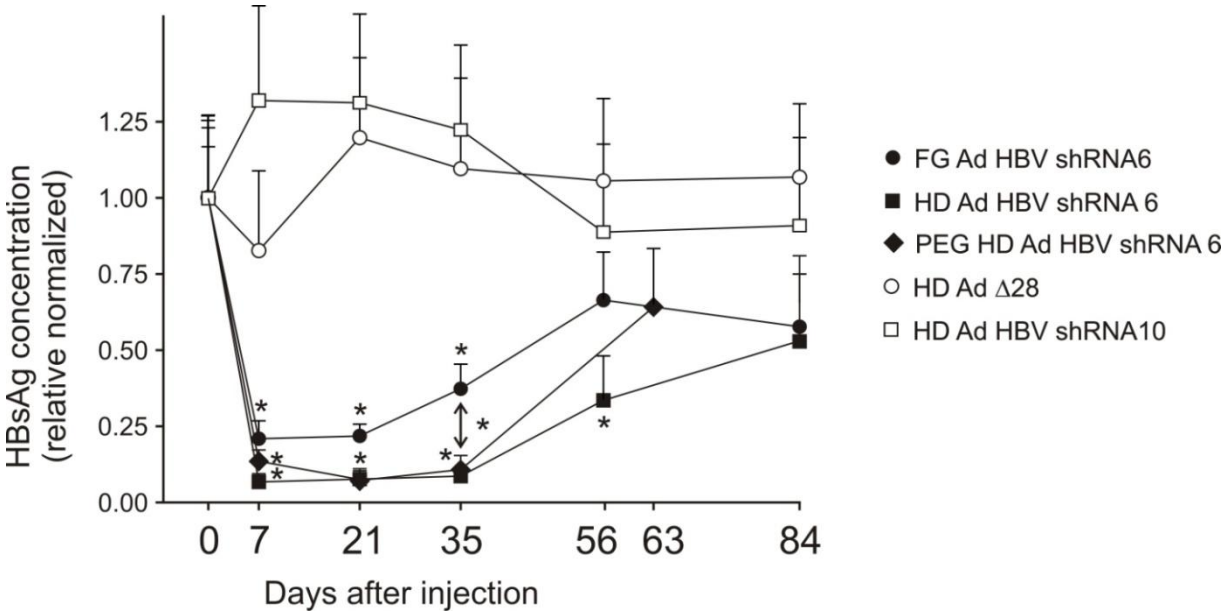


Figure 3.4: Effects of intravenously administered first generation and HD Ads on circulating HBV surface antigen levels in HBV transgenic mice. Animals received a single administration of infectious Ad particles at commencement of the experiment. Control animals were either injected with FG Ad shRNA 6, backbone HD Ads lacking RNAi expression cassettes or a previously described ineffective anti-HBV sequence (shRNA 10). Titres of HBsAg were determined using ELISA. Results are expressed as the mean (±SEM) from 8 mice. Statistically significant differences (* $p<0.05$) between shRNA 6-expressing vectors and controls are indicated and were determined according to the Student's 2 tailed paired t-test.

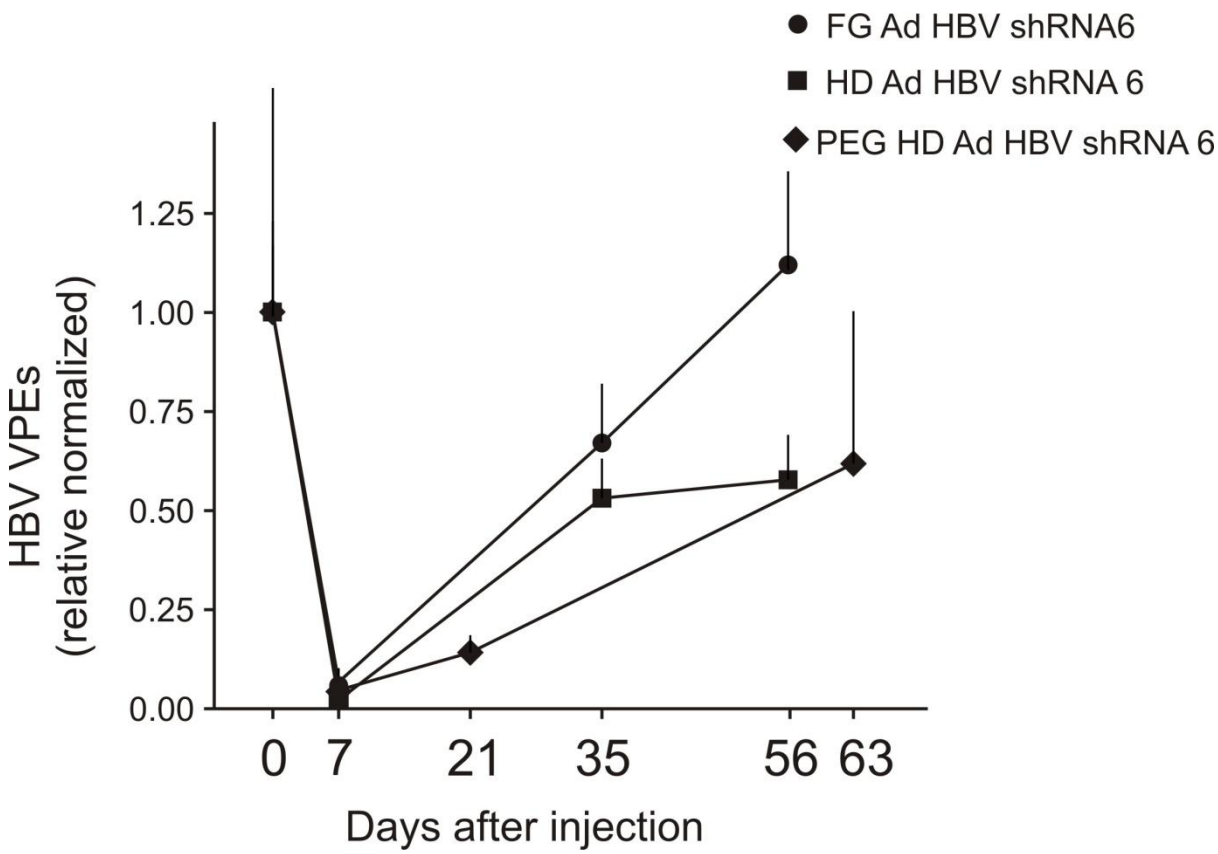


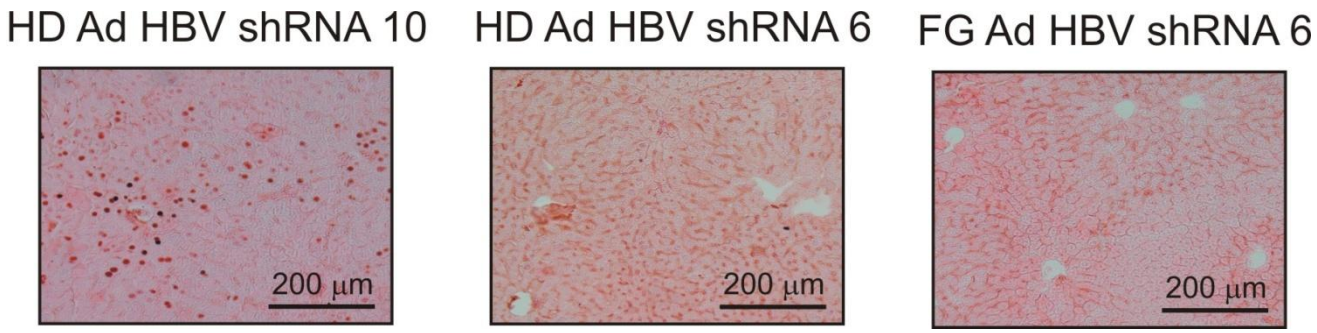
Figure 3.5: Effects of intravenous administration of FG Ad and HD Ads on circulating HBV VPEs. Following administration of Ads, titres of VPEs at the indicated time points were measured using Q-PCR. Representative results are expressed as the mean (±SEM) from 8 mice. Statistically significant differences (* $p<0.05$) between shRNA 6 expressing vectors are indicated and were determined according to the Student's 2 tailed paired t-test.

Representative stained low power fields from sections prepared from animals that had been injected with the Ad vectors one week previously showed that HBcAg was barely detectable in animals that received HD Ad HBV shRNA 6. However, cells staining positive for HBcAg were abundant in mice that received the HD Ad HBV shRNA 10 control vector.

To assess concentrations of HD Ad genome equivalents in the livers of mice during the time course of the experiments, Q-PCR was carried out on DNA extracts from livers at various time points after the vector injection. The results show that for mice treated with either the control (HD Ad Δ28) or HD Ad HBV shRNA6 vectors, concentrations of HD Ad genome equivalents in the livers rapidly decreased at time points beyond 1 week after injection (Figure 3.6.B). These data suggest that immunostimulatory effects of the *LacZ* reporter gene [237] may account for the elimination of the Ad genomes. In accordance with this, others have reported more sustained transgene expression following HD Ad-mediated delivery with vectors lacking a reporter gene [185, 238]. Thus, although presence of the reporter gene is convenient for tracking infection of hepatocytes in vivo, durability of the silencing effect may be diminished by presence of the *LacZ* cassette and further studies need to be done to clarify this issue.

Northern blot hybridisation analysis of RNA, which had been extracted from mouse livers seven days after injection, was performed to assess processing of anti-HBV shRNAs *in vivo* (Figure 3.7). Hybridisation was carried out using radiolabeled probes complementary to HBV-targeting U6 shRNA 6- or U6 shRNA 10- derived mature guides. The expected 21 nt fragment was detectable in the livers of animals that had been infected with HD Ad HBV shRNA 6, PEG HD Ad HBV shRNA 6 and FG Ad HBV shRNA 6 (Figure 3.7, left panel).

A



B

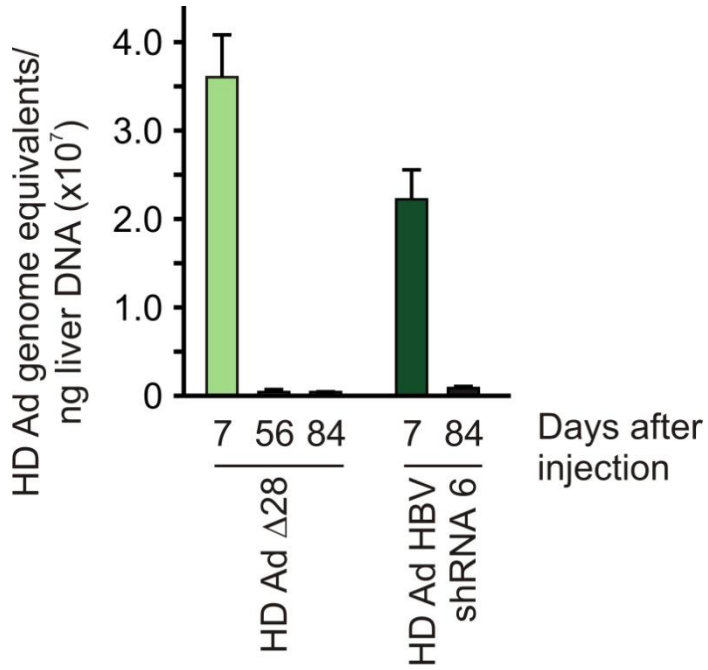


Figure 3.6: Anti-HBV efficacy of first generation and HD Ads. **A**. Representative low power fields of liver sections following immunohistochemical staining for HBcAg. **B**. Quantitative-PCR was carried out on DNA extracted from livers of mice that had been sacrificed at the indicated time points after vector administration. Hepatic concentrations of Ad genome equivalents per ng of liver DNA are indicated.

There was no evidence of incompletely processed higher molecular weight shRNA intermediates. Also, absence of a signal in the lanes loaded with RNA extracted from HD Ad HBV shRNA 10 and HD Ad Δ28 LacZ indicated that the band was specific to livers transduced with U6 shRNA 6 cassettes. Similar analysis carried out with a probe complementary to the shRNA 10-derived guide revealed a low concentration of the 21 nt fragment (Figure 3.7, right panel). Some higher molecular weight bands were also detectable, which are likely to represent incompletely processed shRNA10 sequences. HBV silencing that is achievable with shRNA6-containing Ads correlates with the efficient processing of this cassette in vivo. In contrast, the poor maturation of shRNA 10 reflects poor functional efficacy of HD Ad HBV shRNA 10.

3.3.3 Hepatotoxicity and cytokine secretion following intravenous injection of HD Ad HBV shRNA 6

As a general indicator of toxicity, HBV transgenic mice receiving HD Ad HBV shRNA 6 and saline as a control were weighed regularly during the time course of the investigation. All mice tolerated administration of the vectors well. There were no observable adverse effects, and animals receiving the Ads gradually gained weight in a manner that was similar to that of the control mice receiving saline (Figure 3.8 A). Activity of ALT, a more sensitive indicator of Ad-mediated hepatotoxicity, was measured 48 hours after vector administration (Figure 3.8 B). The results showed that ALT activity was significantly higher in animals that had received the FG Ad shRNA 6 vector when compared to the mice receiving the HD Ad panel of vectors or saline.

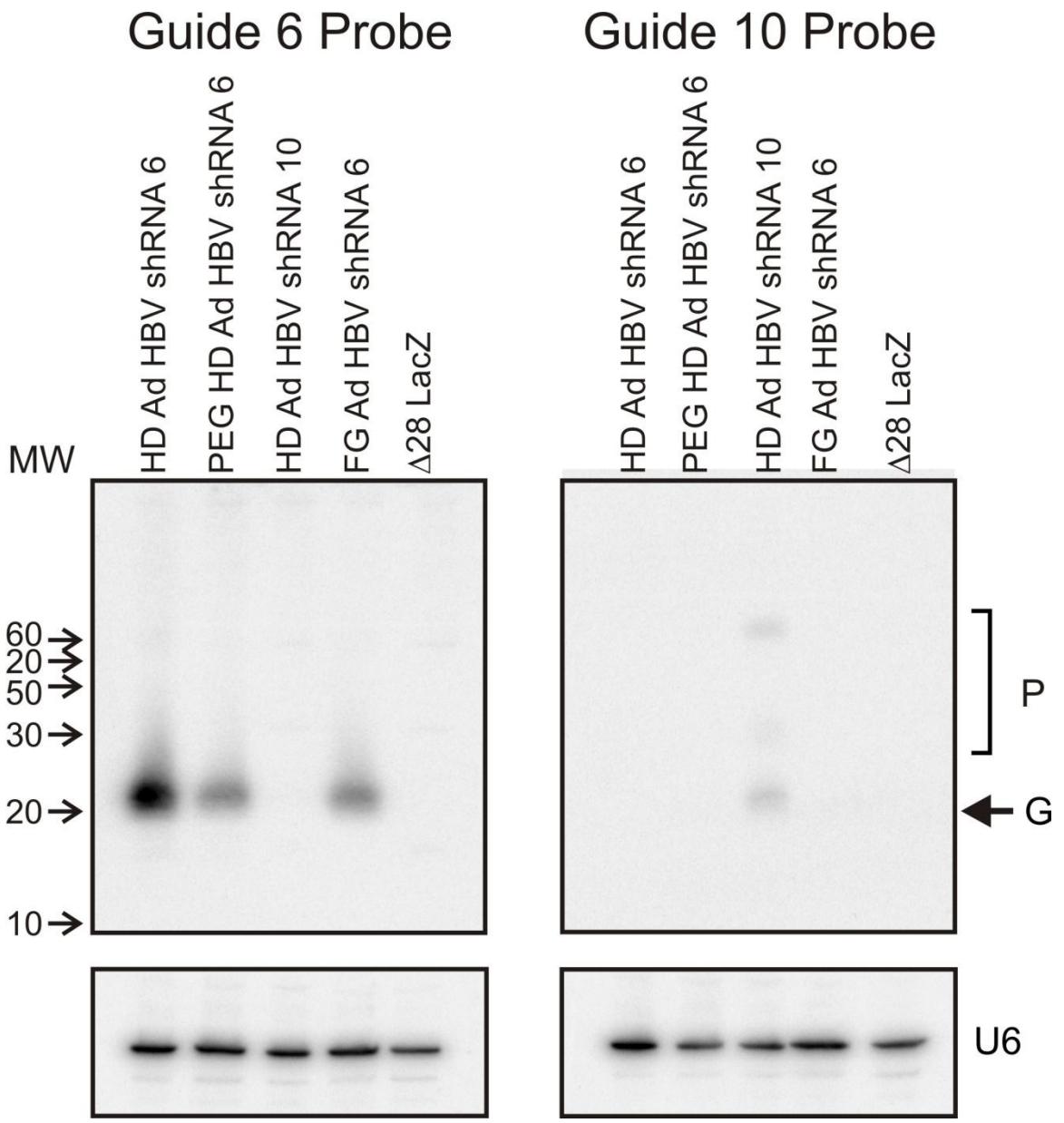
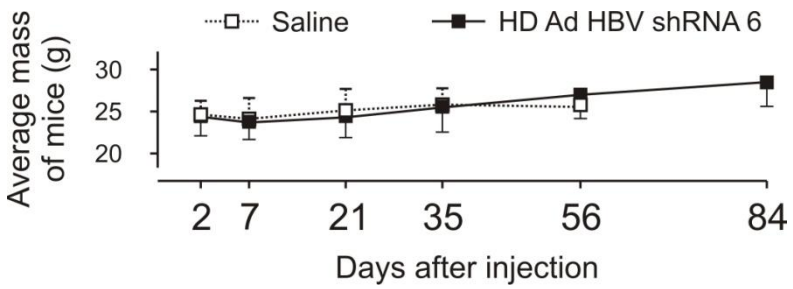


Figure 3.7: Northern blot hybridization analysis of RNA extracted from mice livers that had been injected with indicated Ad vectors 7 days previously. The probe used for the blot shown on the left panel was complementary to the predicted guide sequence derived from the shRNA 6 antiviral sequence, and that on the right for the shRNA 10 sequence. Equal loading of the lanes was verified by stripping and reprobing the blot with a labeled oligonucleotide complementary to U6 small nuclear RNA.

A



B

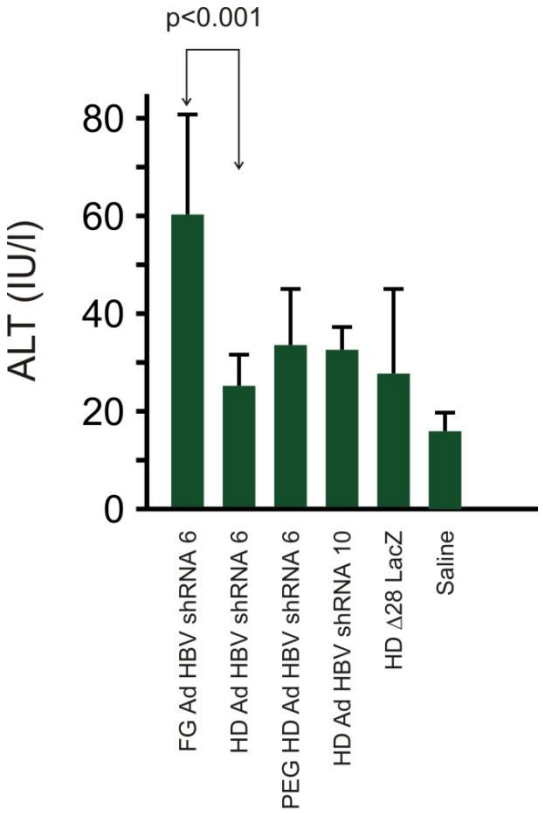


Figure 3.8: Weight of mice and markers of hepatic inflammation following Ad administration. A. Average mass (±SEM) of groups of mice receiving HD Ad HBV shRNA 6 or saline during the period of the experimental investigation. **B**. Serum activities of ALT were measured 2 days following injection with saline, indicated HD Ads or FG Ad preparations. Mean values are represented together with ±SEM.

Since activation of the innate immune response is primarily responsible for Ad-mediated toxicity (reviewed in [163, 233, 239]), we set out to measure markers of innate immune-stimulation following HD Ad administration. Innate immunostimulation is triggered by both infectious and non-infectious Ad capsids particles, and there is no requirement for transgene expression [149, 155]. Ideally Ad vector preparations compared in a study should have an equal number of infectious particles and total viral particles which is technically very challenging. Theoretically, preparations of Ads that are enriched with intact gene transducing infectious particles should achieve the intended therapeutic effect with less immunostimulation than formulations containing the same number of infectious particles, but a higher number of viral particles. To assess this and determine the safety of the anti-HBV HD Ads described here, release of proinflammatory cytokines triggered by similar numbers of anti-HBV Ad infectious particles but different titers of total Ad particles were compared.

Innate immunostimulatory effects of the HD Ad shRNA 6 injections containing 5×10^9 infectious and 8.44×10^{10} total particles, were compared to immunostimulation by FG Ad shRNA 6 preparations containing 5×10^9 infectious and 6.23×10^{11} total particles. Importantly, the dose of infectious particles was the same as that used to inhibit viral replication and express anti-HBV sequences in the transgenic model (Figures 3.4 to 3.6). Serum concentrations of a selection of cytokines and chemokines were determined at baseline and at time points of 6 hours and 48 hours after vector administration. The panel of cytokines, comprising TNF-α, IL-6, IL-12p70, MCP-1, IL-10, IFN-γ, were measured using a CBA assay (Figures 3.9 and 3.10). Six hours after injection of the FG Ad preparation containing an excess of total Ad particles, IFN-γ, IL-12p70, IL-6 and MCP-1 were significantly elevated. Administration of the HD Ad vector preparations resulted in attenuated release of IFN-γ, IL-12p70, IL-6 and MCP-1. By 48 hours after injection, serum IFN-γ, IL-12p70, IL-6 and

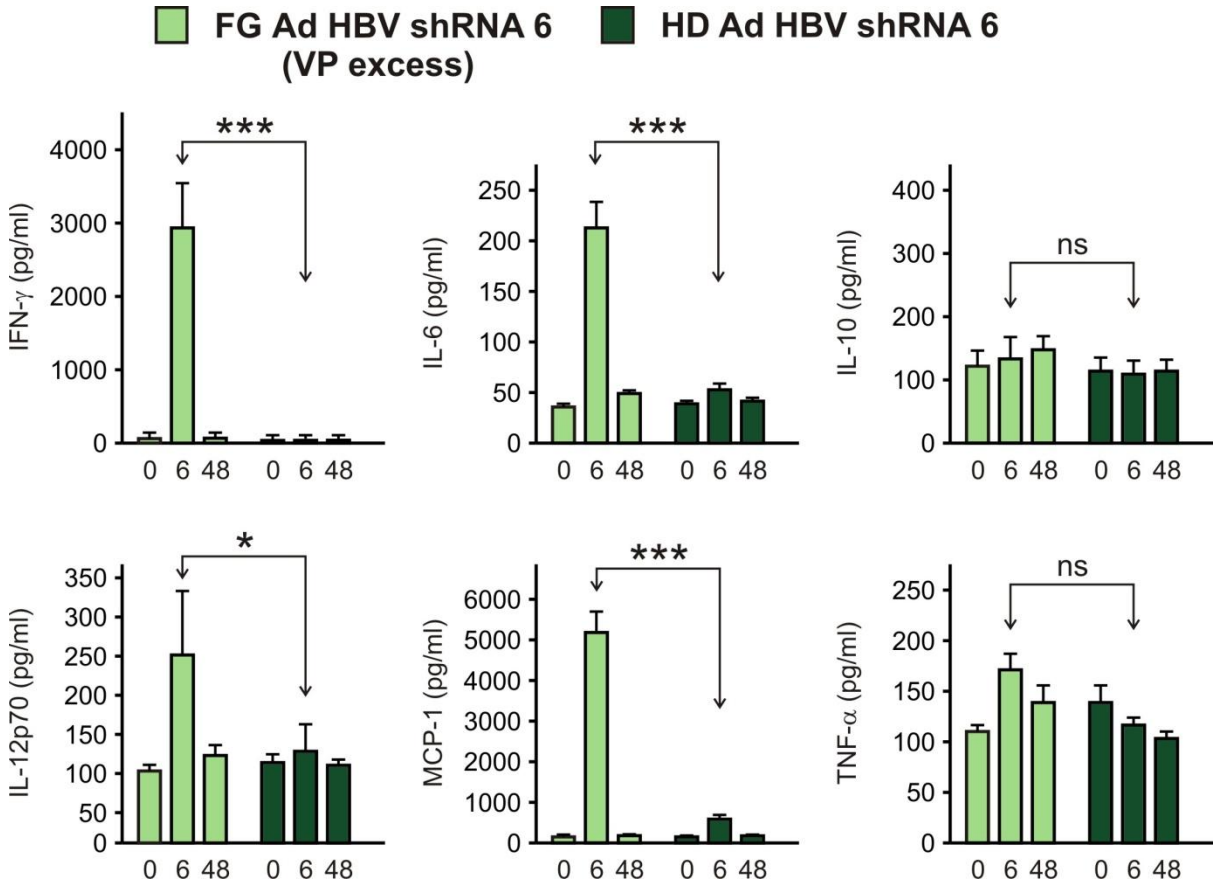


Figure 3.9: Effects of HD Ads on serum cytokine concentrations following vector administration to HBV transgenic mice. Mean concentrations of TNF-α, IL-12, MCP-1, IL-10, IL-6 and IFN-γ in groups of mice at baseline (time 0), 6 and 48 hours following administration of unmodified HD Ads. FG Ad preparations, which contained similar numbers of infectious particles (5×10^9) but an approximately ten-fold excess of viral particles, were used as a positive control to induce an innate immune response. Results are expressed as the mean (±SEM) from 8 mice. Statistical significance was determined according to the Mann Whitney paired t-test.

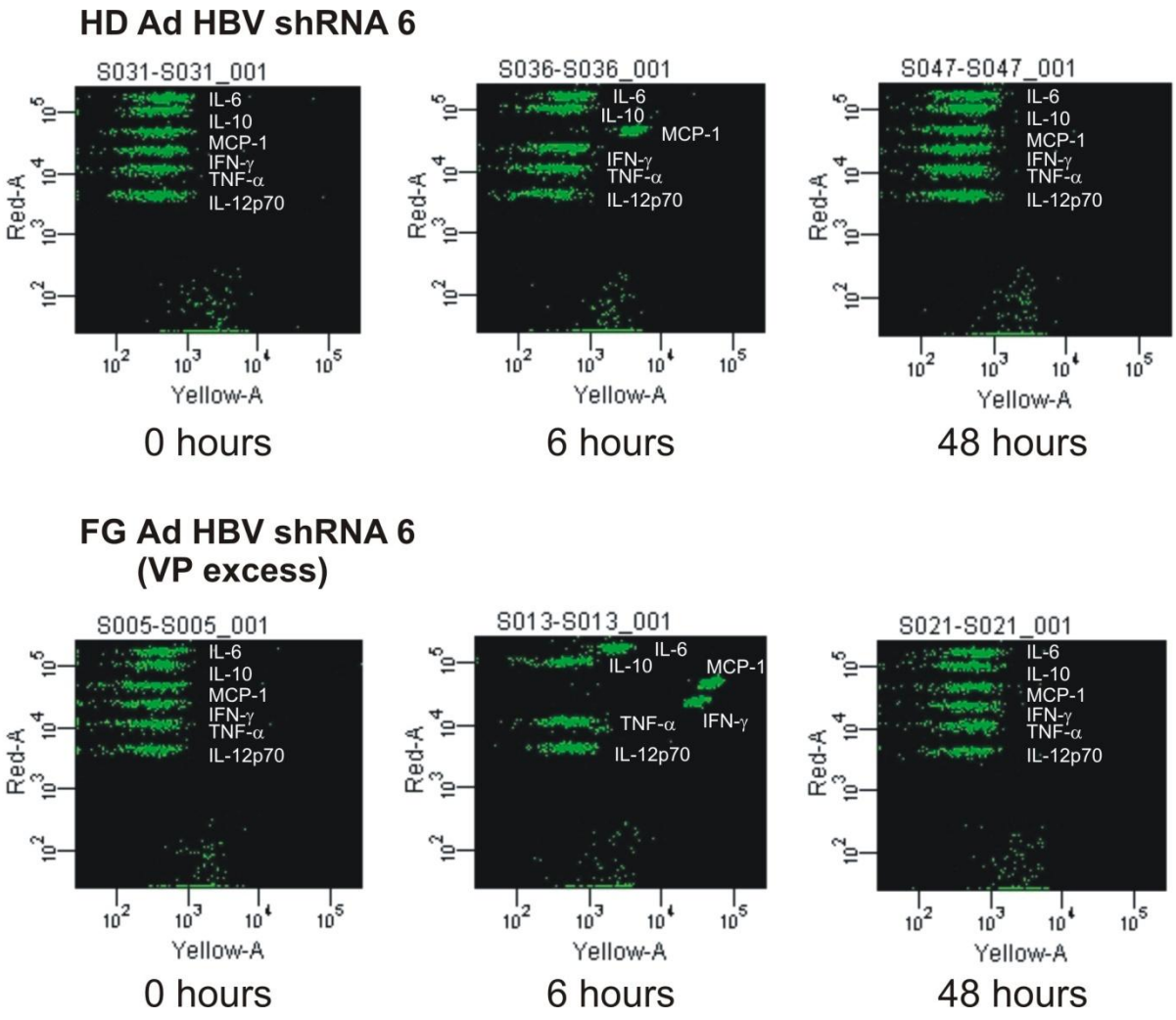


Figure 3.10: Flow cytometry plots showing effects of Ads on serum cytokine concentrations following vector administration to HBV transgenic mice. Representative data from CBA assay of TNF-α, IL-12p70, MCP-1, IL-10, IL-6 and IFN- γ concentrations following HD Ad and FG Ad injections.

MCP-1 concentrations reverted to baseline control levels in animals receiving the FG vectors. Results from analysis of PEG HD Ad HBV shRNA 6 were similar to those of the unmodified vector (not shown). The cytokines that were elevated following FG Ad administration contribute to innate immune response activation that potentially results in toxicity caused by Ads (reviewed in [239]), and this corresponds to the release of transaminase markers of hepatotoxicity (Figure 3.8 B). Although our observations may reflect specific characteristics of the HBV transgenic mice studied here, and other analyses have demonstrated Ad-mediated activation of additional proinflammatory cytokines [92, 240, 241], the data support the notion that administration of a dose of total HD Ad particles containing sufficient infectious particles to exert a therapeutic effect, but with a lower concentration of total Ad particles, results in an attenuated effect on innate stimulation.

3.4. Discussion

Currently available treatment for HBV infection is capable of suppressing viral replication in chronic carriers, but rarely eliminates the virus completely [25]. A requirement for sustained inhibition of HBV replication poses significant challenges and alternative more effective methods of countering the virus remain a priority. Demonstration that HBV replication is susceptible to silencing by reprogramming the RNAi pathway (reviewed in [45, 101, 242-244] has prompted exploration of the use of exogenous anti-HBV RNAi activators for therapeutic effect. Both expressed and synthetic RNAi activators have been used successfully to inhibit HBV replication. Although synthetic siRNAs have advantages of easier dose control, these RNAi effectors have a requirement for repeated administration to achieve sustained silencing. This is potentially problematic for the treatment of the chronic infection caused by HBV. When transcribed from a DNA template, expressed RNAi activators are

generated in a manner that may be longer lasting and better suited to inhibition of pathology-causing genes of chronic diseases. Nevertheless, achieving efficient and safe delivery of expression cassettes remains challenging. In this study, we have explored the utility of HD Ads for delivery of an anti-HBV Pol III expression cassette that efficiently silences viral replication in a stringent murine model of the human condition. The efficient hepatotropism of these vectors is useful for delivery of HBV silencers to the liver. After infection of liver-derived cells, the shRNA was expressed and processed according to the intended design. Transgene delivery to liver cells *in vivo* by HD Ads was highly efficient, and markers of HBV replication were safely silenced. Although silencing of HBV replication was sustained for a period of eight weeks, more durable expression has been achieved when using HD Ads to deliver a transgene to hepatic tissue *in vivo* [184, 185, 245]. Elimination of the vector genome sequences mediated by an immune response to the β-galactosidase reporter may have contributed to shortening the period of expression of antiviral sequences in this study. Utility of liver specific anti-HBV expression cassettes in HD Ads that lack reporter cassettes are therefore currently being investigated.

A previous study, also aiming to assess inhibition of HBV replication using U6 Pol III RNAi-activating expression cassettes in HD Ads, reported unimpressive silencing of HBV replication [209]. A reason for the inadequate efficacy may have been that the anti-HBV sequences were not potent and emphasises the importance of selecting optimal antiviral sequences for therapeutic application. A further concern of using RNAi expression cassettes is their potential for disruption of the endogenous microRNA pathway. This was shown to cause lethal toxic effects in livers of mice that received recombinant AAVs that transduced hepatocytes with U6 Pol III shRNA expression cassettes [232]. We observed that HD Ad-mediated transduction of hepatocytes with similar cassettes was well tolerated and the

dramatic toxic effects reported by Grimm and colleagues could not be discerned. A factor contributing to this may have been the low dose of HD Ad infectious particles that was required to effect HBV silencing reported here.

The strong innate immunostimulatory effects of Ads [92, 149, 229, 240, 241, 246, 247] are a significant concern, and this has impeded development of these vectors for therapeutic application. Negative consequences of immunostimulation include toxicity, limited duration of transgene expression and reduced efficacy after repeated vector administration. Although FG Ad and HD Ad particles are capable of inducing an innate immune response [149, 240, 241], removal of ORFs to form HD Ad vectors is useful to diminish longer term humoral and cell mediated adaptive immunity [239]. Compared to preparations containing an excess of total Ad particles, we found that markers of hepatotoxicity and release of proinflammatory cytokines were diminished following administration of the HD Ad preparations. This result may have been enhanced by the reduced number of total viral particles in the HD Ad preparations. Results also showed that release of proinflammatory cytokines and the duration of silencing efficacy were not influenced by PEGylation. Although PEGylation of Ads by the covalent attachment of mPEG-SPA did not reveal obvious advantages in this study, the variability in the PEGylation efficiency may be the cause and this needs to be clarified in future studies. The production of HD Ad vectors involves the use of TRIS-containing buffers which are not ideal as they contains primary amines which reacts with the N-hydroxysuccinimide groups of the PEGylation reagents and decrease the reaction efficiency. The use of maleimides which readily react with the thiol groups on cysteine can eliminate this problem. Site directed PEGylation of genetically manipulated HVR sequences having cysteines inserted in hexon capsid proteins may yield modified Ad vectors that are more stable with predictable PEGylation. HD Ad vectors having cysteine residues which are

amenable to maleimide PEG modification are presently been investigated. Polymer modification is also potentially important to evade interaction with Coxsackie Adenovirus receptors (CARs) and complement 1 receptors on human erythrocytes [248].

The high capacity of HD Ads for incorporation of transgenes is a useful property. In addition to RNAi activating cassettes, other antiviral or immunomodulatory cassettes may be utilised to generate multifunctional vectors for HBV therapy. Recent advances in the use of transcription activator like effector nucleases (TALENs) and Zinc finger nucleases have been impressive [249]. These engineered gene-modifying enzymes may be particularly useful in combination with HBV-targeting RNAi activators to disable the stable HBV cccDNA minichromosome. Ability of HD Ads to accommodate additional antiviral sequences is potentially an advantage over other vectors that have a limited capacity for insert size. Although refinements in vectorology are needed before the full potential of RNAi-based HBV therapy is realised, our data show that HD Ads potentially have utility for hepatotropic delivery of gene silencers.

4 CONCLUSIONS

The limited success of existing HBV treatment and the demonstration that HBV replication is susceptible to RNA interference prompted investigations aimed at utilising this pathway for therapeutic application in HBV-infected individuals. Success of this therapy will ultimately be determined by a vector's ability to deliver the RNAi effectors safely to the liver. The use of viral vectors and in particular Ad vectors are proving to be promising vehicles for delivering the RNAi effectors to HBV infected livers. In recent years a deeper understanding of Ad-host interaction has emerged, enabling the development of strategies to overcome Ad vector immunogenicity and toxicity. Employing the strategy of PEG modification of the Ad vector in combination with the use of third generation HD Ad vectors we were able to demonstrate safe and efficient delivery of anti-HBV RNAi effectors *in vivo*, in a murine transgenic model of HBV replication.

Chemical modification of Ads by the covalent attachment of PEG to immunostimulatory capsid proteins has become a common approach to improve efficacy of therapeutic drugs. In this study, monofunctional PEG was employed which reacts with the ε-amino terminal of lysine residues on Ad capsids. Conjugation of Ads with 5 kDa PEG does not compromise hepatic transduction in mice and does not interfere with Ad's ability to interact with blood factors, such as clotting factor X, which are essential for efficient liver delivery (reviewed in [203]). The PEG modified FG Ads described here successfully transduced mouse livers, had attenuated innate and adaptive immune responses and efficiently expressed RNAi effectors. The benefits of PEGylating Ads carrying RNAi effectors targeting HBV in an *in vivo* mouse model has not been previously described.

The random attachment of PEG to the lysine amino acid residues on the Ad capsid has the disadvantage of yielding heterogeneous conjugates, which may cause inconsistencies between production lots. There is also an abundance of lysines on capsid proteins so if an excessive number of these moieties are PEG-modified, crosslinking occurs between PEG molecules and aggregates may form which can inhibit Ad function (reviewed in [134, 250]). In recent years a more targeted and controlled approach to PEGylation has been adopted whereby specific immunogenic sites are modified. Thiol groups are introduced by inserting cysteine residues within immunogenic sites of the Ad capsid [190, 251, 252]. The use of maleimide-PEG which reacts with the thiol groups on the inserted cysteines, does not compromise the Ad vector function and improved *in vivo* liver delivery is achieved. Difficulty was experienced in achieving consistently PEGylated HD Ads which might be attributed to the heterogeneous covalent attachment of PEG to lysine residues on Ads. Future work will include the generation of HD Ad vectors where the HVR of the hexon protein has cysteine residues inserted, which will make them amenable to the more efficient maleimide-PEGylation.

Data from small animal models makes it difficult to predict the outcome in human trials as a result of some fundamental species differences. Mice do not express CAR on their red blood cells (RBCs), however human RBCs express high levels of this receptor [182, 183, 253] which plays a significant role in Ad5 sequestration and reduced liver transduction. Several groups have shown that PEGylation can disrupt Ad5 interacting with CAR (reviewed in [177]) so future work will investigate whether PEG modification of our Ad vectors will inhibit interaction and subsequent sequestration of the Ads by human RBCs in an *in vitro* model [182].

HD Ad vectors have a distinct advantage over FG Ad vectors as they induce a limited cytotoxic T-cell response and may achieve prolonged transgene expression. HD Ad transgene expression exceeding 2 years has been described in mouse and rat livers [168, 205]. In the study presented here, HBV inhibition resulting from shRNA 6 expression was sustained for 8 weeks with a low dose of HD Ad administration as compared to 3 weeks when the same RNAi effectors were delivered with FG Ads. The HD vector utilized in the study has a β-galactosidase reporter sequence included which has been documented to be immunogenic [254] and this might account for the shorter duration of the anti-HBV effects. Work presently being conducted in our lab involves testing HD Ad vectors lacking the β-galactosidase reporter sequence to assess whether more sustained inhibition of HBV can be achieved using these vectors. Recent preliminary data would suggest that the HD Ad vectors β-galactosidase is immunostimulatory as mice injected with these vectors produced Abs against β-galactosidase in an ELISA assay (see Appendix A1-7).

Strategies other than the manipulation of capsid proteins have been used to avoid Ad sequestration and interaction with the host immune system. Clinically relevant approaches include the administration of anti-inflammatory drugs such as methylprednisolone or dexamethasone ([176]. A strategy that has been successfully employed in non-human primates is the use of balloon occlusion catheters for non-systemic delivery of HD Ads. A catheter is inserted into the inferior vena cava and then inflated thereby preventing outflow of blood from the hepatic vein which increases intrahepatic pressure allowing for improved HD Ad transduction. The HD Ad is administered locally to the hepatic artery resulting in highly efficient liver delivery enabling the use of lower, less toxic doses of HD Ad vector and long-term expression of the transgene [185]. This technique however, is invasive and technically difficult. Importantly, we were able to demonstrate that by using low doses of HD Ad

expressing anti- HBV shRNA 6, sustained knockdown of HBV was achieved with minimal hepatic toxicity. Although host immunostimulation by recombinant Ads still remains an obstacle in the clinical use of these vectors, the future success of Ad vectors in gene therapy will be governed by the optimisation of immune evading strategies such as those employed in the *in vivo* studies described in this thesis.

An added advantage of developing safer HD Ad vectors is their large transgene carrying capacity as compared to other viral vectors. Multifunctional cassettes for HBV therapy could be incorporated which carry the RNAi effector together with expressed immunomodulating elements. Anti-HBV therapies are largely ineffective as they target the replicating virus and not the 'latent' episomal cccDNA. Transcription activator like effector nucleases (TALENs) and Zinc finger nucleases which effectively cleave the cccDNA have recently been developed [249, 255]. These enzymes are generated from large expression cassettes which could be accommodated by HD Ad vectors. As techniques improve and relevant human models become available, the utility of Ad vectors in delivering RNAi effectors that target HBV could become a clinical reality.

5 APPENDIX A

A1 Standard laboratory techniques

A1-1 Tissue culture

Reagents

RPMI medium (RPMI)

One litre of RPMI was made as follows: 10.4 g RPMI-1640 (Life Technologies, Paisley, UK) and 2 g $NaHCO_2$ were dissolved in ddH_20, the pH was adjusted to 7.2 and the media was then sterilised by filtration.

Dulbecco's modified Eagle media (DMEM)

One litre of DMEM was made as follows: 13.38 g DMEM (Life Technologies, Paisley, UK) and 3.7 g $NaHCO_2$ were dissolved in ddH_20, the pH was adjusted to 7.2 and the media was then sterilised by filtration.

Eagle's minimum essential medium (EMEM) (without $CaCl_2$)

One litre of EMEM was made as follows: 11 g EMEM (Sigma-Aldrich, MO, USA) was dissolved in ddH_20, the pH was adjusted to 7.2 and the media was then sterilised by filtration.

Minimal essential medium (MEM) (with Earle's salts)

One litre of MEM was made as follows: 9.53 g MEM (Life Technologies, UK) and 2.2 g $NaHCO_2$ were dissolved in ddH_20, the pH was adjusted to 7.2 and the media was then sterilised by filtration.

1000× Pen/Strep

One gram Streptomycin and 0.61g Penicillin (both Sigma-Aldrich, MO, USA) were dissolved in 10 ml dH_20 and the solution was sterilised by filtration.

Hygromycin B (Sigma-Aldrich, MO, USA)

Foetal bovine serum (FBS), heat-inactivated (Life technologies, Paisley, UK; Biochrom, Berlin, Germany)

Trypsin/EDTA (Life Technologies, Paisley, UK)

Protocol for routine propagation of cells

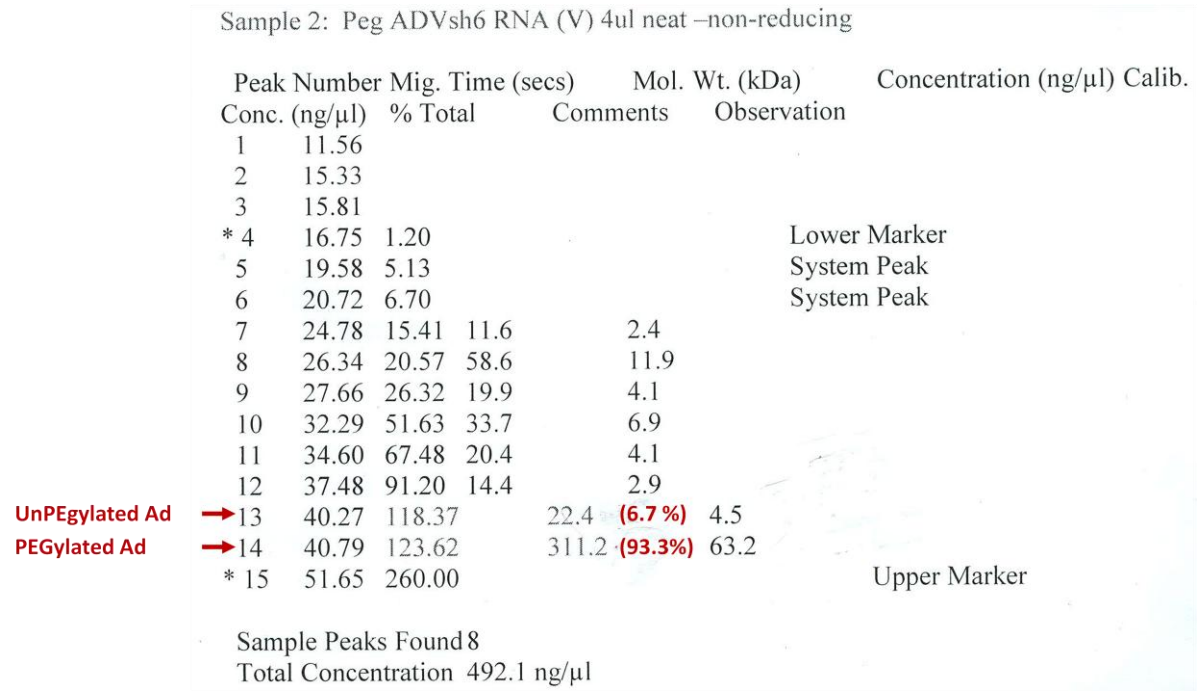
Huh7 and HEK 293 cells were maintained in RPMI and DMEM growth media respectively, supplemented with 5-10% FBS and antibiotics. The cells were maintained in a humidified incubator at 37 $^{\circ}$C and 5% CO_2 and sub-cultured upon reaching a density of 90-100%. Huh7 cells were washed with saline and subsequently incubated for 5 minutes at 37 $^{\circ}$C in saline containing 0.01% EDTA. The saline/EDTA solution was removed and an appropriate volume (depending on culture surface) of 0.5× trypsin was added and the cells were incubated for an additional 5 minutes. Trypsinisation was not necessary for HEK 293 cells and the cells were gently tapped off the culture surface. Dislodged cells were aspirated from the flask and a third of the volume was transferred to a sterile flask of the same size containing fresh culture media. The flasks were returned to the incubator and the media was replenished every 48 hours until the cells needed to be passaged.

The 116 cell line was maintained in 150 cm^2 flasks supplemented with 10% FBS, 0.1 mg/ml hygromycin B, 100 units/ml penicillin/streptomycin, and 2 mM L-glutamine. Cells were passaged twice a week by detaching the cells from the culture surface by gentle tapping, followed by dividing the aspirated cells between three sterile flasks. The cells were incubated at 37 $^{\circ}$C in 5% CO_2.

Adenoviral infection of cells

For Northern blot experiments 2×10^6 Huh7 cells were plated into 25 cm^2 flasks in antibiotic-free RPMI media supplemented with 5% FBS. The cells were immediately infected with Ad at a moi of100 pfu/ml and transferred to a 37 $^{\circ}C$, 5% CO_2 incubator for 48 hours. The media was then aspirated from the cells and RNA extracted.

A1-2 Experion microchip electrophoresis analysis



Sample 2: Peg ADVsh6 RNA (V) 4ul neat –non-reducing

	Peak Number	Mig. Time (secs)		Mol. Wt. (kDa)		Concentration (ng/µl)	Calib.
	Conc. (ng/µl)	% Total		Comments	Observation		
	1	11.56					
	2	15.33					
	3	15.81					
	* 4	16.75	1.20			Lower Marker	
	5	19.58	5.13			System Peak	
	6	20.72	6.70			System Peak	
	7	24.78	15.41	11.6	2.4		
	8	26.34	20.57	58.6	11.9		
	9	27.66	26.32	19.9	4.1		
	10	32.29	51.63	33.7	6.9		
	11	34.60	67.48	20.4	4.1		
	12	37.48	91.20	14.4	2.9		
UnPEgylated Ad →	13	40.27	118.37		22.4 (6.7 %)	4.5	
PEGylated Ad →	14	40.79	123.62		311.2 (93.3%)	63.2	
	* 15	51.65	260.00			Upper Marker	

Sample Peaks Found 8
Total Concentration 492.1 ng/µl

Figure A.1: Experion microchip electrophoresis. Representative result from capillary electrophoresis analysis performed on PEGylated and unPEGylated Ad shRNA 6 samples. An estimated 93.3% of Ad virus was PEGylated in this sample.

A1-3 p28ΔE4LacZ

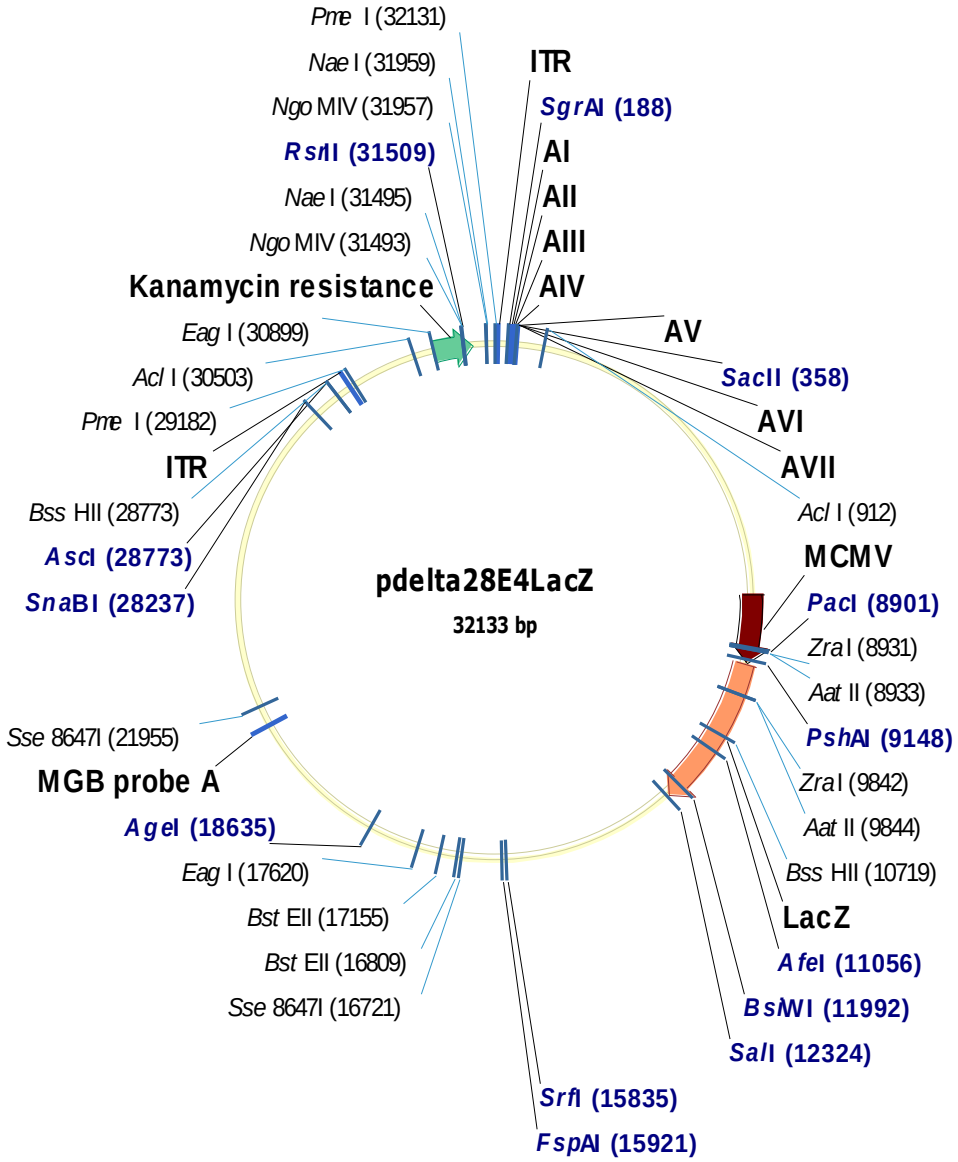


Figure A.2: Plasmid map of p28ΔE4LacZ used for cloning of RNAi effector sequences to generate HD Ads. The 32133 bp plasmid is kanamycin resistant for selection and has a LacZ marker sequence inserted.

A1-4 HBV Q-PCR standard curve

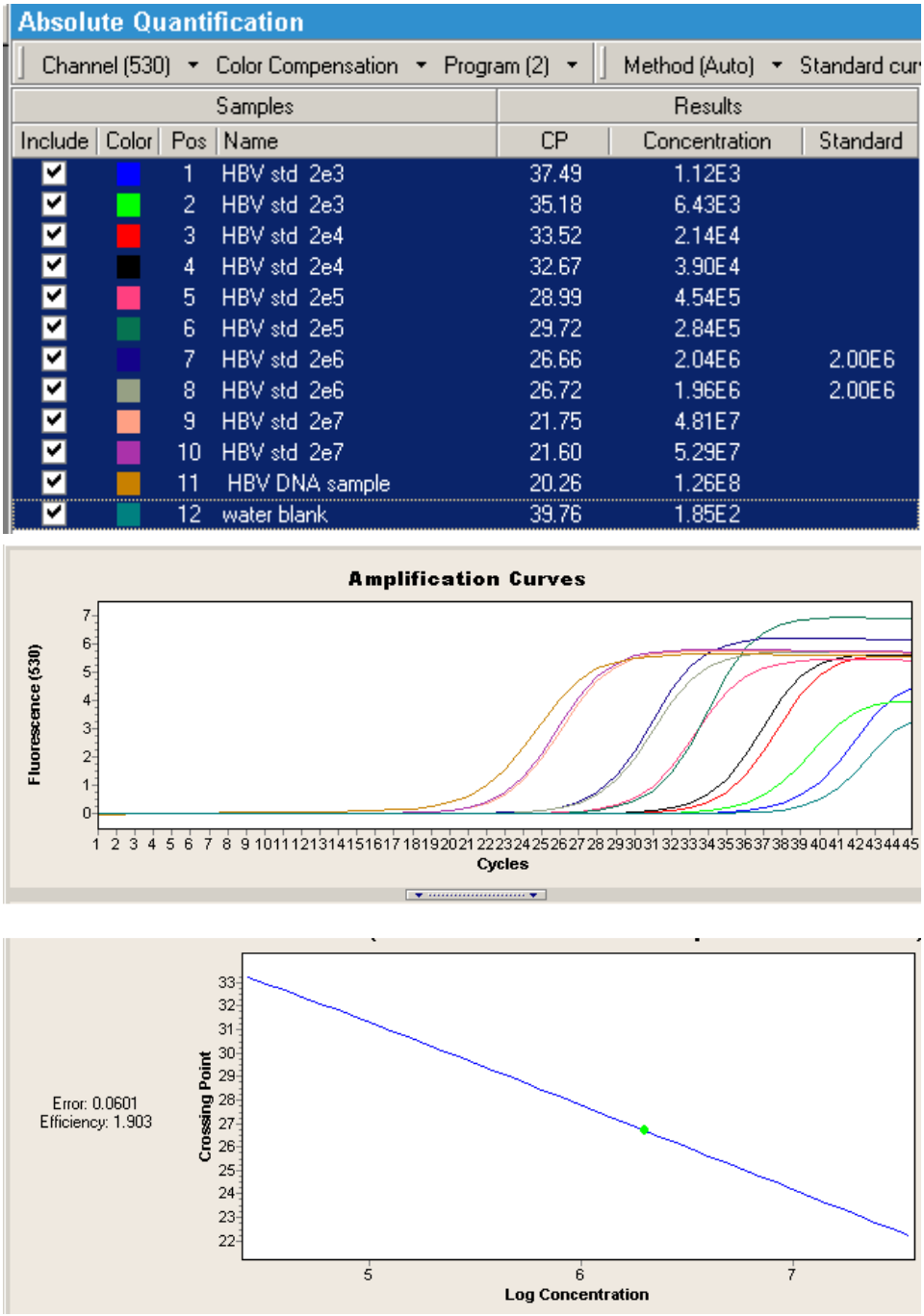


Figure A.3: HBV Q-PCR standard curve. Representative data of Q-PCR standard curve generated from a commercial HBV standard obtained from the National Institute of Biological standards and Controls (NIBSC, Hertfordshire, UK). Absolute VPEs in mouse serum samples could be calculated from the curve.

A1-5 Helper virus Q-PCR standard curve

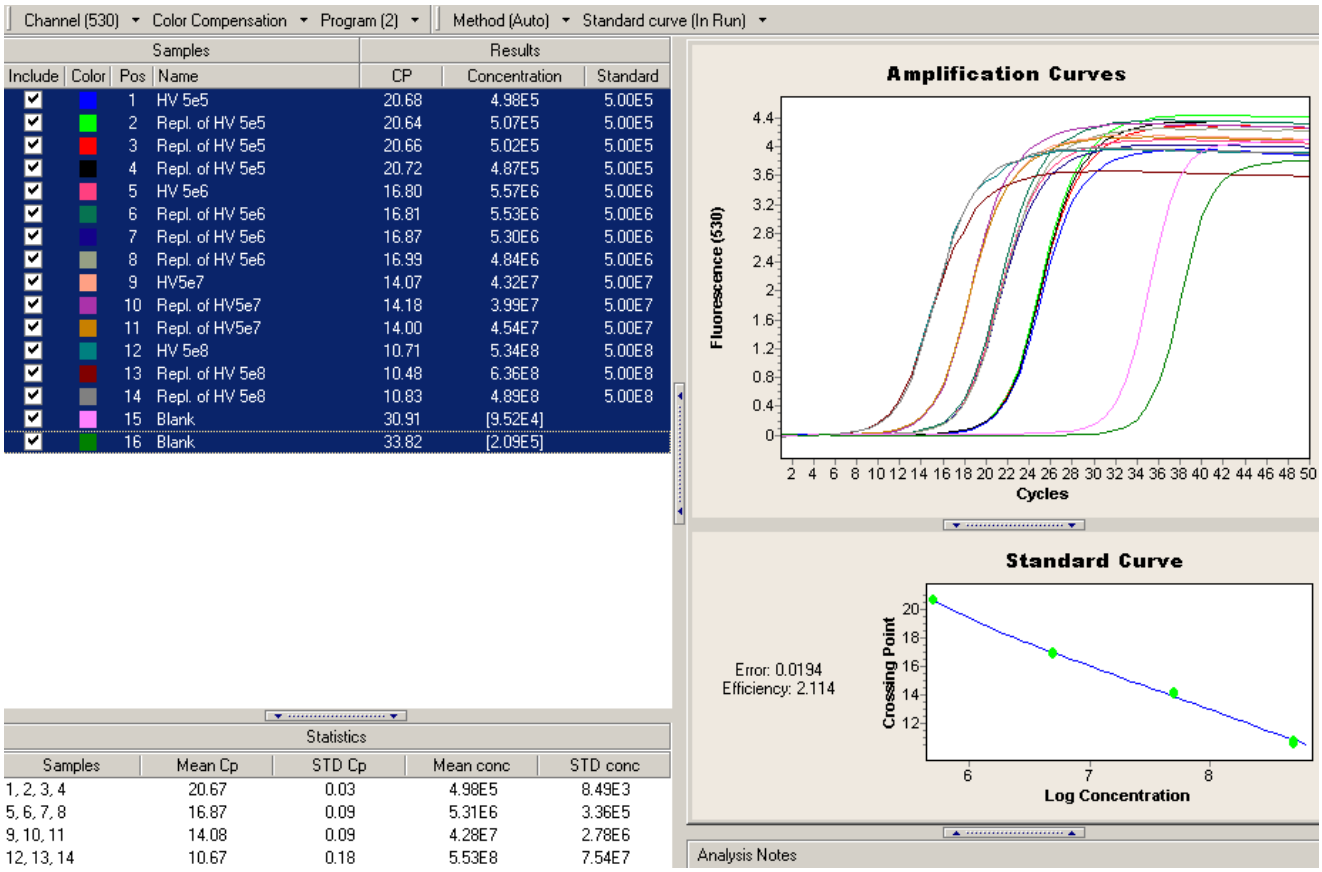


Figure A.4: HV Q-PCR standard curve. The curve was generated from HV of known concentration. The absolute amount of contaminating HV in viral preparations could be calculated using the standard curve.

A1-6 HD Ad Q-PCR standard curve

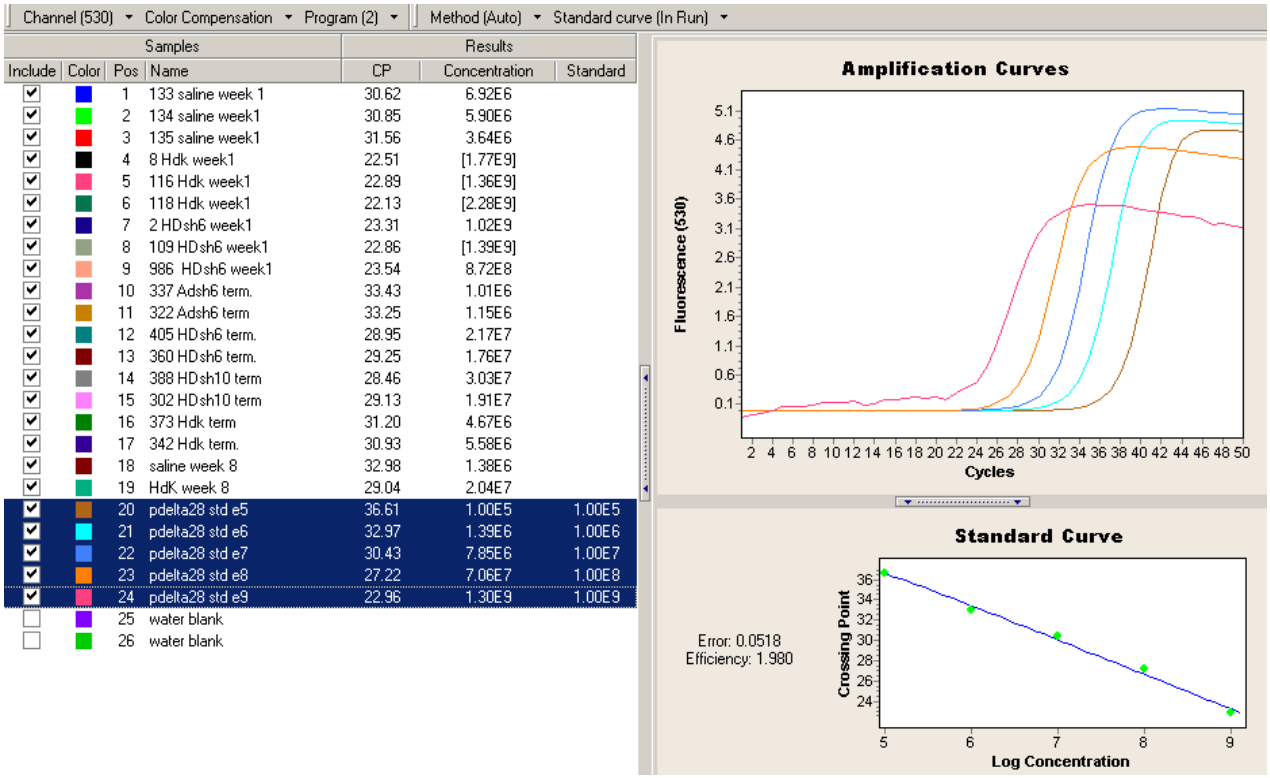


Figure A.5: HD Ad Q-PCR standard curve. The curve was generated from HD Ad of known concentration. The absolute amount of HD Ad in viral preparations could be calculated using the standard curve.

A1-7 β-galactosidase ELISA

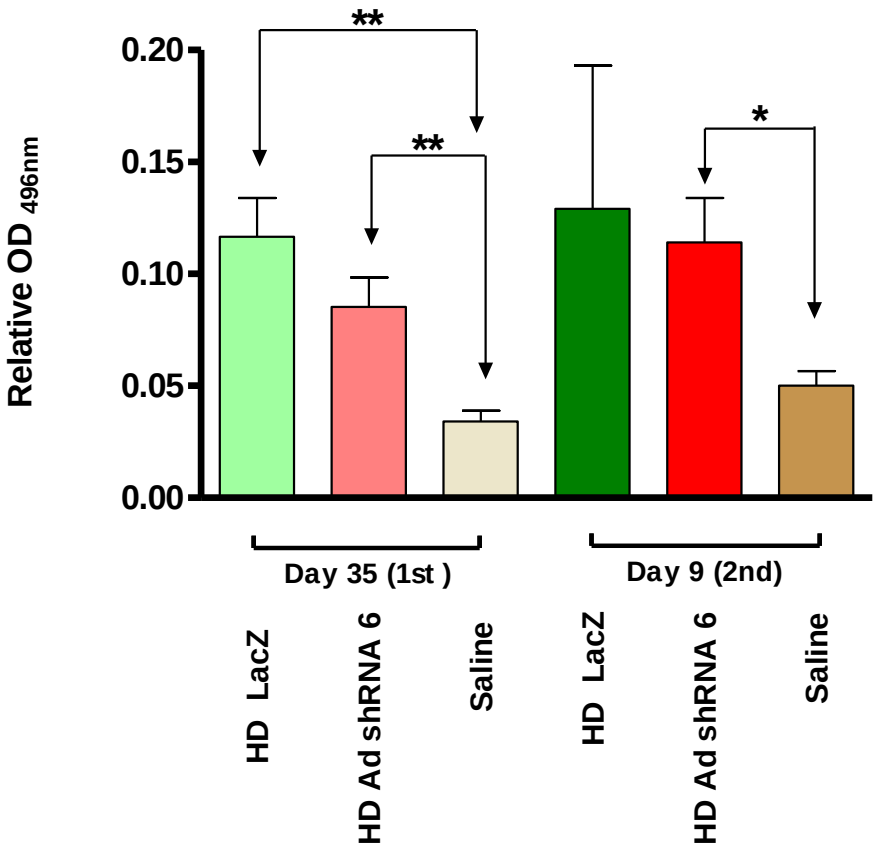


Figure A.6: Relative serum anti-β-galactosidase immunoglobulin concentrations after vector administration to mice. Total anti-β-galactodidase protein-specific antibodies were measured by ELISA 35 days after first administration (left) and 9 days after the second administration (right) of vectors. Mean relative optical density readings (±SEM) are given. Statistically significant differences (* $p < 0.05$, ** $p < 0.005$) are indicated and were determined using the Student's 2 tailed paired t-test.

A2 Animal ethics clearance certificate

AESC 2007

Please note that only typewritten applications will be accepted. Should additional space be required for section "I" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Patrick Arbuthnot

b. Department: Antiviral Gene Therapy Research Unit, Department of Molecular Medicine & Haematology

c. Experiment to be modified / extended **AESC NO:**

Original AESC number	2008/24/03		
Other M&E's :			

d. Project Title: Testing anti hepatitis B virus efficacy of RNA interference activators in a transgenic mouse model of viral replication.

e. Number and species of animals **originally approved**:		834
f. Number of additional animals **previously allocated on M&Es**:		0
g. Total number of animals **allocated** to the experiment to date:		314
h. Number of animals **used** to date:		314
i. Specific modification / extension requested: Extension for use of allocated animals approved under original application		
j. Motivation for extension: Experiments originally described in the approved submission to the AESC are ongoing and have been delayed because of technical difficulties encountered in the development of the antiviral sequences. Our intention to carry out the investigations remain a priority and this request is to allow use of the remaining 520 mice that have been allocated to the study during the coming 2 years.		

Date: 03 March 2010 Signature: Patrick Arbuthnot

RECOMMENDATIONS:

Approved

Date: 03/03/2010 Signature: ..... Chairman, AESC

A3 Publications

A3-1 Selected research publications derived from work presented in this thesis

doi:10.1016/j.ymthe.2005.10.013

ARTICLE

Effective Inhibition of HBV Replication *in Vivo* by Anti-*HBx* Short Hairpin RNAs

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Available online 5 December 2005

Exploiting the RNA interference pathway has shown promise for developing novel and effective treatment of hepatitis B virus (HBV) infection. To advance this approach, we analyzed the antiviral efficacy of a panel of 10 Pol III U6 promoter-encoded short hairpin RNAs (shRNAs) that target conserved sequences of the oncogenic *HBx* open reading frame. To facilitate intracellular processing, the shRNAs included mismatches in the 25-bp stem region and a terminal loop of miRNA-23. Two shRNAs (shRNA 5 and shRNA 6) showed knockdown of HBV markers by 80–100% in transfected hepatocytes and also in a murine hydrodynamic injection model of HBV replication. Intracellular processing of hairpin RNA with the intended strand bias correlated with antiviral efficacy. Moreover, markers of HBV replication were inhibited without inducing genes associated with the nonspecific interferon response. To assess the antiviral efficacy of the shRNAs in a context that is similar to natural HBV infection, shRNA-encoding cassettes were tested against the virus in a HBV transgenic murine model. When delivered using recombinant adenovirus vectors, U6 shRNA 5 and U6 shRNA 6 mediated significant HBV knockdown. Collectively, these observations indicate that U6 shRNA 5 and U6 shRNA 6 are promising candidates for therapy of chronic HBV infection.

Key Words: RNAi, short hairpin RNA, HBV, hydrodynamic injection, HBV transgenic mice, recombinant adenovirus, interferon response

INTRODUCTION

Persistent hepatitis B virus (HBV) infection remains an important global public health problem with an estimated 6% of the world's population chronically infected with the virus [1–3]. The clinical course of HBV infection is not constant and may be influenced by properties of individual viral variants [4,5]. For example, chronic infection with HBV subgenotype A1, which is hyperendemic to South Africa, is associated with a particularly high risk of hepatocellular carcinoma [6]. Licensed treatments for HBV infection, which include interferon-α and nucleoside (lamivudine) and nucleotide (adefovir) analogues, produce a long-term response in only a minority of chronic carriers [7,8]. The continued search for an effective therapy to prevent life-threatening complications thus remains a priority. HBV has a compact genome (Fig. 1) that makes it well suited to developing antiviral therapies that are based on nucleic acid hybridization. Overlapping open reading frames (ORFs) cover the entire viral genome [9] and conserved regions may encode more than one protein as well as HBV *cis* elements required for viral replication. *HBx* is an example of a multifunctional HBV sequence. It encodes the HBx protein, which has been implicated in HBV-mediated hepatocarcinogenesis (reviewed in [1]) and is required for HBV replication [10,11]. *HBx* also overlaps with the 3′ end of the polymerase ORF, direct repeats that are required during virus reverse transcription, and regions important for transcription control (basic core promoter and negative regulatory elements). HBV transcripts are usually unspliced and have a common 3′ end that includes *HBx* (Fig. 1). The key role of HBx in viral replication and hepatocarcinogenesis, together with the presence of the conserved *HBx* sequence in all of the HBV transcripts, makes it a good target to develop nucleic acid hybridization-based therapy.

Exploiting the RNA interference (RNAi) pathway has shown exciting promise for the development of novel

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doi:10.1016/j.ymthe.2005.10.013

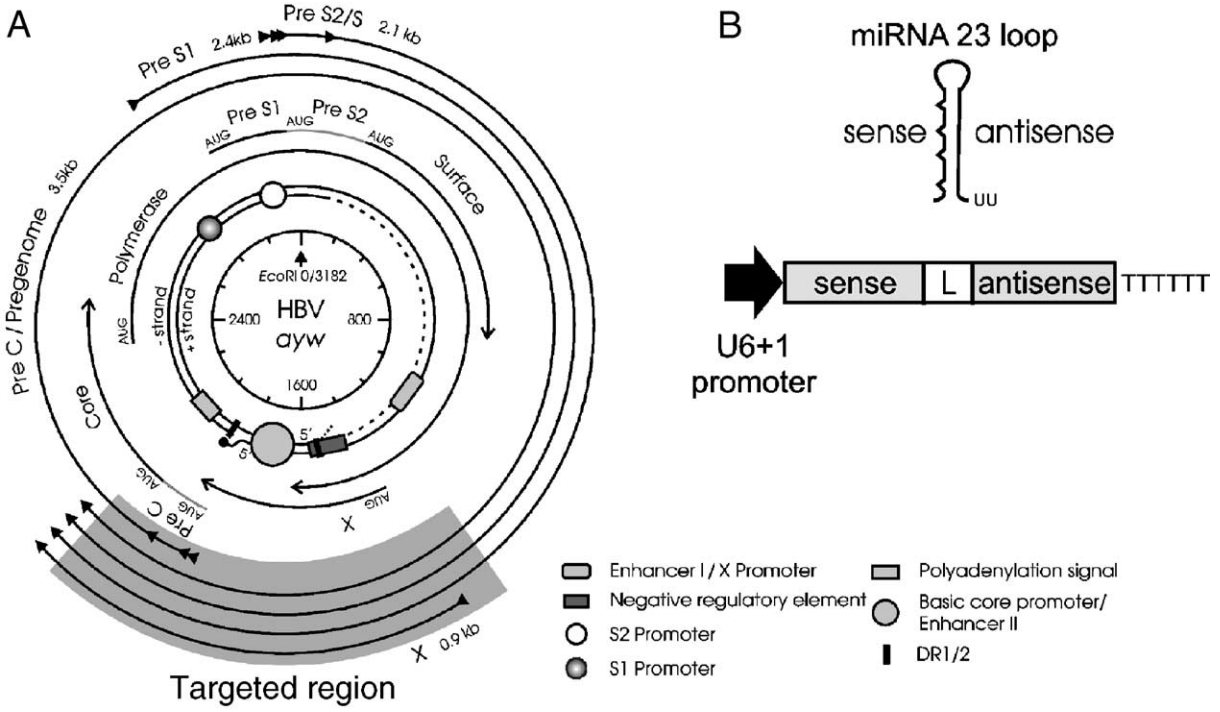


FIG. 1. HBV target sites and shRNA-encoding vectors. (A) Organization of the hepatitis B virus genome showing sites targeted by shRNA sequences. Coordinates of the genome are given relative to the single *Eco*RI restriction site. Partially double-stranded HBV DNA comprises + and − strands with cohesive complementary 5′ ends. The *cis* elements that regulate HBV transcription are represented by the circular and rectangular symbols. Immediately surrounding arrows indicate the viral open reading frames (with initiation codons) that encompass the entire genome. Four outer arrows indicate the HBV transcripts, which have common 3′ ends that include *HBx*. (B) (Top) Schematic illustration of anti-HBV shRNA indicating mismatches in the sense strand, miRNA 23 loop, and sequence of two U residues that are derived from the transcription termination signal. The DNA cassette with U6 promoter, sense, miRNA 23 loop (L), and antisense-encoding sequences is indicated below.

antiviral therapy. Naturally, the mechanism involves processing of larger dsRNA by Dicer to form short interfering RNA (siRNA) duplexes [12–14]. One of the strands of the siRNA is incorporated into the RNA-induced silencing complex (RISC) and acts as a guide to target degradation of complementary cytoplasmic RNA. Exogenous silencing is typically induced by synthetic RNA or transcripts produced from Pol III cassettes [12,15–17]. A number of studies have demonstrated that activation of RNAi has potential for the development of novel treatments of HBV infection [18–27]. As the pathway of RNAi becomes better understood, more rational approaches to the design and characterization of potentially therapeutic RNAi inducers are being sought. Avoidance of the interferon response, efficacy at a low concentration of RNAi-inducing sequences, specificity for the viral cognate, and processing of the duplexes with appropriate strand bias are important considerations. In this study, we report that anti-subgenotype A1 short hairpins RNAs (shRNAs) targeted to *HBx*, which have features of endogenous micro RNA (miRNA) that allow for influencing strand bias, are powerful and specific inhibitors of HBV replication. Inhibition of HBV replication was observed *in vitro* in transfected cells, *in vivo* after gene transfer using hydrodynamic DNA injection, and in HBV transgenic models. Sequences found to be most effective also have homology to other HBV genotypes and potentially have broad therapeutic applications.

RESULTS AND DISCUSSION

HBV Targets and Design of DNA Encoding Antiviral shRNA Sequences

We generated a panel of 10 U6 shRNA cassettes, which target the most conserved sites of the *HBx* sequence from South African genotype A1 isolates of the virus [28]. We designed hairpins to comprise a 25-bp stem with four GU or CA mismatches in the shRNA strand of HBV sense polarity, and the *HBx* antisense sequence was perfectly complementary to the viral target (Fig. 1 and Table 1). The 10-nt loop sequence was common to all of the hairpins and was derived from miRNA-23 [29]. These features were incorporated to facilitate intracellular processing and may also diminish non-specific effects of activating the interferon response [30,31].

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TABLE 1: Nucleotide sequences of PCR reverse primers used to generate HBV U6 shRNA cassettes

HBV target[a]	Name	Primer sequence
1168–1192	U6 shRNA 1.1	5′-**TGACG**TGACAGGAAGCGTTAGCAGACACTTGGCATAGGCCCGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 1.2	5′-CCCAGATCTACGCGTAAAAAAGGTCTGTGCCAAGTGTTTGCTGACGTGACAGGAAGCGTTA-3′
1432–1456	U6 shRNA 2.1	5′-**GGACG**TGACAGGAAGCGTTCGTGGGATTCAGCGTCGATGGCGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 2.2	5′-CCCAGATCTACGCGTAAAAAACCGTCGGCGCTGAATCCCGCGGACGTGACAGGAAGCGTTC-3′
1514–1538	U6 shRNA 3.1	5′-**CTTTA**TGACAGGAAGCAAAGAGAGATGCGCCCCATGGCCGCGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 3.2	5′-CCCAGATCTACGCGTAAAAAACGACCACGGGGCGCACCTCTCTTTATGACAGGAAGTAAAG-3′
1518–1542	U6 shRNA 4.1	5′-**ACGCG**TGACAGGAAGCGTGTGAAGAGAGGTGTGCCCTGTGCGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 4.2	5′-CCCAGATCTACGCGTAAAAAACACGGGGCGCACCTCTCTTTACGCGTGACAGGAAGCGTGT-3′
1575–1599	U6 shRNA 5.1	5′-**CTCTG**TGACAGGAAGCAGAGGCGAAGCAAAGCGCACACGACGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 5.2	5′-CCCAGATCTACGCGTAAAAAACCGTGTGCACTTCGCTTCACCTCTGTGACAGGAAGCAGAG-3′
1580–1604	U6 shRNA 6.1	5′-**CACGT**TGACAGGAAGATGTGTAGAGGTGAAGCGAGGTGTACGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 6.2	5′-CCCAGATCTACGCGTAAAAAATGCACTTCGCTTCACCTCTGCACGTTGACAGGAAGATGTG-3′
1640–1664	U6 shRNA 7.1	5′-**GGACT**TGACAGGAAGAGTTCTTTTATGTAGGACTTTGGGCCGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 7.2	5′-CCCAGATCTACGCGTAAAAAAGCCCAAGGTCTTACATAAGAGGACTTGACAGGAAGAGTTC-3′
1678–1702	U6 shRNA 8.1	5′-**GAGGC**TGACAGGAAGGCTTCAAGGTTGGTTGTTGACGTTGCGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 8.2	5′-CCCAGATCTACGCGTAAAAAACAATGTCAACGACCGACCTTGAGGCTGACAGGAAGGCTTC-3′
1774–1798	U6 shRNA 9.1	5′-**TTGGT**TGACAGGAAGACTAATTTGTGCCTACAGCTTCTTACGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 9.2	5′-CCCAGATCTACGCGTAAAAAATAGGAGGCTGTAGGCATAAATTGGTTGACAGGAAGACTAA-3′
1863–1887	U6 shRNA 10.1	5′-**CTTGG**TGACAGGAAGCCAAAGCACAACTCGGAGGCTCGAACGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 10.2	5′-CCCAGATCTACGCGTAAAAAATTCAAGCCTCCAAGCTGTGCCTTGGTGACAGGAAGCCAAG-3′

Overlapping sequences are underlined. Mismatches incorporated into the sense strand of the hairpin stems are indicated with a double underline. The region complementary to the U6 promoter is italicized. Sequences encoding *HBx* antisense RNA are bold.

[a] HBV coordinates are relative to the single *Eco*RI site as indicated in Fig. 1.

Effects of shRNA on HBsAg Secretion from Transfected Cells and Assessment of Efficacy *in Situ*

Initially, to assess efficacy against HBV, we cotransfected Huh7 cells with hairpin-encoding sequences and the pCH-9/3091 HBV target plasmid [32]. Variable efficacy of knockdown of viral antigen secretion was achieved by each of the sequences (Fig. 2A). U6 shRNA 5 and U6 shRNA 6 were most effective and plasmids encoding these hairpins decreased HBsAg concentration in the culture supernatant to less than 5% of the mock-treated cells' level. Transfection of the vector encoding U6 shRNA 10 had little if any effect on HBsAg secretion. We corroborated these data using a reporter gene plasmid (pCH-eGFP) to measure knockdown *in situ* (Fig. 2B) [33]. In pCH-eGFP, the preS2/S sequence of pCH-9/3091 was replaced with the enhanced green fluorescent protein (eGFP) ORF, with the targeted *HBx* ORF remaining intact. Cotransfection of pCH-eGFP with shRNA-encoding vectors allows for the convenient measurement of anti-HBV shRNA efficacies using flow cytometry and fluorescence microscopy. Analysis showed that the number of cells expressing eGFP was diminished significantly by U6 shRNA 5 and U6 shRNA 6, while U6 shRNA 10 was least effective (Fig. 2C). The sites targeted by these shRNAs 5 and 6 overlap each other, which suggests that this region of *HBx* is particularly susceptible to RNAi-mediated knockdown. Activators of the RNAi pathway can potentially stimulate nonspecific inflammatory effects by activating the interferon response, which in turn is capable of inducing cell death by apoptosis [34,35]. To exclude this effect, we measured mRNA from three genes that are key components of the interferon response: IFN-β, OAS1, and MxA (Supplemental Fig. 1). Expression of interferon response genes was not increased in Huh7 and HEK293 cells that were transfected with shRNA-expressing plasmids. These observations indicate that the knockdown of HBsAg secretion and eGFP marker expression was specific to activation of RNAi and not a result of causing interferon response-mediated programmed cell death.

Effects of shRNAs on HBV RNA Concentrations

We extracted total cellular RNA from Huh7 cells that had been transiently transfected with shRNA-encoding plasmids together with HBV target DNA. Analysis using Northern blot hybridization confirmed that shRNA 5 efficiently reduced the concentration of HBV RNA (Fig. 3A). When normalized for loading differences using the GAPDH mRNA band intensity, U6 shRNA 5 diminished HBV transcript concentration to approximately 35% of the control value. Concentrations of HBV transcripts in cells cotransfected with the U6 shRNA 10 vector were 90–100% of the controls. Cotransfection of Huh7 cells with U6 shRNA 9 had an intermediate knockdown effect on HBV RNA and diminished concentrations by approximately 40%. These data correlate with the observations on the shRNA effects on HBsAg secretion and *in situ* marker gene expression.

shRNA Processing

To assess processing of expressed shRNA sequences, we carried out primer extension analysis on total RNA that was extracted from the transfected Huh7 liver cell line.

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doi:10.1016/j.ymthe.2005.10.013

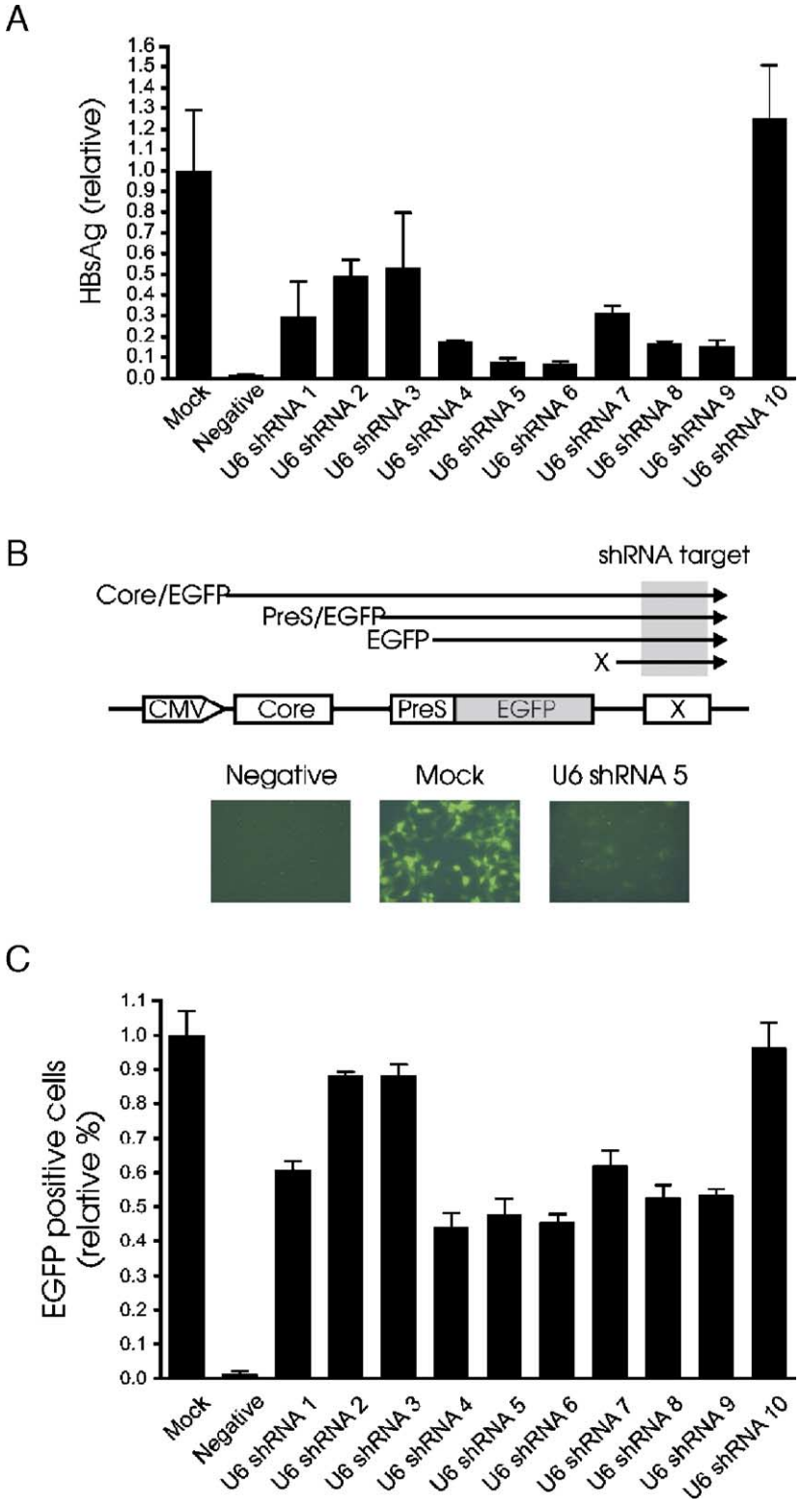


FIG. 2. shRNA-mediated inhibition of HBsAg secretion and HBV–eGFP fusion marker protein expression in transfected cells. (A) Measurement of HBsAg secretion from Huh7 cells cotransfected with indicated shRNA-encoding plasmids together with HBV target plasmid. HBsAg measurements from quantitative ELISA are given as a normalized mean relative to the mock-treated cells. Results are from four independent transfections and the bars indicate the standard error of the mean (SEM). (B) Schematic illustration of plasmid construct pCH-eGFP showing open reading frames, respective transcripts, and sites targeted by shRNAs. The disrupted polymerase ORF is not indicated. Representative fluorescence microscopy fields of Huh7 cells transfected with pCH-eGFP and indicated shRNA-expressing construct are also shown. (C) Quantitative comparison of the percentage of eGFP-positive Huh7 cells detected using flow cytometry after transfection with indicated shRNA-encoding expression vectors. Number of eGFP-positive cells is given as a normalized mean relative to the mock-treated cells, which represents approximately 45% eGFP-positive cells in the total population. Results are depicted as means from four independent transfections (each counting 100,000 events) with the SEM indicated.

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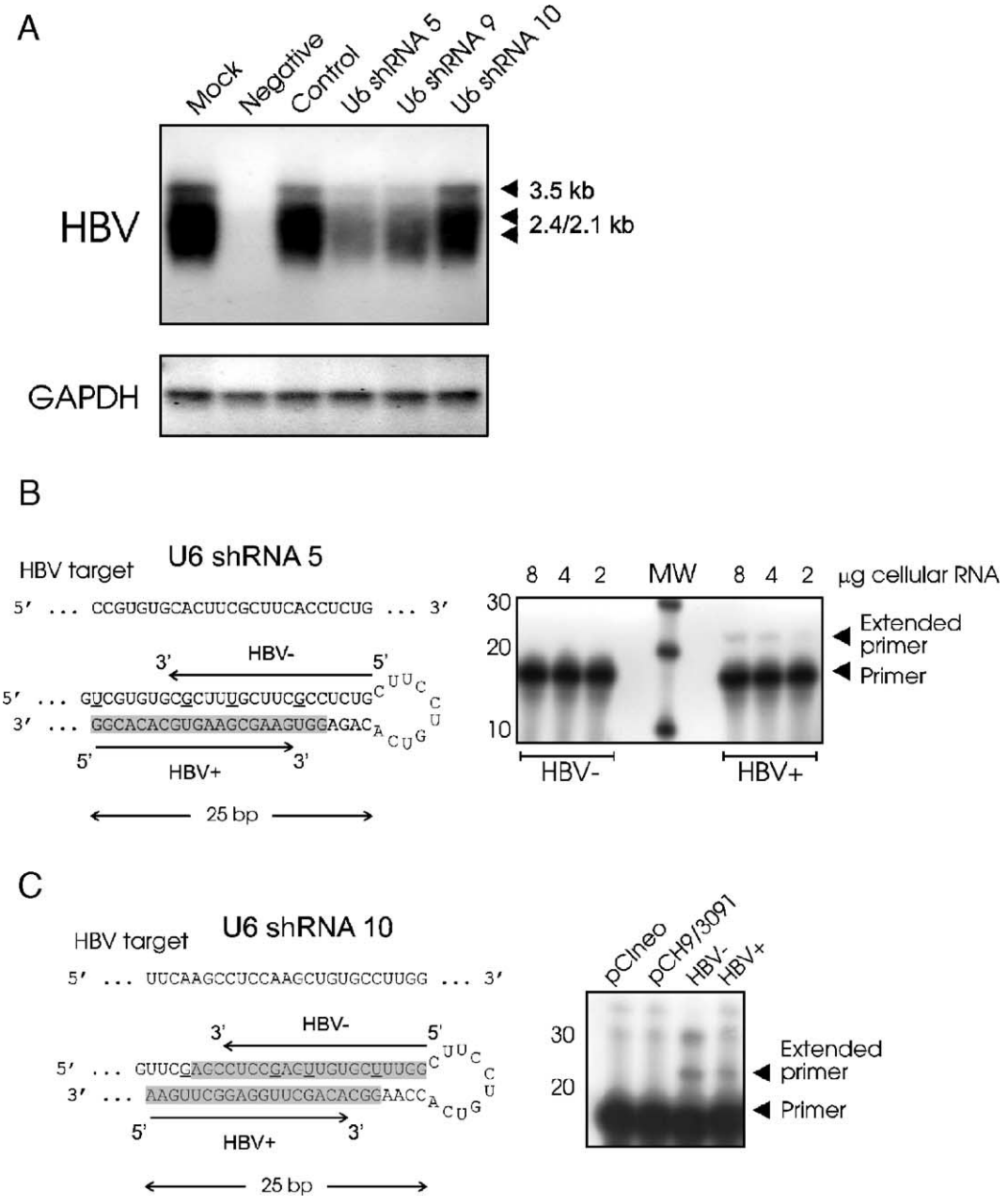


FIG. 3. RNA analysis. (A) Huh7 hepatocytes were transfected with the indicated shRNA-encoding plasmids. Two days after transfection, total RNA was extracted from the cells and analyzed by Northern blot hybridization. The control lane represents analysis of RNA extracted from cells that had been transfected with a plasmid encoding shRNA 5 sequences under transcriptional regulation of the Pol II CMV immediate early promoter/enhancer. Blots were probed for HBV RNA and also GAPDH as a loading control. (B and C) Either radiolabeled HBV^- or radiolabeled HBV^+ oligonucleotides targeting the relevant sequences of shRNA 5 or shRNA 10 were hybridized to total RNA extracted from transfected Huh7 cells and then subjected to primer extension analysis using reverse transcriptase. Sizes of extended fragments were then detected using autoradiography after resolution with denaturing PAGE. The amount of template cellular RNA subjected to reverse transcription is indicated above the lanes and RNA molecular weight marker sizes (nt) are indicated on the left of the autoradiographs.

We radiolabeled oligonucleotide primers that were complementary to the HBV sense or antisense sequence of the hairpin RNA encoded by U6 shRNA 5 or U6 shRNA 10 at their 5′ end, hybridized them to complementary cellular RNA from transfected cells, and then extended them using reverse transcriptase. Extension of end-labeled primers complementary to the U6 shRNA 5 antisense strand generated a 21-nt product that corresponds to the

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putative guide sequence that effects HBV gene silencing (Fig. 3B). A similar primer extension to detect a shRNA-derived *HBx* sense sequence did not generate a signal, and this was confirmed in an overexposed autoradiograph from the same gel. Conversely, when we subjected RNA extracted from U6 shRNA 10-transfected cells to primer extension analysis, both sense and antisense labeled oligonucleotides generated extended products 21 nt in length (Fig. 3C). Moreover, the U6 shRNA 10-encoded sense strand sequence is present at a slightly higher concentration compared to the intended antisense guide. Thus, intracellular processing of the hairpin encoded by U6 shRNA 10 has a slight bias for the nonhybridizing sequence with the same polarity as its viral target. Conversely, the only single-stranded RNA sequence that is detectable from U6 shRNA 5-transfected cells is that which corresponds to the intended antisense guide RNA. Several factors that affect processing and functionality of RNAi effectors have been identified. Included among these is a GC content of 30–52% that is thought to be optimal for efficient unwinding of the duplex while at the same time retaining sufficient stability of interaction of the siRNA guide strand with its target [36–38]. Based on primer extension analysis of shRNA 5, the siRNA derived from this hairpin has 47% GC pairs (9 of 19 pairs in the duplex region). However, the proportion of GC pairs between the shRNA 5-derived guide sequence and the HBV target is higher (62%), and this is a result of the GU mismatch pairs of the shRNA stem that are not present in the hybrid with the HBV target. Thus, it is likely that the relatively lower GC content of the Dicer-processed product facilitates unwinding of the duplex, while the higher GC content of the guide and target pair stabilizes this interaction. Another important factor that determines the processing of RNA duplex is the stability at the 5′ end of the intended guide sequence within the double-stranded RNA [36–38]. With shRNA 5, there is a 5′ GU mismatched base pair at the 5′ end of the antisense strand, and this is likely to facilitate the intended strand bias. Together, these factors are likely to improve processing of shRNA 5 and advance our understanding of the properties of effective anti-HBV RNA sequences.

Efficacy of shRNA-Encoding Plasmids *in Vivo* Using the Murine Hydrodynamic Injection Procedure

To determine anti-HBV efficacy of shRNA *in vivo*, we initially used the hydrodynamic injection procedure to deliver target and shRNA-encoding DNA simultaneously. Using this approach, shRNA 5-encoding plasmid DNA continuously knocked down HBsAg in the serum of mice to a background level over a period of 4 days (Fig. 4A). We also observed an inhibitory effect when we measured HBV core antigen (HBcAg) using immunohistochemical staining in liver sections from mice that had been subjected to hydrodynamic injections (Fig. 4B). The control for DNA delivery efficiency showed that uptake and expression of the *lacZ* marker gene were similar in treated and untreated mice. Comparing efficacies of U6 shRNAs 5, 6, and 10 on day 4 after injection, the production of HBsAg was suppressed completely in mice treated with shRNA 5- or shRNA 6-encoding plasmids (Fig. 4C). However, as was observed in transfected cells, HBsAg concentrations in the serum of mice that were injected with shRNA 10-encoding DNA were not significantly diminished. Similarly, on day 4 HBV viral loads were knocked down to background levels in the mice treated with DNA encoding shRNA 5 or shRNA 6 (Fig. 4D). Again U6 shRNA 10 was less effective, indicating that the knockdown is a sequence-specific phenomenon and is unrelated to generic features of the anti-*HBx* hairpin cassettes. Efficient inhibition of viral replication markers was confirmed when we administered shRNA 5-encoding DNA at a fifth of the molar concentration of HBV target DNA (not shown). Simultaneous injection of 25 μg of synthetic siRNA 5, which has a sequence equivalent to that encoded by U6 shRNA 5, inhibited secretion of HBsAg and viral particle concentration in the serum significantly ($P < 0.05$) (Figs. 4C and 4D). However, the synthetic RNA was also significantly less effective against markers of viral replication than U6 shRNA 5 and U6 shRNA 6 ($P < 0.05$). Sustained production of shRNA from a constitutively active DNA cassette as well as the shorter half-life of RNA compared to plasmid DNA is likely to account for this. Moreover, improved silencing by coupled Dicer processing prior to loading of guide sequences onto the RISC has been reported [39] and may contribute to the enhanced silencing effected by DNA-encoded shRNA compared to synthetic siRNA.

Efficacy of shRNA-Encoding Recombinant Adenovirus Vectors *in Vivo* in HBV Transgenic Mice

We assessed the efficacy of shRNAs against HBV in the context of established constitutive replication of the virus in HBV transgenic mice [40], which mimic natural infection more closely than does the hydrodynamic murine injection model. We incorporated U6 shRNA 5, 6, and 10 expression cassettes into recombinant adenovirus vectors to produce ADV shRNA 5, ADV shRNA 6, and ADV shRNA 10, respectively. Control adenoviruses included a U6 shRNA cassette targeted to the β-galactosidase marker (ADV shRNA LacZ) or no U6 shRNA sequence (ADV eGFP). The recombinant vectors coexpressing eGFP and fluorescence microscopy revealed that approximately 60–80% of hepatocytes expressed adenovirus DNA after tail vein injection with 5×10^9 infectious particles (not shown). Higher doses of adenovirus vectors caused significant hepatic toxicity. Compared to controls and ADV shRNA 10, a single dose of ADV shRNA 5 and ADV shRNA 6 significantly diminished the concentration of HBsAg concentration in the serum over a period of 12

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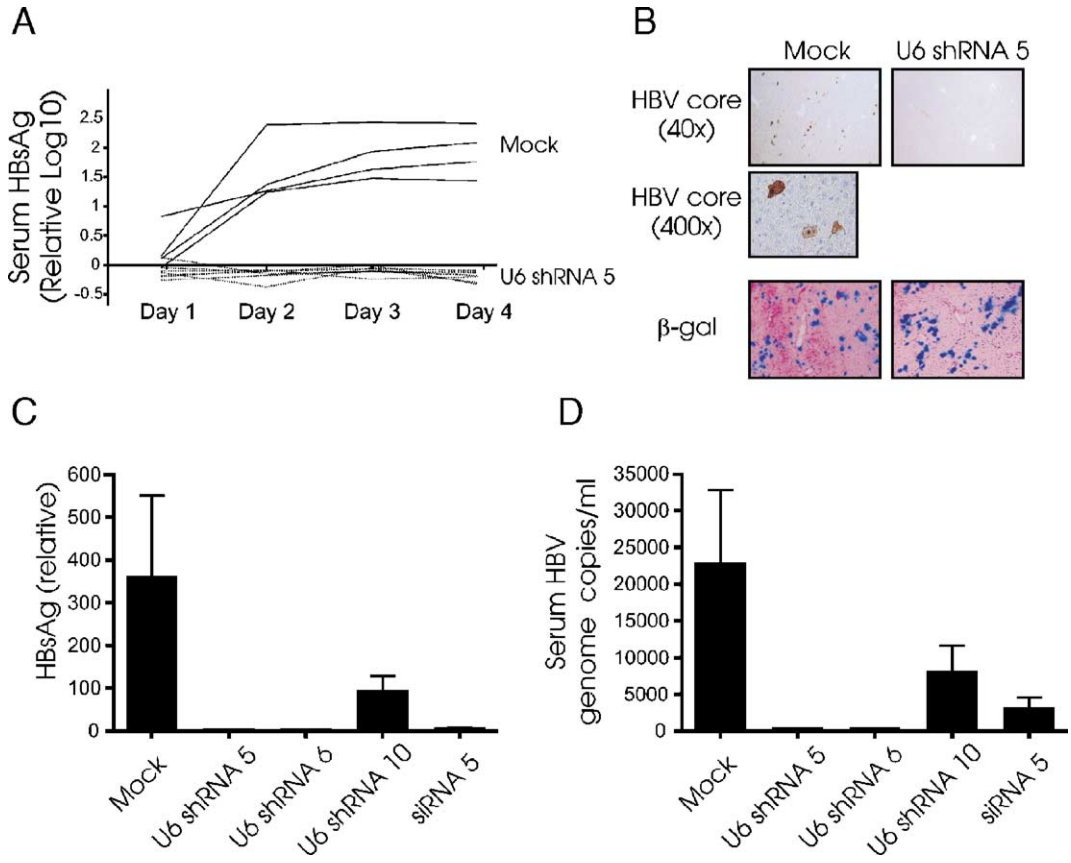


FIG. 4. Effects of shRNA sequences on HBV antigen production in the hydrodynamic injection model of HBV replication. (A) Time course of relative serum HBsAg concentrations, measured using quantitative ELISA, in mice treated with hydrodynamic injection. Graphical depictions of individual measurements of the viral antigen are given on a log scale for four mock- and four U6 shRNA 5-treated animals. (B) Mice subjected to the hydrodynamic injection procedure were sacrificed after 4 days and the livers analyzed using immunohistochemistry to detect the HBcAg. Representative low- and high-power fields are shown for livers from mock- and U6 shRNA 5-treated animals. Frozen sections from the same animals were stained for LacZ activity to confirm similar delivery of DNA to hepatocytes. (C) Serum HBsAg concentrations and (D) viral loads on day 4 after injection of mice using the hydrodynamic injection procedure. Mice were injected with the indicated shRNA-encoding plasmids or synthetic RNA duplex equivalent to shRNA 5 (siRNA 5) together with pCH/9-3091 HBV target DNA. Mock-treated animals received backbone plasmid (pGEM-T Easy) without shRNA-encoding sequences. Viral loads were determined using real-time quantitative PCR and included EuroHep standards to calibrate the virion copy numbers. Groups comprised six to eight animals and the graphs indicate the mean and SEM for each group.

days ($P < 0.05$) (Fig. 5A). Preliminary data also indicate that the inhibition of HBsAg secretion persists for at least 28 days (not shown). This is comparable to a recently reported observation that adenovirus vectors expressing shRNA sequences effected a sustained inhibition of markers of HBV replication [25]. Similarly, compared to control adenovirus vectors, serum HBeAg concentrations were significantly diminished by both ADV shRNA 5 and ADV shRNA 6 at day 12 after administration of the viral vectors ($P < 0.05$) (Fig. 5B). Interestingly, the effects of ADV shRNA 5 and ADV shRNA 6 on HBeAg secretion were less marked than those on HBsAg (approx 2-fold compared to 10-fold inhibition, compare Figs. 5A and 5B). This correlates with the previously reported observation that suggests the 3.5-kb HBV transcript encoding HBeAg is relatively more resistant to RNAi-mediated knockdown [25]. Circulating virion counts were diminished in animals treated with ADV shRNA 5 and ADV shRNA 6, although the effect was less significant than on serum HBeAg and HBsAg concentrations (Fig. 5C). Although target sites of shRNA 5 and shRNA 6 are conserved in the transgenic mice, silencing is less marked than that observed in the hydrodynamic model. There are two possible explanations for this observation. First, levels of HBV in the transgenic mice greatly exceed those of the hydrodynamic injection model (compare viral loads of Figs. 4D and 5C) and complete silencing may be more difficult to achieve when HBV replication is high. Second, under experimental conditions described here, the adenovirus vectors infected 60–80% of hepatocytes. Incomplete delivery of the shRNA-encoding cassettes to all of the HBV-producing cells is likely to account for

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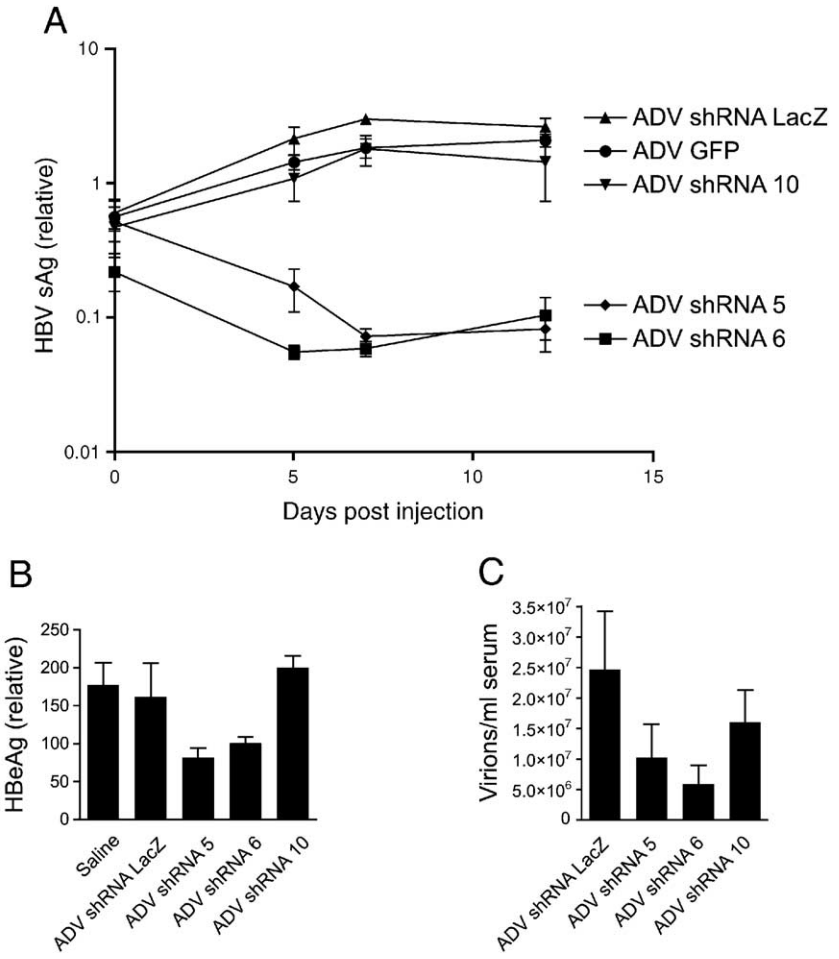


FIG. 5. Effects of recombinant adenovirus-mediated shRNA delivery on viral replication markers in the HBV transgenic mouse model. (A) Time course of relative mouse serum HBsAg concentrations, measured using quantitative ELISA, after tail vein injection of indicated recombinant adenovirus vectors. Relative serum HBsAg concentrations over a period of 12 days are shown. Comparison of (B) serum HBeAg and (C) viral loads at day 12 after injection of animals with saline or indicated recombinant adenovirus.

lower silencing efficiency. Taken together, these data indicate that recombinant adenoviruses incorporating U6 shRNA 5 and U6 shRNA 6 are capable of sustained inhibition of gene expression *in vivo* during constitutive replication of HBV. Moreover, HBV RNA is susceptible to RNAi-mediated silencing and is not protected from silencing by viral proteins.

The demonstration that the anti-*HBx* shRNAs used in this study can successfully inhibit HBV replication indicates that this target sequence is potentially useful for therapeutic application. Effective inhibition of HBV antigen production by U6 shRNA 5 and U6 shRNA 6 is an important advantage over available inhibitors of HBV reverse transcription, which do not reduce viral antigen secretion directly. HBV antigenemia may attenuate the host immune response and compromise eradication of the virus by these drugs [41]. RNAi-based inhibition of viral antigenemia may thus induce a more vigorous anti-HBV immune response to eliminate HBV during chronic infection. Unlike many other viruses, plasticity of the HBV genome is restricted because of its compact arrangement. Target sites of shRNA 5 and shRNA 6 are conserved in most genotypes, suggesting that these RNAi effectors may have broad applicability against different HBV genotypes. Although the results reported here and elsewhere augur well, there are concerns that need to be addressed before effective RNAi-based therapy of HBV infection will be realized. In particular, off-target effects of anti-HBV RNA sequences, which include activation of the interferon response and nonspecific interactions between guide sequences and alternative cellular targets, remain a concern. These effects require further characterization, and optimal therapeutic concentrations need to be defined. An important technical hurdle related to developing RNAi-based antiviral therapy is the efficient delivery of nucleic acid sequences to infected cells in the liver. Although DNA expression cassettes have been shown to be effective in the context of recombinant adenoviruses tested in this study and by others [25], these vectors are unlikely to be suitable for clinical application. Potential toxicity and the limited number of administrations caused by adenovirus immunity are of concern.

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Incorporation of U6 shRNA 5 and U6 shRNA 6 into hepatotropic nonviral vectors with limited immunogenicity is an objective of current work.

MATERIALS AND METHODS

shRNA expression cassettes and synthetic siRNA. Conserved target sequences within the *HBx* open reading frame of the HBV A1 subgenotype were identified by aligning sequences of 27 viral isolates of South African origin. To generate the panel of 10 shRNA expression constructs, oligonucleotides were designed to produce Pol III U6 shRNA cassettes from a U6 DNA template [42] in a two-step amplification reaction. The reverse oligonucleotide sequences used during PCR are indicated in Table 1. U6 shRNA X.1 primers were complementary to part of the U6 promoter and included the mismatched *HBx* sense sequences of the short hairpin, together with the hairpin loop. U6 shRNA X.2 primers included the overlapping loop, *HBx* antisense sequence, and transcription termination sequence. Each PCR step was performed with a U6 universal forward primer (5′-CTAACTAGTGGCGCGCCAAGGTCGGGCAGGAAGAGGG-3′). The PCR products from the final amplification step were purified and ligated to a PCR cloning vector (pGEM-T Easy, Promega, WI, USA) to generate pG-U6shRNA plasmids (e.g., pG-U6shRNA 5 and pG-U6shRNA 6). The sequences were confirmed by standard manual or automated sequencing procedures involving dideoxy chain termination. siRNA 5, which has a sequence equivalent to that of the duplex stem of shRNA 5, was synthesized using 2′-O-ACE-RNA phosphoramidites (Dharmacon, CO, USA). The sequences of the oligoribonucleotides were 5′-UCGUGUGCGCUUUGCUUCGCCUCUG-3′ (sense) and 5′-CAGAGGUGAAGCGAAGUGCACACGG-3′ (antisense).

Target vectors. pCH-9/3091 has been described previously [32]. It contains a greater than genome length HBV sequence, which is similar to the HBV A1 subgenotype consensus. pCH-eGFP is derived from pCH-9/3091 and has the eGFP sequence substituting for the preS2/S ORF [33].

Cell culture. Huh7 cells were maintained in RPMI medium supplemented with 2.5% fetal calf serum (FCS), penicillin (50 IU/ml), and streptomycin (50 μg/ml) (Gibco BRL, UK). HEK293 cells were propagated in DMEM supplemented with 10% FCS, penicillin (50 IU/ml), and streptomycin (50 μg/ml) (Gibco BRL). On the day prior to transfection, 250,000 HEK293 cells or 150,000 Huh7 cells were seeded in wells 2 cm in diameter. Transfection was carried out using Lipofectamine (Invitrogen, CA, USA) according to the manufacturer's instructions. To determine the effects of shRNA-encoding plasmids, Huh7 cells were transfected with a combination of 6 μg of pCH-9/3091 and 2 μg of hairpin-encoding pGEM-derived plasmid or control plasmid lacking the shRNA cassette. HBsAg secretion into the culture supernatants was measured daily using the Monolisa (ELISA) immunoassay kit (Bio-Rad, CA, USA). To determine *in situ* effects of the pG-U6 shRNA series of plasmids, Huh7 cells were cotransfected with 6 μg pCH-eGFP instead of pCH-9/3091. Cells labeled with eGFP were detected using flow cytometry and confirmatory fluorescence microscopy 48 h after transfection. The mean number of fluorescent cells as well as the standard error of the mean was calculated from four independent experiments. A plasmid vector that constitutively produces β-galactosidase [43] was included in each cotransfection and equivalent transfection efficiencies were verified by staining for activity of this marker gene [44].

Northern blot hybridization. Huh7 cells were harvested 4 days after transfection and total RNA was extracted using Tri Reagent (Sigma, MI, USA) according to the manufacturer's instructions. The RNA was resolved using formaldehyde agarose gel electrophoresis and blotted onto nylon membranes. An HBV sequence from the surface region was radiolabeled with [α-^{32}P]dCTP using the multiprime technique (Megaprime kit; Amersham, UK) and then hybridized to blotted RNA and detected using autoradiography. As a control for equal loading, the same blot was stripped and rehybridized to a radiolabeled GAPDH-specific probe.

Expression and processing of shRNA. DNA oligonucleotides, which were complementary to 18 nucleotides of each of the strands of the U6-encoded hairpins, were labeled at their 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase. After purification using standard procedures, labeled DNA oligonucleotides were hybridized to 2 to 8 μg of total Huh7 cellular RNA and then extended for 20 min at 42°C with AMV reverse transcriptase (Promega) according to the conditions recommended by the supplier. Products were analyzed by autoradiography after resolution using 8 M urea denaturing 15% polyacrylamide gel electrophoresis. The oligonucleotides used in the primer extension assays were as follows: shRNA 5 HBV+, 5′-CCGTGTGCACTTCGCTTC-3′; shRNA 5 HBV−, 5′-CAGAGGCGAAGCAAAGCG-3′; shRNA 6 HBV+, 5′-TGCACTTCGCTTCACCTC-3′; shRNA 6 HBV−, 5′-ATGTGTAGAGGTGAAGCG-3′; shRNA 10 HBV+, 5′-TTCAAGCCTCCAAGCTGT-3′; shRNA 10 HBV−, 5′-CCAAAGCACAACTCGGAG-3′.

Quantitative PCR. To measure the effects of shRNA sequences on circulating virion DNA (see below), total DNA was isolated from 50 μl of mouse serum using the Total Nucleic Acid Isolation Kit and MagNApure instrument from Roche Diagnostics. Controls included water blanks and HBV-negative serum. DNA extracted from the equivalent of 8 μl of mouse serum was amplified using SYBR Green *Taq* Readymix (Sigma, MO, USA). Crossing-point analysis was used to measure virion DNA concentrations and standard curves were generated using EuroHep calibrators [45]. The HBV surface primer set was HBV surface forward, 5′-TGCACCTGTATTCCATC-3′, and HBV surface reverse, 5′-CTGAAAGCCAAACAGTGG-3′. PCR was carried out using the Roche Lightcycler v.2. Capillary reaction volume was 20 μl and thermal cycling parameters consisted of a hot start for 30 s at 95°C followed by 50 cycles of 57°C for 10 s, 72°C for 7 s, and then 95°C for 5 s. Specificity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis.

Assessment of in vivo efficacy of anti-HBV shRNA constructs. The murine hydrodynamic tail vein injection method was initially employed to determine the effects of shRNA plasmid vectors on the expression of HBV genes *in vivo*. Experiments on animals were carried out in accordance with protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee. A saline solution comprising 10% of the mouse's body mass was injected via the tail vein over 5–10 s. The saline solution included a combination of three plasmid vectors: 10 μg target DNA (pCH-9/3091) or 10 μg pCI-neo plasmid DNA (Promega), which lacks HBV sequences; 10 μg anti HBV sequence (shRNA-encoding plasmid) or mock (pGEM backbone); and 10 μg pLTR β-gal [43] (a control for hepatic DNA delivery, which encodes the β-galactosidase marker gene under control of an LTR promoter). In some investigations, the amount of shRNA-encoding plasmid was reduced to 5 and 1 μg. To test the efficacy of synthetic siRNA 5 against HBV, 25 μg of this duplex was coinjected via the tail vein. Blood was collected daily from the tail vein over a period of 5 days and HBsAg was measured using the electrochemiluminescence assay (ECLIA) from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's instructions. Animals were sacrificed after 4 days. Fixed and unfixed frozen liver sections were processed respectively for immunohistochemical HBcAg detection or for β-galactosidase staining [44]. A rabbit polyclonal antibody against HBcAg (Signet Laboratories, Inc., MA, USA) and horseradish peroxidase-conjugated secondary antibody (Dako, Denmark) were used to detect the viral antigen in paraffin-embedded sections according to standard procedures.

Adenovirus vectors. The procedure described by He *et al*. [46] was followed for the preparation of the ADV shRNA 5, ADV shRNA 6, ADV shRNA 10, ADV eGFP, and ADV shRNA LacZ adenovirus vectors. ADV shRNA LacZ is a control adenovirus that includes a U6 shRNA cassette with hairpin that targets the β-galactosidase reporter gene. ADV eGFP is another control that includes the backbone sequence without U6 hairpin cassette. The pG shLacZ vector was propagated using the two-step PCR procedure described above. The U6 universal forward primer, with 5′-CTGTTTGACAGGAAGAACAAGTATCCGCTAGTCACTTCGACGGTGTTTCGTCCTTTCCACA-3′ and 5′-CCCAGATCTACGCGTAAAAAATCGAAGTGACCAGCGAATACCTGTTTGACAGGAAGAACAA-3′ as reverse primers, was used in sequential amplifications. To generate adenovirus shuttle vectors containing the shRNA cassettes, pG-U6shRNA vectors were

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digested with *Not*I and *Hin*dIII. The U6 shRNA-containing fragment was purified and ligated to equivalent sites of pAdTrack to generate pAd-Track U6 shRNA. After verifying the sequence of the inserts, pAdTrack U6 shRNAs were digested with *Pme*I and homologous recombination with the pAdEasy-1 adenoviral backbone plasmid was carried out in *Escherichia coli* BJ55183 cells. Propagation of the adenovirus in HEK293 cells was then done according to the described procedures [46]. Absence of E1A sequences from each adenovirus was confirmed using PCR. Failure to observe a cytopathic effect in A549 cells, which support adenovirus replication, but which do not provide E1 sequences *in trans*, verified that wild-type revertants were not present in any of the adenovirus preparations.

HBV transgenic mice. HBV transgenic mice with greater than genome length HBV sequence stably integrated into their genomes, which constitutively generates HBV particles [40], were used to assess the antiviral efficacy of shRNA-encoding adenoviral vectors. All procedures were approved by the Animal Care Committee at Stanford University. A dose of 5×10^9 adenovirus infectious particles was injected via the tail vein. Serum HBsAg was measured using a quantitative sandwich ELISA from Abbott Laboratories, and HBeAg was determined using the ECLIA from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's instructions. Viral loads were determined using real-time PCR according to procedures described above. Adenovirus gene transduction, assessed by detection of eGFP, was determined using fluorescence microscopy of liver sections.

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Statistical difference was considered significant when $P < 0.05$ and was determined according to the Dunnett multiple comparison test and calculated with the GraphPad Prism software package (GraphPad Software, Inc., CA, USA).

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymthe.2005.10.013.

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Brief Report

Efficient Inhibition of Hepatitis B Virus Replication *In Vivo*, Using Polyethylene Glycol-Modified Adenovirus Vectors

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Abstract

Achieving safe delivery of anti-hepatitis B virus (HBV) RNA interference (RNAi) effectors is an important objective of this gene-silencing technology. Adenoviruses (Ads) have a natural tropism for the liver after systemic administration, and are useful for delivery of expressed anti-HBV RNAi sequences. However, a drawback of Ad vectors is diminished efficacy and toxicity that results from stimulation of innate and adaptive immunity. To attenuate these effects we used monomethoxy polyethylene glycol-succinimidyl propionate (mPEG-SPA) to modify first-generation vectors that express an anti-HBV RNAi effector. Efficient hepatocyte transduction and knockdown of HBV replication were achieved after intravenous administration of 5×10^9 PEGylated or native recombinant Ads to HBV transgenic mice. After the first injection, circulating HBV viral particle equivalents (VPEs) remained low for 3 weeks and began to increase after 5 weeks. A second dose of PEGylated anti-HBV Ad caused a less sustained decrease in circulating VPEs, but no silencing after a second dose was observed in animals treated with unmodified vector. Release of inflammatory cytokines, including monocyte chemoattractant protein-1 (MCP-1), interferon-γ, interleukin-6, and tumor necrosis factor-α, was elevated in animals receiving unmodified vectors. However, only a modest increase in MCP-1 was observed in mice that received a second dose of PEG Ads. Also, polymer-conjugated vectors induced a weaker adaptive immune response and were less hepatotoxic than their unmodified counterparts. Collectively, these observations show that PEG modification of Ads expressing RNAi effectors improves their potential for therapeutic application against HBV infection.

Introduction

It is estimated that globally there are 387 million carriers of hepatitis B virus (HBV) and persistent infection with the virus places individuals at high risk for the life-threatening complications of cirrhosis and hepatocellular carcinoma (HCC) (Arbuthnot and Kew, 2001; El-Serag and Rudolph, 2007). Licensed anti-HBV agents, which include interferon (IFN)-α, nucleoside (lamivudine), and nucleotide (adefovir) analogs, are only partially effective (Hanazaki, 2004). Serious complications of infection with HBV are thus likely to remain significant global medical problems and the development of new effective therapy continues to be an important objective. The demonstration that HBV replication is susceptible to RNA interference (RNAi)-mediated silencing (Giladi *et al*., 2003; Klein *et al*., 2003; McCaffrey *et al*., 2003; Shlomai and Shaul, 2003; Uprichard *et al*., 2005; Carmona *et al*., 2006) has prompted investigations aimed at harnessing this pathway for therapeutic application. Exogenous activators of RNAi are typically expressed primary or precursor microRNA (miR) mimics or synthetic small interfering RNAs (siRNAs). Both classes of RNAi effector have been used to silence HBV replication and each has distinct advantages. Although the dose and delivery of synthetic siRNAs are relatively easy to achieve, DNA expression cassettes have better stability, sustained silencing effects, and are compatible with incorporation into highly efficient viral vectors. To date, the most promising viral vectors that have been used to de-

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liver anti-HBV sequences are recombinant adenoviruses (Ads) (Uprichard *et al*., 2005; Carmona *et al*., 2006) and adeno-associated viruses (AAVs) (Moore *et al*., 2005). Ads have the useful properties of efficient targeting of hepatocytes *in vivo* after systemic administration, lack of integration into host genome sequences, stability, and ease of preparation (reviewed in Bangari and Mittal, 2006). However, widespread use of these vectors has been limited by preexisting immunity and their powerful induction of innate and adaptive immune responses. After intravenous administration, adenoviruses are readily taken up by Kupffer cells in the liver. Ad capsid proteins mediate signal transduction via mitogen-activated protein kinases (MAPKs), which leads to activation of an innate immune response. Associated release of cytokines, for example, interleukin (IL)-6 and IL-12 (Zhang *et al*., 2001), causes local inflammation and toxicity (Guidotti and Chisari, 2001; Schnell *et al*., 2001; Taniguchi *et al*., 2003; Chen *et al*., 2008). Resulting damage to healthy tissues with decreased transgene expression is thus a significant concern for therapeutic use of recombinant Ads. Given the importance of Ad capsid proteins in mediating immune responses, methods have been devised to attenuate immunostimulation by modifying Ad capsid proteins with polymers such as polyethylene glycol (PEG) and poly-*N*-(2-hydroxypropyl)methacrylamide (Kreppel and Kochanek, 2008). Although decreased immunostimulation has been shown by this approach (Croyle *et al*., 2000, 2002, 2005; Eto *et al*., 2004; Mok *et al*., 2005), there have been few studies that measured the efficiency of polymer-modified Ad vectors *in vivo* in disease models. In this study, we assessed PEG-modified first-generation vectors that express an RNAi activator that we have previously shown to silence HBV replication efficiently (Carmona *et al*., 2006). We observed that PEGylation improved silencing of HBV *in vivo* in a murine transgenic model of HBV replication. This chemical modification also significantly suppressed immunostimulation and hepatotoxicity.

Materials and Methods

Adenoviral vectors

First-generation adenoviral vectors expressing enhanced green fluorescent protein (eGFP) together with anti-HBV short hairpin RNA 6 (shRNA 6) or shRNA 10 (Carmona *et al*., 2006) were generated according to the AdEasy procedure (He *et al*., 1998). The methods used to modify the viral vectors with monomethoxy polyethylene glycol-succinimidyl propionate (mPEG-SPA) were based on previously described protocols (Croyle *et al*., 2000; Eto *et al*., 2004). A change in molecular weight of the hexon protein from 105 to approximately 140 kDa, determined by polyacrylamide gel electrophoresis (PAGE) or microchip analysis, was used to verify PEGylation. Determination of the number of infectious particles in PEGylated or unPEGylated preparations was carried out with the Adeno-X rapid titer kit (Clontech, Mountain View, CA) with minor modifications.

Cell culture and Northern blot analysis

Culture and transfection of Huh7 and HEK293 lines was carried out as described (Carmona *et al*., 2006). For Northern blot hybridization (Ely *et al*., 2008), liver-derived Huh7 cells were infected with an approximate multiplicity of infection of 100 recombinant adenovirus vectors per cell and harvested 2 days thereafter, before extracting total RNA with TRI reagent (Sigma-Aldrich, St. Louis, MO). Blots were hybridized to digoxigenin (DIG)-labeled oligonucleotides, using methods that were recommended by the suppliers (Roche Applied Science, Mannheim, Germany). An oligonucleotide sequence complementary to U6 small nuclear RNA (snRNA) was used as a control for equal loading of the cellular RNA (Ely *et al*., 2008). The oligonucleotide sequence of the shRNA 6 probe was 5′-TGCACTTCGCTTCACCTC-3′.

Adenoviral vector administration to HBV transgenic mice

HBV transgenic mice (Marion *et al*., 2003) were used to determine the effects of RNAi-activating adenoviral vectors on markers of HBV replication *in vivo*. These experiments were carried out in accordance with protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee. A dose of 5×10^9, 1×10^9, or 5×10^8 adenovirus infectious particles was injected as a bolus via the tail vein and blood was collected by retroorbital puncture. In all experiments, groups of mice comprised eight animals each. Circulating viral particle equivalents (VPEs) and hepatic HBV mRNA were determined by real-time polymerase chain reaction (PCR) according to previously described methods (Carmona *et al*., 2006; Ely *et al*., 2008). Adenovirus gene transduction was assessed by detecting eGFP microscopically in frozen liver sections taken 48 hr after initial adenovirus injection of separate animals.

Cytokine assays

A cytometric bead array (CBA) mouse inflammation kit (BD Biosciences, San Jose, CA) was employed to measure IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1), IFN-γ, tumor necrosis factor-α (TNF-α), and IL-12p70. Recombinant standards were also included to calibrate the assay.

Anti-adenovirus immunoglobulin assay

To measure mouse antibodies against complete Ad virions, a previously described protocol was followed (Jiang *et al*., 2004). The anti-Ad mouse antibodies were detected with a peroxidase-conjugated secondary anti-mouse immunoglobulin (Dako, Carpinteria, CA) with *o*-phenylenediamine dihydrochloride as the chromogenic substrate. To detect mouse antibodies to the Ad hexon protein, the RIDASCREEN kit (R-Biopharm Rhône, Pfungstadt, Germany) was used. The protocol was done according to the manufacturer's instructions except that a 1:10,000 dilution of mouse serum was used and the kit secondary antibody was substituted with peroxidase-conjugated anti-mouse immunoglobulin (Dako).

Serum transaminase assay

Aspartate transaminase (AST) and alanine transaminase (ALT) activities were measured by a kinetic assay with an automated photometric analyzer (Roche Diagnostics, Rotkreuz, Switzerland).

Statistical analysis

Means $\pm$ standard error of the mean (SEM) were calculated and a statistical difference was considered significant

when $p < 0.05$ and was determined according to the Student two-tailed t test. Calculations were done with the GraphPad Prism software package (GraphPad Software, San Diego, CA).

Results

HBV target sequence

The HBV genome has a compact arrangement with overlapping open reading frames (ORFs) and *cis* elements embedded within protein-coding sequences. There is a single transcription termination signal, which results in all HBV transcripts having a common 3′ end that includes *HBx*. This arrangement limits sequence flexibility of the virus and makes *HBx* a particularly good target for nucleic acid hybridization-based gene silencing. Previous work from our laboratory demonstrated that Ads expressing hairpin sequences targeting *HBx* (HBV coordinates 1580–1604) are efficient silencers of HBV gene expression and replication (Carmona *et al.*, 2006). To assess potential improvement by polymer modification, we selected the recombinant adenovirus vector that expresses HBV shRNA 6 (Ad HBV shRNA 6) and subjected this to analysis after PEG conjugation. After infection of Huh7 cells in culture with Ad HBV shRNA 6, the intended anti-HBV guide sequence was detected (Fig. 1A), which verified that transcripts of the expression cassette were indeed processed by the RNAi pathway.

Anti-HBV efficacy of PEG-modified Ad vectors

Ad HBV shRNA 6 vectors were modified with mPEG-SPA, and efficient addition of PEG to the Ad hexon protein was verified by microchip electrophoretic analysis (data not shown). Titration of PEG-modified vectors did not result in an appreciable decrease in infectivity, and suitable concentrations for *in vivo* application were routinely achieved. Hepatic delivery of transgenes was assessed after injection of mice with 5×10^9 infectious recombinant virus particles. Fluorescence microscopy of frozen liver sections showed that approximately 80% of liver cells were transduced 48 hr after injection, and that this was achieved with both native and modified vectors (Fig. 1B and C). eGFP was also not detectable in any significant amounts in nonhepatic tissue (data not shown). Our PEG modification procedure therefore did not compromise delivery efficiency and liver targeting of these vectors after systemic administration.

HBV transgenic mice were then injected with PEGylated and native Ads expressing HBV shRNA 6 and anti-HBV efficacy was measured by determining the number of circulating VPEs (Fig. 1D). Initial effects of PEGylated and unmodified vectors on circulating VPEs were similar in mice treated with both vectors. There was a significant drop in the circulating VPEs in each of the groups of animals 1 week after administration of the virus. This effect persisted for approximately 4 weeks, and at week 5 the VPE titer had increased to approach baseline levels. At week 5, a second dose

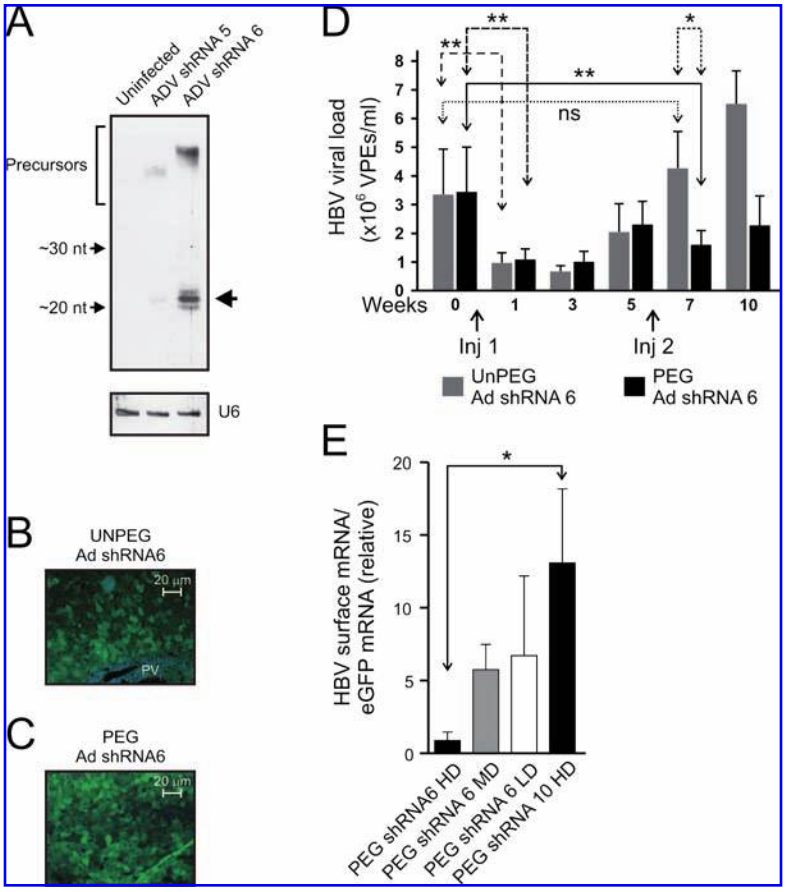


FIG. 1. Anti-HBV efficacy of recombinant Ads. (**A**) Northern hybridization analysis of expressed anti-HBV sequences. RNA was extracted from Huh7 cells after infection with recombinant viruses expressing the indicated shRNA cassettes or from uninfected cells. Blots were hybridized to a probe complementary to the intended shRNA 6 guide strand and then stripped and rehybridized to an endogenous U6 snRNA probe to confirm equal loading of cellular RNA (*bottom*). Shown is a representative fluorescence microscopy analysis of frozen sections from mouse liver that was processed 48 hr after administration of 5×10^9 native (**B**) and PEG-modified (**C**) Ad vectors. The location of the portal vein (PV) is indicated. (**D**) Circulating VPEs in HBV transgenic mice after administration of PEG-modified and native Ads. Animals received two injections of 5×10^9 infectious Ad particles, which were administered at commencement of the experiment and 5 weeks after the initial injection. Titers of VPEs were determined by real-time quantitative PCR. (**E**) Effects of Ad dose on hepatic HBV mRNA. Animals were killed 1 week after intravenous administration of PEG-modified vectors at doses of 5×10^9 (HD), 1×10^9 (MD), and 5×10^8 (LD) vector particles. Mean ratios of HBV surface mRNA (±SEM) were measured relative to eGFP mRNA, using quantitative real-time PCR. *$p < 0.05$; **$p < 0.01$.

of Ad vectors was administered to the mice via the tail vein. At week 7, the PEG-modified vectors effected a decrease in VPEs, which was not observed after treatment with the unmodified Ads. By 3 weeks after the second PEG Ad vector injection the number of circulating VPEs had returned to baseline concentrations, which indicates that silencing efficacy after the second Ad injection is less sustained than after the initial vector administration. Knockdown of intrahepatic HBV mRNA was also measured 1 week after administration of various doses of Ad HBV shRNA 6 or control Ad HBV shRNA 10 (Fig. 1E). Quantitative reverse transcription PCR, using primers that target the HBV *surface* ORF, showed that intrahepatic HBV mRNA was diminished in animals that had been treated with 5×10^9 Ad HBV shRNA 6 particles. This effect was not observed when mice received lower doses of the PEG-modified recombinant vector. Moreover, PEGylated Ad HBV shRNA 10 did not diminish intrahepatic HBV mRNA concentration, which is in keeping with the previously reported observations showing that this unmodified Ad vector had no effect against HBV replication (Carmona *et al*., 2006).

Serum cytokine concentrations after injection of PEGylated and unmodified Ad vectors

The improved anti-HBV efficacy of PEGylated vectors is likely to result from an attenuated immune response to the polymer-coated Ads (Croyle *et al*., 2002, 2005; Eto *et al*., 2004; Mok *et al*., 2005). To assess the effects of PEGylated and unmodified Ad HBV shRNA 6 on the release of inflammatory cytokines, serum concentrations of a selection of cytokines and chemokines were determined. Initially, blood was collected 6 and 24 hr after the first injection of mice with recombinant vectors and the cytokines were measured by a CBA assay. The panel of cytokines, comprising TNF-α, IL-12, MCP-1, IL-10, IL-6, and IFN-γ, included markers that give a broad indication of innate and adaptive immune response activation. At the time point of 6 hr after injection of the first dose of Ad HBV shRNA 6, MCP-1 was elevated in mice receiving the unPEGylated vector but not in the serum of animals receiving PEG-modified recombinant virus. By 24 hr after injection, the serum MCP-1 concentration reverted to baseline control levels (data not shown). MCP-1 is produced from a variety of cells and functions as a monocyte chemoattractant (Furutani *et al*., 1989; Yoshimura *et al*., 1989) and mediator of inflammation (Jones *et al*., 1992; Koch *et al*., 1992). Although other studies have reported stimulation of secretion of additional proinflammatory cytokines after Ad administration, our observations may be specific to the line of HBV transgenic mice studied here and also the low dose of Ad that these animals received.

Serum concentrations of cytokines were again measured in animals that received a second dose of native and PEG-modified Ad. PEG-modified vectors caused modest elevation of only MCP-1 at 6 hr, and the concentration of this chemokine reverted to baseline 24 hr after injection (Fig. 2). Because a raised serum MCP-1 concentration was also the only marker of immunostimulation after initial administration of native Ad, secretion of this chemokine may be the most sensitive indicator of Ad-induced immunostimulation

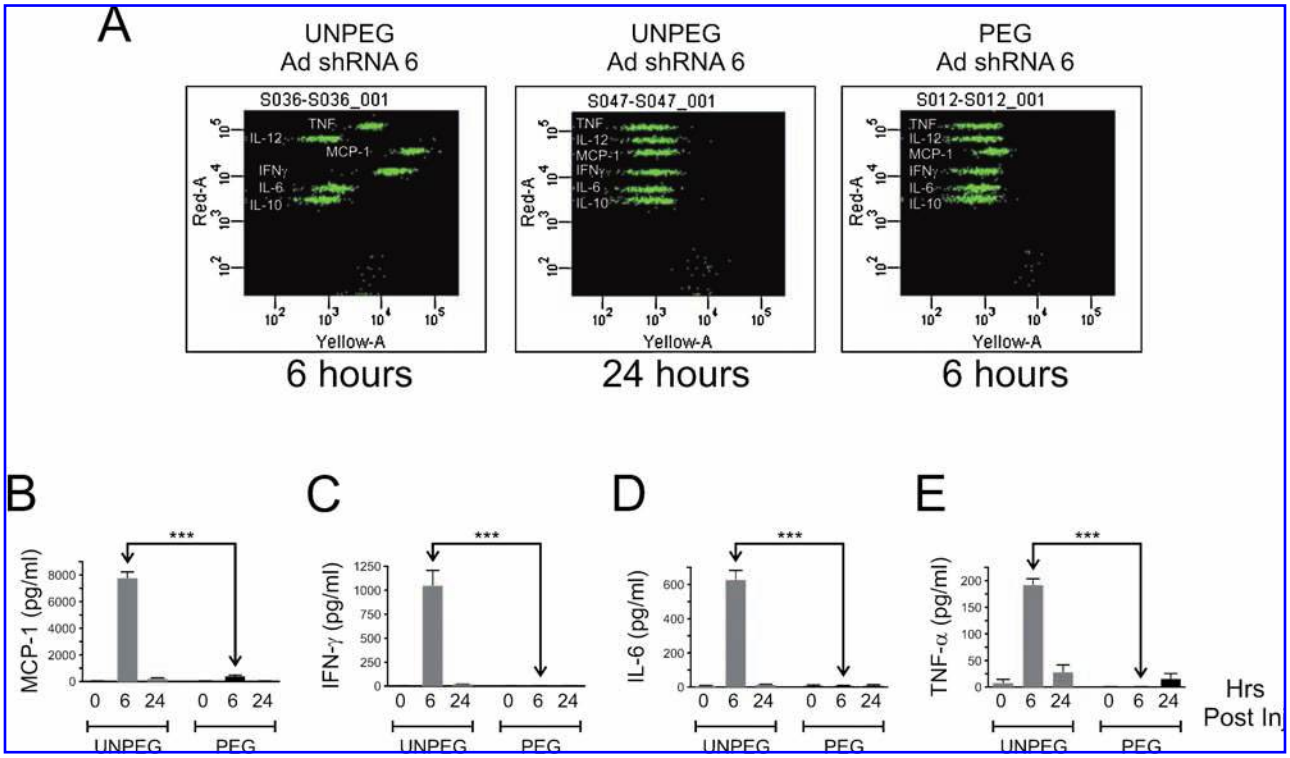


FIG. 2. Serum cytokine concentrations after second administration of Ads to HBV transgenic mice. (**A**) Representative flow cytometry data from CBA assay for TNF-α, IL-12, MCP-1, IL-10, IL-6, and IFN-γ 6 and 24 hr after adenovirus injection. Shown are mean concentrations of MCP-1 (**B**), IFN-γ (**C**), IL-6 (**D**), and TNF-α (**E**) in mice at baseline and 6 and 24 hr after administration of PEG-modified and native adenoviral vectors. ***$p < 0.005$.

under the experimental conditions described here. CBA analysis revealed that concentrations of TNF-α, IFN-γ, MCP-1, and IL-6 were markedly elevated in mice 6 hr after receiving the unmodified vector. By 24 hr, the concentrations had returned to normal baseline levels. These cytokines are involved in crucial steps of humoral and cell-mediated immunity and their elevation indicates potent activation of both arms of the adaptive immune response after administration of unmodified vectors. This observation contrasts with the modest acute elevation in serum MCP-1 concentration after injection of PEGylated Ads and indicates that this modification diminishes immunostimulatory properties of the anti-HBV vectors.

Assay of anti-Ad vector immunoglobulin titers and markers of hepatocyte toxicity

To measure the concentrations of humoral immune response to Ad vectors, enzyme-linked immunosorbent assays (ELISAs) were performed to detect antibodies interacting with Ad hexon proteins and also complete Ad particles. Comparison of the relative optical density (OD) readings indicated that there was a significant difference in immunoglobulin titers in animals 5 weeks after receiving either unmodified and PEGylated vectors (Fig. 3A). Lower values observed after administration of PEGylated vectors confirm that the humoral immune response is attenuated and correlates with diminished cytokine release, as well as data reported by others (Croyle *et al*., 2002, 2005; Eto *et al*., 2004; Mok *et al*., 2005). Hepatotoxic effects of the Ad administration were determined by measurement of ALT and AST activities in the serum of mice 1 day and 1 week after receiving a second dose of the PEG-modified or unmodified Ad vectors (Fig. 3B). Compared with mice receiving the PEGylated Ads, the serum concentrations of both transaminases were significantly increased at 24 hr in animals receiving unmodified vectors. Taken together, our data support the interpretation that PEG modification improves anti-HBV efficacy, attenuates immunostimulatory properties, and improves the safety profile of recombinant Ad vectors in a stringent model of HBV infection.

Discussion

Recombinant adenovirus vectors have several properties that make them useful for therapeutic transfer of anti-HBV sequences (Bangari and Mittal, 2006). These include efficient infection of nondividing cells, hepatocyte targeting after systemic administration *in vivo*, and transient transgene expression that is sustained for weeks without integrating into host DNA. However, immunostimulatory effects of Ads may be toxic and also diminish the efficiency of transgene expression. In addition to modification with synthetic polymers, as was used here, a number of approaches have been employed to diminish activation of host immune responses. These include immunosuppression (Smith *et al*., 1996; Ye *et al*., 2000), silencing mediators of hepatocyte injury (Chen *et al*., 2008), serotype switching (Vogels *et al*., 2003), genetic manipulation of capsid-encoding sequences (Roberts *et al*., 2006), and use of high-dose vectors (Parks *et al*., 1996). Although these strategies have had success, there are limitations to their general applicability. For example, immunosuppression has side effects that would not be desirable in a clinical setting of HBV treatment. Importantly, Ad immunostimulation is largely mediated by viral capsid proteins, and is not dependent on viral gene expression or transduction (Schnell *et al*., 2001). Modification of viral capsid proteins with PEG is therefore an attractive method to control Ad immunostimulatory properties.

PEG has been widely used in various therapeutic applications and has well-characterized pharmacologic properties. The polymer has low immunogenicity, is nontoxic, and improves the water solubility of PEG complexes (reviewed in Kreppel and Kochanek, 2008). In addition, PEG has other advantageous properties such as simultaneous modification of many surface proteins without the need for genetic manipulation, improvement of vector stability, and diminishment of nonspecific interactions. Covalent attachment of PEG to Ads can be carried out under mild reaction conditions to preserve vector bioactivity. The modification employed in this study entailed use of a semitelechelic mPEG-SPA, which reacts with lysine residues of the vector hexon, fiber, and penton proteins (O'Riordan *et al*., 1999). These are

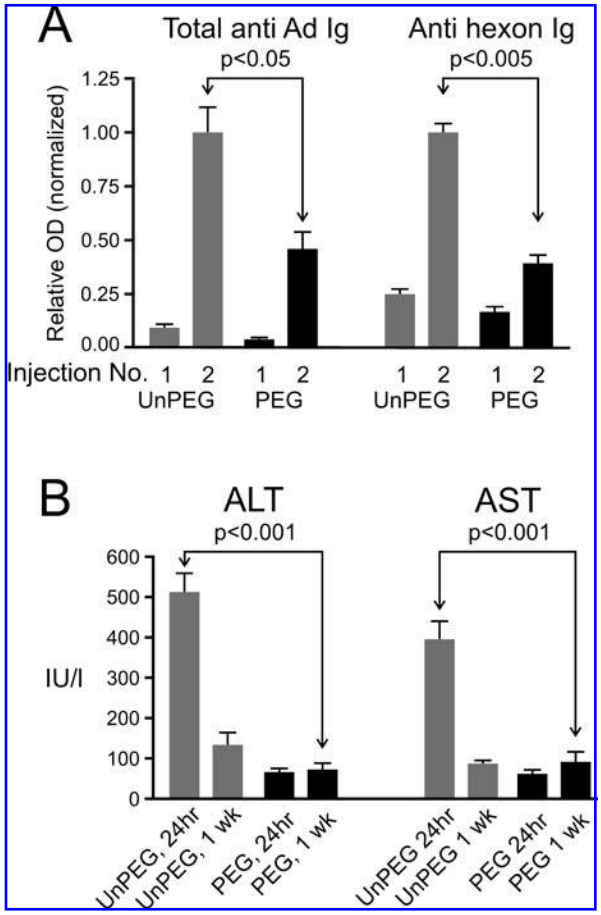


FIG. 3. Relative serum anti-Ad immunoglobulin concentrations and transaminase activities after vector administration to mice. (**A**) Total anti-Ad and anti-hexon protein-specific antibodies were measured by ELISA. (**B**) Transaminase (ALT and AST) activities were measured 24 hr and 1 week after the second injection of PEGylated or unmodified adenoviral vectors. Mean relative optical density readings (±SEM) and enzyme activities are given.

also the main targets of host neutralizing antibodies and PEG conjugation is therefore ideal to counter Ad interaction with host antibodies.

The bolus of 5×10^9 Ad infectious particles administered systemically to mice in this study represents a low dose compared with that used in other studies. Ad activation of dendritic cells and macrophages with resultant release of IL-6 and IL-12 was observed in mice treated with $0.3–5 \times 10^{11}$ particles per animal (Zhang *et al*., 2001). In primates, intraportal administration of up to 5×10^{12} particles/kg led to a self-limiting hepatitis, but higher doses caused massive hepatic necrosis and coagulopathy within days of vector administration (Schnell *et al*., 2001). This dose-dependent effect has also been reported on readministration of Ad vectors (Nunes *et al*., 1999). The significantly lower dose that was used in this study (approximately 2×10^{10} particles/kg) is likely to contribute to our observation of attenuated immunostimulation and improved safety. However, it appears that efficacy of RNAi-activating Ads is dose dependent, as administration of lower amounts of Ads did not cause significant HBV gene silencing. Although complete silencing of HBV replication was not observed in this study, a higher Ad dose may well silence HBV replication more effectively. However, higher doses have lethal toxic effects (data not shown), which are likely to result from Ad immunostimulation and saturation of the endogenous miR pathway (Grimm *et al*., 2006). The low dose of vector that was required to be effective as well as the favorable effects of PEG modification of Ad vectors are important means of improving the safety profile of RNAi-activating recombinant Ads for potential therapeutic application. To enhance the sustained efficiency of RNAi-inducing anti-HBV vectors, our current research is aimed at investigating PEG-modified HD RNAi-activating vectors and their long-term use in conjunction with existing licensed anti-HBV agents (e.g., lamivudine).

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Author Disclosure Statement

The authors declare no conflicting interests.

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Efficient Silencing of Hepatitis B Virus by Helper-dependent Adenovirus Vector-mediated Delivery of Artificial Antiviral Primary Micro RNAs

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Abstract: Hepatitis B virus (HBV) infection is endemic to southern Africa and parts of Asia where approximately 350 million individuals are chronically infected. Persistent infection increases risk for the serious complications of cirrhosis and hepatocellular carcinoma. Licensed HBV treatments rarely eradicate the virus, which makes developing new strategies for the treatment of chronic HBV a priority. Pol II-transcribed mono- and trimeric primary micro RNAs (pri-miRNAs) have previously been used to activate RNA interference (RNAi) and inhibit HBV gene expression, indicating that this approach holds promise for HBV therapy. Nevertheless, achieving safe and efficient delivery of anti-HBV RNAi expression cassettes remains an important objective before therapeutic application of this gene silencing technology is realized. Recombinant adenoviruses (Ads) are amongst the most efficient hepatotropic gene delivery vehicles, but a drawback of their use is transient transgene expression and toxicity that results from induction of host immune responses. To diminish immunostimulation of anti-HBV RNAi-activating vectors, helper-dependent (HD) Ads with all viral protein-encoding sequences removed from their genomes, were generated. A CMV Pol II promoter element was used to transcribe antiviral pri-miRNAs that target HBV. Processing of the anti-HBV pri-miRNA RNAi activators occurred according to intended design. Assessment in cultured cells and in a HBV transgenic model of the infection demonstrated that HD Ads delivered the silencing sequences efficiently and replication of the virus was inhibited without causing overt toxic effects. Collectively these data augur well for clinical use of HD Ads to deliver Pol II HBV-silencing cassettes to counter the persistent infection.

Keywords: HBV, Helper dependent adenovirus, Micro RNA, Pol II, RNAi.

INTRODUCTION

Approximately 6% of the world's population are chronic carriers of the hepatitis B virus (HBV) and at risk for complicating hepatocellular carcinoma and cirrhosis [1]. Persistent infection with the virus is endemic to sub Saharan Africa, east and southeast Asia where it is a significant cause of public health problems. Currently available therapies are capable of suppressing HBV replication but rarely eliminate the virus [2]. Improved HBV treatment is therefore important to limit carriers' risk for the associated life-threatening complications. Dependence of HBV on a RNA pregenomic replication intermediate, as well as compact genome arrangement, make the virus susceptible to potentially therapeutic RNA interference- (RNAi-) based gene silencing. Several studies have demonstrated successful inhibition of HBV replication by harnessing this gene knock down technology (reviewed in [3-6]).

Although synthetic short interfering RNAs (siRNAs) have been widely used as exogenous RNAi activators, expressed intermediates of the pathway potentially have greater utility for treatment of chronic infections such as are caused by HBV. Antiviral expression cassettes typically comprise a Pol II or Pol III transcription regulatory element, down stream sequence encoding mimics of precursor micro RNA (pre-miRNA) [7] or primary miRNA (pri-miRNA) [8] hairpin-containing elements, and a transcription termination signal. To date, the most commonly used RNAi expression cassettes have incorporated Pol III regulatory elements, e.g. U6 and H1. These promoters have advantages of small size, ability to transcribe defined short transcripts constitutively and presence of most of the regulatory elements upstream of the transcription termination site. However, a disadvantage is their limited range of transcriptional control. Although regulatable U6 promoter activity has been achieved with tetracycline responsive elements [9], toxicity resulting from saturation of the endogenous RNAi pathway remains a concern [10]. To address this problem, previous work has entailed the use of Pol II promoters to express antiviral pri-miRNAs [11-15]. Pol II elements have better transcription capabilities to enable tissue specific and precisely controlled expression of silencing sequences. Artificial pri-miRNAs are also compatible with a polycistronic configuration to enable simultaneous targeting of multiple viral sites. This should improve silencing efficacy and also limit the emergence of viral escape. Moreover, use of RNAi activators that enter the pathway at a proximal step, may improve efficacy of silencing. This is based on the concept that steps of the RNAi pathway may be functionally linked.

Previous work from our laboratory demonstrated highly efficient silencing of HBV replication by monomeric and trimeric anti-HBV pri-miRNAs [12, 13]. The cassettes were propagated using backbone sequences from natural pri-miR-

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31, pri-miR-122 and pri-miR-30a. Although these results are promising, safe and efficient hepatotropic delivery of expressed antiviral miRNAs following systemic administration *in vivo* remains challenging. To date, recombinant adenoviruses (Ads) [16-18], adeno associated viruses [10] and lentiviruses [19] have been used to deliver RNAi cassettes to the liver. The natural tropism of Ads is a very useful property, but toxic immunostimulation is problematic (reviewed in [20]). Third generation helper dependent (HD) Ads, which have viral protein coding sequences stripped from their genomes, are less immunostimulatory and therefore safer. Also, the durable transgene expression that may be achieved with HD Ads is well-suited to treatment of a chronic infection such as is caused by HBV. To date only one study has reported on use of HD Ads to deliver anti-HBV RNAi activators [18]. Modest HBV silencing was reported and is likely to have been the result of poor efficacy of the U6 Pol III anti-HBV expression cassette. To explore the utility of HD Ads using known effective anti-HBV RNAi activators, we have characterized anti-HBV efficacy in cell culture and a murine transgenic model. Results demonstrate that anti-HBV pri-miRNAs are expressed and processed according to the intended design. Efficient silencing of viral replication was achieved *in vivo* without obvious evidence of toxicity.

METHODS AND MATERIALS

Generating Anti-HBV HD Ad Genomic DNA

Previously described cytomegalovirus (CMV) promoter-containing anti-HBV mono- or trimeric pri-miRNA mimics [13] (pri-miR-122/5, pri-miR-31/5 and pri-miR-31/5-8-9) were initially amplified using forward (5'GATCGG CGC GCCCTATGGAAAAACGCCAGCAA 3') and reverse (5'G-ATCGG CGC GCCGAAAGGAAGGGAAGAAAG-CGA3') primers that incorporated *Asc* I restriction digestion sites (underlined). Amplicons were then inserted into the PCR cloning vector, pTZ57R/T (InsTAclone™ PCR cloning Kit, Fermentas, MD, USA) to generate pTZ pri-miR-31/5HD, pTZ pri-miR-122/5HD and pTZ pri-miR-31/5-8-9HD. After confirming correct sequences of the inserts, they were excised using *Asc* I and inserted at the equivalent restriction site of pΔ28E4LacZ [21]. Resulting plasmids, named pΔ28E4 HBVpri-miR-31/5,pΔ28E4 HBVpri-miR-122/5 and pΔ28E4 HBVpri-miR-31/5-8-9, were then digested with *Pme* I to release the HD Ad viral genome as linear DNA.

Preparation of Anti-HBV HD Ads

HEK293-derived 116 HD Ad packaging cells [22] were propagated in Eagle's or Joklik's minimum essential medium (MEM). Production, amplification and purification of anti-HBV HD Ads were carried out according to published methods with minor modifications [22]. Briefly, 116 cells were transfected with linear HD Ad genome DNA. Transfected cells were then infected with helper virus (AdNG163), and incubated for 48 hours to initiate production of anti-HBV HD Ads. To amplify anti-HBV HDAds, freeze-thawed transfected cell suspensions, together with helper virus, were used repeatedly to co-infect increasing numbers of 116 cells. For large scale preparation of anti-HBV HDAds, 116 cells were cultured in 2 L suspensions in 3 L Spinner flasks. Harvested cells were concentrated using centrifugation then lysed. Anti-HBV HDAds were purified using CsCl gradient centrifugation. Yield of total viral particles and helper virus contamination were assayed using quantitative PCR. HD Ad infectious units were determined using dilutions of virus preparations to infect HEK293 cells followed by staining for beta galactosidase activity [22]. Aliquots of pure anti-HBVHDAds particles were stored in 10% glycerol at -80^{o}C. Vectors used in the experiments were a control HD Ad Δ28, which lacked a RNAi expression construct, HD Ad HBV pri-miR-31/5, HD Ad HBV pri-miR-122/5 and HD Ad HBV pri-miR-31/5-8-9.

Maintenance, Transfection and HD Ad Infection of Cells in Culture

Huh7 and HEK293 lines weremaintained as has been described [16]. To assess anti-HBV effects in cell culture, Huh7 cells wereinitially transfected with an HBV replication competent plasmid (pCH-9/3091 [23]) , which contains a greater than genome length HBV sequence. A plasmidthat constitutively expresses eGFP [24] was included as a transfection control. Five hours after transfection, cells were washed with fresh medium and infected with anti-HBV HD Ads at varying multiplicities of infection (MOIs). Knockdown of HBV replication was assessed by measuring the secretion of HBV surface antigen (HBsAg) into the culture supernatants. This was carried out 48 hours after HD Ad infection and performed using the MONOLISA® HBs Ag Assay kit (Bio-Rad, CA, USA).

Northern Blot Hybridization

Liver-derived Huh7 cells were infected with HD Ads at an approximate MOI of 100 HD Ads per cell. The cells were harvested 2 days thereafter before extracting total RNA using Tri Reagent (Sigma, MI, USA). RNA was analyzed using Northern blot hybridization according to procedures that have previously been described [12]. Sequences of the probes used to detectmature 5, 8 and 9 guides were 5' CCG TGT GCA CTT CGC TTC 3', 5' CAA TGT CAA CGA CCG ACC 3' and 5' TAG GAG GCT GTA GGC ATA 3' respectively. To confirm equal loading of lanes with RNA, blots were stripped and probed with a labelled a U6 snRNA oligonucleotide (5' TAGTATATGTGCTGCCGAAG-CGAGCA 3').

Assessment of *In Vivo* Efficacy of Anti-HBV HD Ads

Experiments using HBV transgenic mice [25] as a model of HBV infection were carried out in accordance with protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee. A dose of 5×10^9 infectious HD Ads was injected as a bolus *via* the tail vein. Groups of mice comprised 4 animals each and blood was collected by retroorbital puncture. ELISA for HBsAg levels was performed on serum samples using the MONOLISA® HBs Ag ULTRA kit from Bio-Rad. Circulating viral particle equivalents (VPEs) were determined using real time PCR according to previously described methods [16]. Using separate groups of animals, adenovirus gene transduction was assessed by detecting *LacZ* expression macro- and micro-

scopically in frozen liver sections taken at 48 hours after initial adenovirus injection.

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). Statistical difference was considered significant when $P<0.05$ and was determined according to the Student's 2 tailed t-test. Calculations were done with the GraphPad Prism software package (GraphPad Software Inc., CA, USA).

RESULTS

Mono- and Trimeric pri-miRNA expression Cassettes Targeting HBV and Structure of HD Ads

The HBV targets of the mono- and trimeric pri-miRNA expression cassettes are located in the *X* open reading frame of HBV (*HBx*) (Fig. **1A**). *HBx* is conserved in HBV genotypes and is also present in all of the viral transcripts, which makes it a suitable target for therapeutic gene silencing. HD Ads containing anti-HBV pri-miRNA expression cassettes were propagated from a genomic vector sequence using standard techniques [21]. In summary, vector DNA was used to transfect 116 packaging cells and helper Ads provided viral sequences in trans. *LoxP* sites flanking the Ψ packaging sequence of the helper virus, and vector propagation in Cre recombinase-expressing 116 cells, facilitated purification of HD Ads. Using this procedure and after CsCl gradient purification, helper virus contamination was routinely less than 0.1%.

The anti-HBV cassettes incorporated into the HD Ads comprised a CMV Pol II promoter and previously described downstream sequences encoding monomeric or trimeric pri-miR-31s or pri-miR-122s (Fig. **1B**) [12,13]. HD Ad HBV pri-miR-31/5 and HD Ad HBV pri-miR-122/5 vectors had monomeric pri-miR-31 or pri-miR-122 backbones, respectively. The intended guide targeted the same HBV site 5 and has previously been shown to be effective against the virus [16] (Fig. **1A**). The HD Ad HBV pri-miR-31/5-8-9 vector included two additional previously described antiviral sequences [16] in a trimeric cassette. This polycistronic sequence was designed to generate three guides simultaneously. In addition to the antiviral sequences, HD Ad vectors included a β-galactosidase cassette (Fig. **1B**). This reporter sequence enabled tracking of HD Ad infection of cells.

Processing of Mono- and Trimeric anti-HBV Expression Cassettes

The scheme that illustrates design and intended processing of anti-HBV pri-miRNA expression cassettes is shown in Figs **2A** and **2B**. The CMV Pol II promoter-generated pri-miRNA sequences should be processed by Drosha/DGCR8 to form pre-miRNA intermediates. After export form the nucleus, pre-miRNA sequences are cleaved by Dicer and an antiviral guide is selected for action against viral targets. Pri-miR-31/5 and pri-miR-122/5 cassettes generate the same antiviral guide 5 sequence. The trimeric cassette from pri-miR-31/5-8-9 results in formation of three different mature antiviral guides that are intended to target HBV sequences simultaneously.

To assess the processing of anti-HBV pri-miRNAs, Huh7 liver-derived cells were infected with the recombinant HD Ads. RNA was extracted from these cells after 48 hours and then subjected to Northern blot hybridization analysis (Fig. **2C**). Blots probed with a labeled oligonucleotide that was complementary to intended guide 5 showed presence of

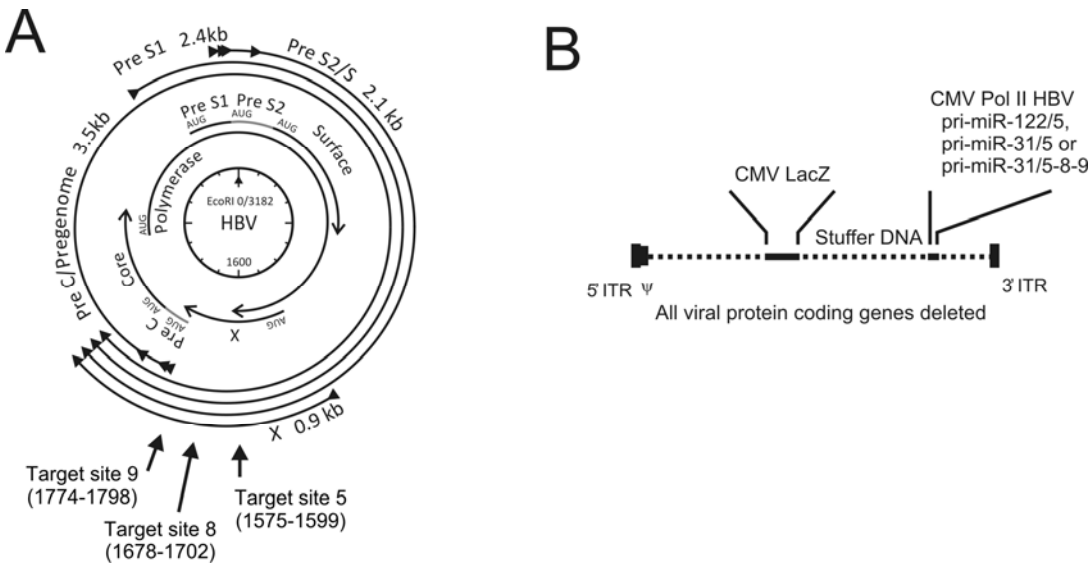


Fig. (1). HBV target sites and antiviral HD Ad vectors. **A.** Hepatitis B virus genome showing sites targeted by pri-miR-31/5, pri-miR-122/5 and pri-miR-31/5-8-9 cassettes. Co-ordinates of the viral genome are given relative to the unique *Eco*RI restriction site. The *surface*, *core*, *polymerase* and *HBx* viral open reading frames (ORFs) (with initiation codons), encompassing the entire genome, are indicated by arrows. Four outer arrows represent the HBV transcripts with common 3′ ends. Specific nucleotide coordinates of the target 5, 8 and 9 sites within the genome are shown. **B.** HD Ads containing the CMV Pol II pri-miR-31/5, pri-miR-122/5 and pri-miR-31/5-8-9 cassettes with CMV β-galactosidase-expressing reporter cassette are illustrated schematically. Apart from ITRs and viral packaging signal (ψ) the remainder of the vector DNA comprises stuffer sequence with all Ad ORFs removed.

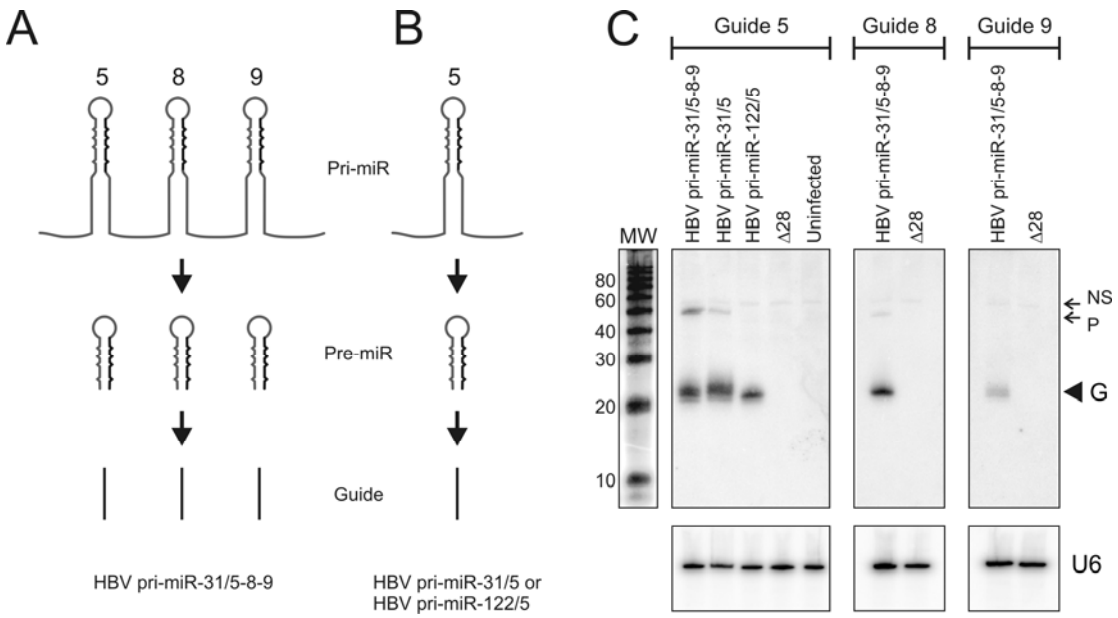


Fig. (2). Processing of anti-HBV pri-miR-31/5, pri-miR-122/5 and pri-miR-31/5-8-9. Schematic illustration of the trimeric (**A**) and monomeric (**B**) pri-miRNAs, derived pre-miRNA and mature guides. The guide strands are indicated as black lines and the remaining pri-miRNA components are in grey. Mature guides targeting sites 5, 8 and 9 are generated from the trimeric cassette, while guides complementary to the target site 5 are formed from pri-miR-31/5, pri-miR-122/5. **C.** RNA extracted from HD Ad-transduced liver-derived Huh7 cells was subjected to Northern blot hybridization using a probe that was complementary to the intended guide 5, 8 or 9 strands. Viral vectors contained the indicated cassettes (pri-miR-31/5, pri-miR-122/5 or pri-miR-31/5-8-9) or no anti-HBV sequence (empty, Δ28). Putative guide (G), precursor (P) and non specific (NS) bands are indicated. A decade RNA molecular weight marker (MW), with size of bands (nt) indicated, was used to determine approximate length of guide strands. After hybridization, blots were stripped and reprobed with an oligonucleotide complementary to U6 snRNA to confirm equal loading of the lanes.

a band of approximately 21 nt in length. This was evident in RNA extracted from cells that had been infected with HD Ad HBV pri-miR-31/5, HD Ad HBV pri-miR-122/5 and Ad HBV pri-miR-31/5-8-9, but not in cells that were uninfected or treated with the empty HD Ad vector (HD Ad Δ28). Hybridization to probes for guide 8 and guide 9 demonstrated presence of a band of approximately 21 nt in the RNA from cells that had been infected with Ad HBV pri-miR-31/5-8-9. Again bands corresponding to the mature guide sequences were not evident in cells infected with the empty vector. As had previously been demonstrated when using the trimeric pri-miR-31/5-8-9 cassette in the context of a plasmid [13], the intensity of the band corresponding to the guide 9 sequence was less intense. This suggests that processing of the guide targeting sequence 9 was less efficient than that of HBV guide sequences 5 or 8. Compared to the concentration of mature miRNA sequences, pri- or pre-miRNAprecursor species were present in low concentrations in HD Ad-infected cells. Collectively these data demonstrate that the anti-HBV pri-miRNA expression cassettes are processed efficiently and according to intended design when delivered to liver-derived cells by HD Ad vectors.

Silencing of HBV Replication in Cultured Cells by Pri-miRNA Expressing HD Ads

As an initial assessment of anti-HBV efficacy of HD Ads, a cell culture model of HBV replication was utilized. Liver-derived Huh7 cells were transfected with the pCH-9/3091 HBV replication competent plasmid and then infected with HD Ads 5 hours thereafter (Fig. **3**). Ads were

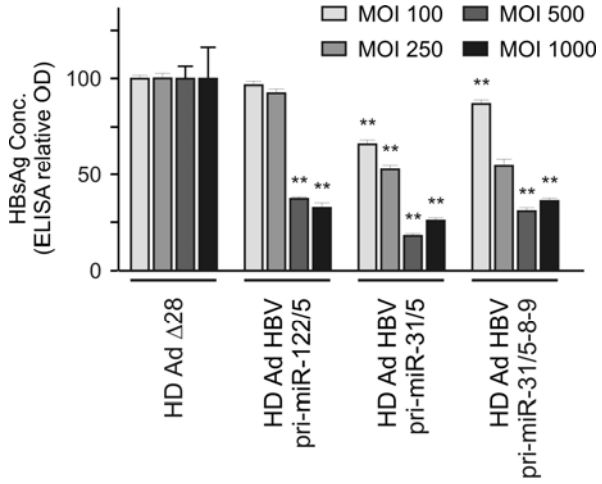


Fig. (3). Efficacy in cell culture of HD Ad HBV pri-miR-31/5, HD Ad HBV pri-miR-122/5 and HD Ad HBV pri-miR-31/5-8-9 against HBV. Huh7 liver derived cells were transfected with the pCH9/3091 HBV replication competent plasmid. Five hours thereafter, the cells were infected with HD Ads containing no insert (HD Δ28) or the indicated anti-HBV cassettes. The cells were infected with HD Ads at MOIs of 100, 250, 500 or 1000. HBsAg was then measured in the culture supernatants 48 hours after Ad infection and the concentrations determined relative to the control values obtained for cells infected with the empty HD Ad. Results are expressed as the mean (±SEM) of 3 or 4 replicates. Statistically significant differences (* $p<0.05$, ** $p<0.01$) between HBV pri-miR-expressing vectors and controls are indicated and were determined according to the Student's 2 tailed paired t-test.

added to the cultured cells at a range of MOIs from 100 to 1000. When HBsAg was measured in the culture supernatants 48 hours after infection, all vectors caused a decrease in this marker of replication at MOIs of 500 and 1000. However, only HD Ad HBV pri-miR-31/5 and HD Ad HBV pri-miR-31/5-8-9 caused a significant decrease in HBsAg concentrations in the culture supernatants at MOIs of 100 and 250. These results indicate that HD Ads expressing pri-miRNAs, albeit at high MOIs, are capable of inhibiting HBV replication in liver-derived cells. This evidence supports the notion that the HD Ads, which typically have utility as hepatotropic vectors *in vivo*, may be capable of countering HBV replication in transgenic mice.

Efficient HD Ad Transduction of Hepatocytes and HBV Replication Silencing *In Vivo*

To determine the efficacy *in vivo* of HD Ads expressing anti-HBV Pol II pri-miRNAs, a HBV transgenic mouse model was employed [25]. Stable and constitutive replication of HBV occurs in these animals in a manner that simulates aspects of the virus chronic carrier state that occurs in humans. There are however differences between the human HBV chronic carrier state and the transgenic mouse model. HBV infection of murine hepatocytes does not occur and the virus is only propagated from the greater than genome length integrant. Also as transgenic animals, the HBV sequences are recognized as 'self'. Consequently an inflammatory cytotoxic immune response, which is a characteristic precursor of cirrhosis and HCC in HBV carriers, is not induced. Moreover, HBV per se is not directly cytotoxic and the mice do not develop cirrhosis and liver cancer as it occurs in humans.

Injection of HD Ads resulted in highly efficient transduction of hepatocytes (Fig. **4**). Mice demonstrated no obvious adverse effects of HD Ad administration. When compared to first generation Ads, histological examination of stained liver sections, measurement of serum transaminases and release of pro-inflammatory cytokines revealed that use of HD Ads had a significantly improved the safety profile (Crowther *et al*, manuscripts under review and in preparation).Analysis of microscopy sections stained for β-galactosidase activity showed that more than 90% of hepatocytes were transduced, which was evaluated to be sufficient to achieve a therapeutic effect by the antiviral sequences.

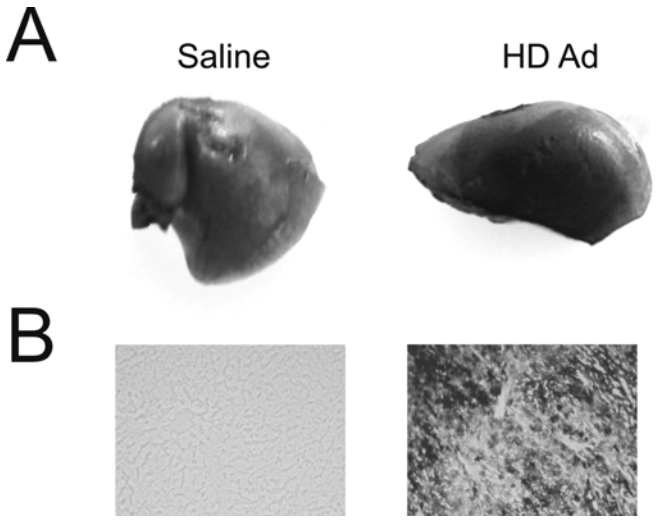


Fig. (4). Efficient HD Ad-mediated reporter gene delivery to the liver. Adult HBV transgenic mice were injected intravenously with saline or 5×10^9 HD Ad particles. Forty eight hours thereafter they were killed and the livers removed. Representative macroscopic samples (**A**) or frozen sections (**B**) of hepatic tissue were subjected to staining for β-galactosidase reporter gene activity.

To assess the inhibition of HBV replication in HBV transgenic mice, the animals received an intravenous dose of 5×10^9 HD Ad infectious particles. The concentrations of HBsAg and circulating VPEs were determined in the serum at the time of HD Ad administration and weekly thereafter for 14 days (Fig. **5 A**&**B**). Analysis was carried out by comparing the normalized values relative to the animals that received the empty HD Ad vector (HD Ad Δ28). In our hands, a characteristic of the HBV transgenic model used here is that variation in markers of viral replication occurs naturally, and this may influence analysis of antiviral efficacy. Never-

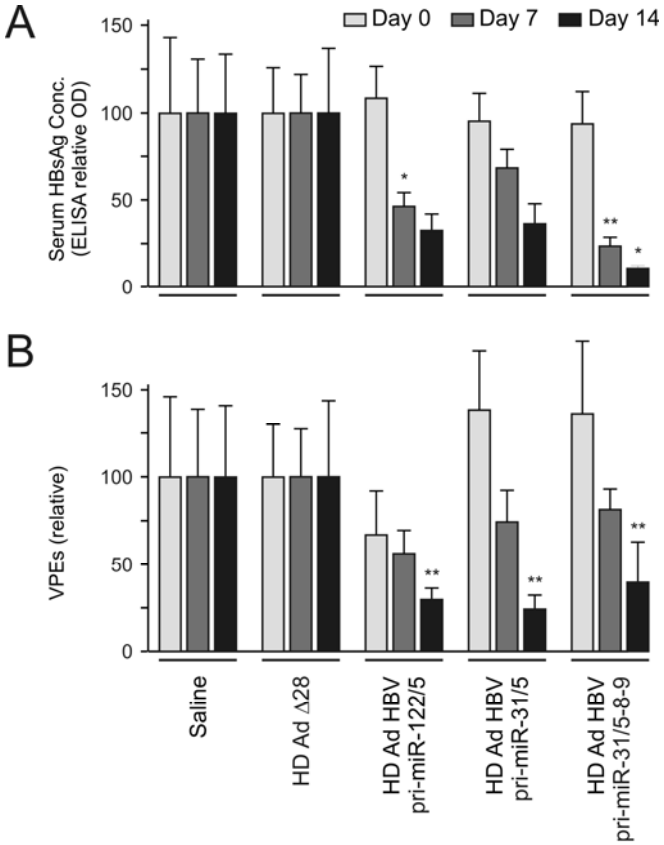


Fig. (5). Anti-HBV efficacy of HD Ads. Effects of intravenous administration of HD Ads on circulating HBV surface antigen (**A**) and VPEs (**B**) in HBV transgenic mice. Each animal received a single bolus injection of 5×10^9 infectious Ad particles at commencement of the experiment. Controls animals were either injected with saline or HD Ad Δ28, which lacked the HBV-targeting sequences. Titers of HBsAg were determined using ELISA and circulating VPEs were measured using real time quantitative PCR. Results are expressed as the mean (±SEM) from groups comprising 4 mice. Statistically significant differences (* $p<0.05$, ** $p<0.01$) between HBV pri-miRNA expressing vectors and controls are indicated and were determined according to the Student's 2 tailed paired t-test.

theless, all three anti-HBV HD Ads tested, HD Ad HBV pri-miR-31/5, HD Ad HBV pri-miR-122/5 and Ad HBV pri-miR-31/5-8-9, caused statistically significant decreases in markers of viral replication. Comparison of the HBsAg concentrations indicated that the trimeric HD Ad was most effective against HBV replication *in vivo*. This observation suggests that a combination of three anti-HBV sequences improves silencing efficacy. Collectively, our results indicate that delivery of pri-miRNAs using HD Ads effectively silences HBV replication *in vivo* and therefore has potential clinical utility.

DISCUSSION

Complications that arise from the chronic carrier state of infection with HBV remain an important global public health problem [1]. Although several drugs have been licensed to treat the infection, elimination of HBV from carriers of the virus remains difficult to achieve [2]. As a result, persistently infected individuals continue to be at risk for complicating cirrhosis and hepatocellular carcinoma. Available therapies effectively suppress HBV replication. However daily long term drug administration is typically required to achieve a sustained therapeutic effect and implementation of such treatment regimens is difficult in resource-poor settings. Demonstration that harnessing the RNAi pathway to inhibit HBV replication potentially has therapeutic benefit may offer a useful approach to improve available HBV therapy. Despite the promising results from preclinical studies, significant hurdles remain before RNAi-based therapy for HBV infection is realized [6]. Obstacles that need to be overcome include the identification of optimal RNAi activators that effect gene silencing at low concentrations, and also delivery of antiviral RNAi activators in a manner that enables sustained silencing of viral replication *in vivo*. Availability of such an HBV therapy that results in a functional cure after single administration would be of considerable benefit.

The artificial pri-miRNAs used in this study have several advantages for countering HBV replication. The notion that each step of the natural miRNA pathway is functionally coupled implies that use of pri-miRNA mimics, which enter the pathway at a proximal stage, should achieve more effective target gene silencing. As expression cassettes, sustained production of viral gene silencers from a DNA template may be utilized more effectively against chronic HBV infection than repeated administration of synthetic siRNAs. Also, the mimics of naturally occurring pri-miRNA sequences may be configured in Pol II cassettes as polycistronic multimers. Multimerization of HBV gene silencers has the benefit of simultaneous targeting of several HBV sites to improve efficacy and limit the chances of viral escape. Evidence indicates that trimeric pri-miRNA sequences produced in this manner have little if any effect on the endogenous miRNA pathway [12,13]. Use of Pol II promoters to generate antiviral sequences provides the means for achieving better regulated and safe control of transcription of RNAi activators. The constitutive high rate of transcription that is characteristic of Pol III promoters, such as U6, is potentially problematic as the endogenous miRNA pathway may be disrupted leading to toxic side effects [10]. Pol II promoters have greater flexibility and tissue-specific or inducible regulatory elements may be employed to control the dose of therapeutic effecters. In this study, the constitutively active CMV promoter was used successfully to regulate transcription of anti-HBV gene silencers. Efficient production of antiviral sequences was demonstrated in liver-derived cells and delivery of these sequences to the liver resulted in silencing of HBV replication *in vivo*. Although these results are encouraging, use of the CMV promoter may not be ideal for therapeutic application. Reasons include the high level of activity of the transcriptional element in non-hepatic tissues, and also the variable long term transgene expression in transduced cells [26]. To improve control of HBV RNAi activators, current research is aimed at using liver specific and inducible promoters that are capable of long term transgene production.

Efficient and safe delivery of RNAi activators remains an important challenge for advancing RNAi-based HBV therapy. Use of recombinant viral vectors such as Ads [16-18], AAVs [10] and lentiviruses [19] have been used. Delivery of transgenes to a high number of hepatocytes without causing unwanted side effects and achieving long term expression are critically important requirements of anti-HBV RNAi vectors. The HD Ads that were used in this study meet some of these requirements. The highly efficient *in vivo* liver tropism is particularly useful for delivery of sequences that silence HBV replication in the liver. Our demonstration that more than 90% of hepatocytes are transduced, together with effective silencing of HBV replication in a stringent murine model of the disease, confirm that these vectors may be used to counter HBV *in vivo*. As the wild type Ad sequences have been removed from the HD Ads, immunostimulation is markedly attenuated. This has benefit for limiting immune-mediated toxic effects as well as improving the duration of transgene expression. Although the length of transgene expression was not fully characterized in this study, evidence from other reports indicates that transgene expression of more than 2 years may be achieved in rats [27].

The demonstration that HBV replication may be silenced *in vivo* using recombinant HD Ads that express single and multimeric HBV gene silencers is potentially very useful for therapeutic application. Although promising, further refinements in the technology will be required for it to advance to a stage of clinical use. Duration of expression of HBV gene silencers, polymer modification or pretreatment with immunosuppressive glucocorticoids to attenuate innate immunostimulation, and incorporation of additional therapeutic cassettes are being investigated. Nevertheless, these data augur well for the clinical utility of using RNAi activators to counter HBV persistent infection.

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Expert Opinion

1. Introduction
2. Activating RNAi for therapeutic application
3. Principles underlying methods of propagating adenovirus vectors
4. HD adenovirus vectors are attractive for achieving sustained RNAi-based therapeutic gene silencing
5. Viral infections as targets for adenovirus vector-based RNAi therapy
6. Conclusion
7. Expert opinion

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Therapeutic potential of adenoviral vectors for delivery of expressed RNAi activators

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Importance of the field: Harnessing RNA interference (RNAi) to silence pathology-causing genes has shown promise as a mode of therapy. The sustained gene inhibition that may be achieved with expressed sequences is potentially useful for treatment of chronic viral infections, but efficient and safe delivery of these sequences remains a challenge. It is generally recognized that there is no ideal vector for all therapeutic RNAi applications, but recombinant adenovirus vectors are well suited to hepatic delivery of expressed RNAi activators.

Areas covered in this review: Adenoviruses are hepatotropic after systemic administration, and this is useful for delivering expressed RNAi activators that silence pathology-causing genes in the liver. However, drawbacks of adenoviruses are toxicity and diminished efficacy, which result from induction of innate and adaptive immune responses. In this review, the advantages and hurdles facing therapeutic application of adenoviral vectors for liver delivery of RNAi effectors are covered.

What the reader will gain: Insights into adenovirus vectorology and the methods that have been used to make these vectors safer for advancing clinical application of RNAi-based therapy.

Take home message: Adenoviruses are very powerful hepatotropic vectors. To make adenoviruses more effective for clinical use, polymer conjugation and deletion of viral vector sequences have been used successfully. However, further modifications to attenuate immunostimulation as well as improvements in large-scale production are necessary before the therapeutic potential of adenovirus-mediated delivery of RNAi activators is realized.

Keywords: adenovirus, helper-dependent adenovirus, hepatitis B virus, hepatitis C virus, RNA interference

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1. Introduction

Demonstration that RNA interference (RNAi) may be activated with exogenous effectors to silence pathology-causing genes has generated considerable interest in using this approach for therapy [1,2]. Harnessing RNAi to counter gene expression has been a particularly active field of research, and many diseases, especially those caused by virus infections and cancer, have been shown to be susceptible to RNAi-mediated inhibition. However, one of the major obstacles to realizing the therapeutic potential of RNAi has been the difficulty with which safe and efficient delivery of gene silencing sequences to intended target cells may be accomplished. A variety of methods has been used to achieve this [1]. These include use of non-viral vectors for delivery of synthetic RNAi activators and viral vectors for transfer of antiviral RNAi expression cassettes. The sustained silencing that may be achieved with expressed sequences makes viral vectors attractive for treatment of chronic

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Article highlights.

- Harnessing RNAi to silence pathology-causing genes has enormous therapeutic potential.
- Expressed RNAi activators are compatible with recombinant adenovirus vectors and have the useful property of achieving sustained gene silencing.
- Adenovirus vectors have many features that make them useful for delivery of potentially therapeutic RNAi activators.
- An important drawback of adenoviruses is their stimulation of innate and adaptive immune responses, which is potentially toxic.
- Development of helper-dependent adenoviruses and also polymer conjugation of adenoviruses has contributed to overcoming problems of immunostimulation.
- The efficient delivery of transgenes to the liver should make adenoviruses useful for treating liver diseases such as those caused by HBV and HCV infection.
- The full potential of adenovirus-mediated delivery of RNAi activators will depend largely on establishing their safety and developing convenient methods of large-scale production.

This box summarizes key points contained in the article.

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diseases. Viral vectors that are under development include recombinant adeno-associated viruses (AAVs), lentiviruses and adenoviruses. The ability of lentiviral vectors to integrate proviral sequences that stably produce antiviral RNAi activators has been particularly useful for *ex vivo* approaches to treating viral diseases that result from chronic infections, for example, AIDS caused by HIV persistence. This is not suitable for all RNAi-based antiviral approaches, and utility of lentiviral vectors is specific to certain clinical conditions. A further potential complication of using lentiviral vectors is that promoters that are responsible for control of expression of RNAi activators may interfere with transcription required for vector propagation [3]. The lack of pathology that is caused by infection with wild-type AAVs and attenuated induction of immunostimulatory pathways have made these vectors particularly appealing [4]. However, induction of an immune response by AAVs does occur and their lower transgene capacity would compromise ability to incorporate large RNAi expression sequences [5]. Recombinant adenoviruses have features that make them suited to delivering potentially therapeutic RNAi activators to the liver. These include efficient hepatic expression of inserted cassettes, well-established methods of propagating the vector, episomal location of the adenovirus genome in infected cells, compatibility with chemical modification to alter biological properties, ability to infect non-dividing cells, and transient or sustained expression of transgenes. A shortcoming of adenoviruses is their powerful stimulation of the innate and adaptive immune responses, which may result in toxicity and attenuation of efficiency of transgene delivery. The death of a patient in a clinical trial who developed complications arising from immunostimulatory effects of adenoviruses provided an important and hard lesson for researchers in the field [6]. In this review, recent advances that are relevant to the potential therapeutic utility of incorporating expressed antiviral RNAi activators into adenoviruses are discussed. Modifications to reduce vector immunostimulation, and improve stability, specificity, control of vector tropism and expression of transgenes are discussed. An opinion on the existing important challenges in the field is provided.

2. Activating RNAi for therapeutic application

RNAi, which was first described in 1998 [7], is a highly conserved pathway in metazoan cells and is essential for regulation of genes involved in a variety of cellular processes [8]. Transcription of duplex-containing RNA molecules initiates the pathway. These transcripts are processed in a stepwise manner to form mature gene silencing sequences. The mammalian micro-RNA (miR) pathway is a well-characterized mechanism of RNAi. RNA Polymerase II (Pol II)-mediated transcription of long mono- or polycistronic primary miRs (pri-miRs) sets off the pathway (**Figure 1**). These sequences contain hairpin-like RNA structures and are cleaved in the nucleus by Drosha, an RNase III enzyme, and its double-stranded RNA binding partner, DiGeorge Critical Region 8 (DGCR8) [9]. The resulting precursor miRs (pre-miRs) are then transported to the cytoplasm by exportin-5 and are then processed further by Dicer to yield mature miR duplexes comprising 21 – 23 bp RNA. One strand of the mature miR is selected as a guide for incorporation into the RNA inducing silencing complex (RISC). The guide directs RISC to effect degradation or translational suppression of target mRNA.

Silencing of target genes with exogenous RNAi activators may be achieved with chemically synthesized short interfering RNAs (siRNA), which mimic mature endogenous miRs [10], or by expression of RNAi intermediates from exogenous DNA templates (**Figure 1**) [2]. Synthetic siRNAs have the advantage of being compatible with chemical modification to improve stability, specificity, cellular delivery and safety [11,12]. Also, the smaller size of synthetic siRNAs and the cytoplasmic, not nuclear, site of action make their dose control and delivery with non-viral vectors easier to achieve. Exogenous expressed RNAi activators have the advantages of prolonged efficacy from sustained intracellular supply of siRNAs, ease of propagation in plasmid DNA, and better stability and compatibility with viral vectors such as recombinant adenoviruses. Typically, DNA expression cassettes encoding mimics of pri-miRs (pri-miR shuttles) or short hairpin RNA (shRNA) analogues of pre-miR are used to activate the RNAi pathway (**Figure 1**). RNA polymerase III promoters, for example U6 small nuclear RNA and human ribonuclease P RNA component H1 promoters, are commonly used to regulate transcription of RNAi activators. However, overexpression from Pol III promoters may be complicated by toxicity that is attributed to saturation of the endogenous

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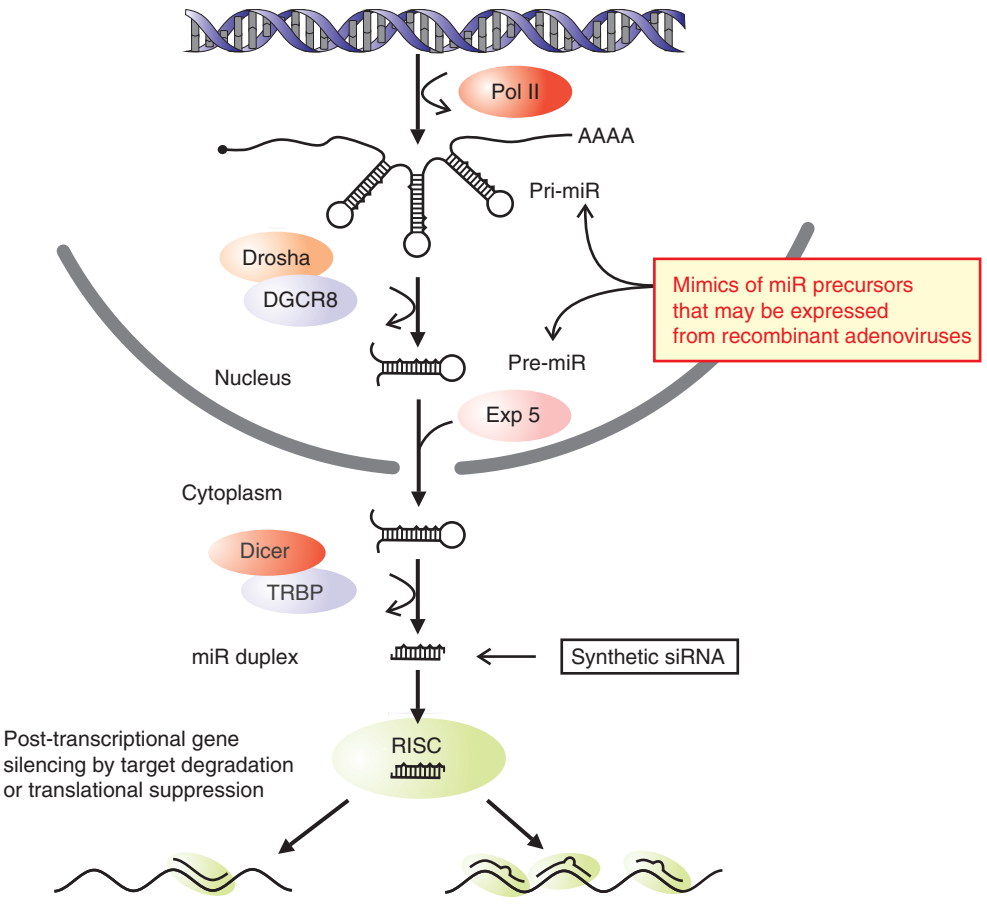


Figure 1. Summarized illustration of miR processing that shows the essential nuclear and cytoplasmic steps of the RNAi pathway. Exogenous expression cassettes that transcribe miR intermediates, which may be pri-miR or pre-miR sequences, are typically incorporated into adenoviruses. Non-viral vectors are normally used to deliver synthetic siRNA analogues of mature miR duplexes.

Adapted from [93,94].

miR: Micro-RNA; pre-miR: Precursor miR; pri-miR: Primary miR; RNAi: RNA interference; siRNA: Short interfering RNA.

RNAi machinery [13-15] or activation of an interferon response [16]. Importantly, unlike the case with delivery of synthetic siRNAs, expressed RNAi activators do not traverse the endosomal compartment and therefore bypass much of the toll-like receptor (TLR)-mediated immunostimulation [17]. Compatibility of pri-miR expression cassettes with versatile Pol II promoters enables improved transcriptional regulation [18] and generation of multimeric RNAi activators [19]. This is particularly useful to achieve tissue-specific expression, regulate the dose of RNAi activators and prevent viral escape. These properties have been utilized effectively to silence hepatitis B virus (HBV) replication *in vivo* [18,19]. In developing RNAi-based antiviral therapy, the focus of research has been on the identification of sequences that are effective at low concentrations (potent), devoid of unintended off-target effects, have limited immunostimulatory effects and can be easily delivered to target tissues in a dose-dependent manner. When used in conjunction with adenoviruses, these considerations are especially important to avoid exacerbating potential undesirable effects of the vectors.

Diseases caused by viral infections that are amenable to RNAi-based therapy are highly varied and this has a bearing on the approach to the development of RNAi-based therapy. Methods of delivery of sequences that silence virus replication and the selection of appropriate RNAi activators need to be tailored to the characteristics of individual infections. Important factors are the tropism of viruses, acute or chronic nature of the infection, whether a virus encodes RNA silencing suppressors (RSSs) and genetic variability as a result of inaccuracies of viral polymerases. Decisions about selection of adenoviruses for delivery of RNAi-activating sequences are guided by these considerations. Adenoviruses have a broad tissue tropism, but are hepatotropic *in vivo* after systemic administration. This means that delivery of anti-HBV or hepatitis C virus (HCV) sequences can be achieved efficiently after intravenous injection of recombinant adenoviruses. The

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duration of adenovirus-delivered transgene expression varies and is largely dependent on the host's immune response to the vector. The development of methods to remove adenovirus sequences and chemical modification of vectors to avoid immunodetection of vectors have therefore been very important. These adaptations improve persistence of transgene expression, which is required for treatment of chronic infections.

3. Principles underlying methods of propagating adenovirus vectors

Wild-type adenovirus virions are non-enveloped and have icosahedral symmetry with a diameter of 70 – 90 nm (reviewed in [20]). They belong to the Adenoviridae family and the genome comprises 26 – 44 kb of linear double-stranded DNA. The capsid contains 240 homotrimeric proteins, 12 pentameric penton proteins that are located at each of the apices of the icosahedral capsid, and homotrimeric fiber monomers that extend from the penton base. The fiber proteins are anchored at their N-terminal regions and the protruding C-terminal globular regions interact with cell surface receptors. Human adenovirus serotype 5 is the most comprehensively studied adenovirus, and together with adenovirus 2 has been used to develop the vectors that are widely used for gene transfer. The initiating event during natural adenovirus infection is the interaction of the fiber knob with its cognate cellular receptor. Several adenovirus receptors have been identified (reviewed in [21]), of which the Coxsackievirus and adenovirus receptor (CAR) is the primary binding site. Subsequent to receptor binding, interaction of the penton base with cellular integrins ($\alpha_V\beta_3$ and $\alpha_V\beta_5$) mediates internalization [22]. With the exception of hepatocytes (see below), the susceptibility of cells to adenovirus serotype 5 infection correlates with their CAR expression and induction of expression of the receptor on cells that normally are refractory to infection show increased adenovirus serotype 5 transduction [23]. In addition to CAR, other lower affinity cellular receptors that are involved with adenovirus attachment include heparin sulfate proteoglycans (HSPGs), intergrins, CD46, CD80/86, sialic acid, major histocompatability complex class I (MHC class I) and vascular cell adhesion molecule 1 (VCAM-1) [21].

Adenoviruses bind to blood cellular components such as erythrocytes [24], macrophages [25] and neutrophils [26], as well as plasma coagulation factors [27]. These interactions may sequester adenoviruses and prevent access to extravascular targets. Interestingly, the hepatotropism of adenovirus serotype 5 has recently been shown to result from interaction of blood clotting Factor X (FX) with the hexon, not fiber, protein of the virus [28]. The exact mechanism by which this interaction causes hepatotropism has not been established. However, it is thought that FX forms a mesh over the virion capsid that sterically inhibits interaction of the fiber protein with other receptors.

The adenovirus serotype 5 genome is organized into regions that are transcribed early or late during the infection (**Figure 2A**) [29]. E1A and E1B are the first to be expressed. Their function is to interact with p53 and Rb to prevent cell cycle arrest, inhibit apoptosis and permit establishment of viral replication. The attenuation of viral replication that results from deletion of E1 has been exploited for the generation of replication-defective adenovirus vectors (discussed below). Replication-competent and conditionally replication-competent adenoviruses, which have direct toxic effects on cells, have been used for the treatment of cancer [30]. Although these vectors show promise for the treatment of various malignancies, they are generally not suitable for delivery of RNAi activators. An important consideration for using adenoviruses to deliver RNAi activators is that adenovirus VA RNAI and adenovirus VA RNAII, which are expressed during the late phase of replication, are capable of inhibiting RNAi [31,32]. Adenovirus VA RNAI and adenovirus VA RNAII transcripts are highly folded and contain hairpin motifs that resemble pri-miRs. They are produced in high amounts, act as substrates and competitive inhibitors of Dicer and are also capable of suppressing RISC function [31]. A further effect that impedes RNAi function is the inhibition of exportin-5-mediated export of shRNAs [32]. Of course, expression of VA RNAs in adenoviruses that deliver RNAi activators would be undesirable. However, as recombinant adenovirus vectors are deficient in E1, they are replication-defective and do not express late genes. Production of late adenovirus VA RNAI and adenovirus VA RNAII transcripts is therefore significantly attenuated and disruption of silencing by adenovirus-delivered RNAi expression cassettes should not occur. This is supported further by the demonstration that first-generation adenovirus vectors do not disrupt endogenous miR biogenesis [33].

First-generation adenoviruses have been in use for gene therapy application for several years. These vectors are deficient in the viral *E1* gene, and the function of this sequence is typically provided *in trans* by HEK293 packaging cells that stably produce *E1* (**Figure 2B**). To propagate RNAi-activating adenoviruses, expression cassettes may be accommodated at sites of adenovirus genome deletions. Limitations of first-generation vectors are that target cells are co-transduced with virus protein encoding sequences together with the transgenes. As a result, immunogenic expression of viral proteins occurs, which shortens the duration of transgene expression and predisposes it to toxicity. Second-generation adenoviruses also have the E2 and E4 sequence removed (**Figure 2C**), which partially diminishes immunostimulatory effects and provides extra transgene capacity. Helper-dependent (HD) or 'gutless' vectors have been widely used for the delivery of transgenes. These third-generation adenoviruses have all of the viral protein coding sequences stripped from the genome (**Figure 2D**). Only the packaging signal and inverted terminal repeat (ITR) elements at the ends of the genome are retained. Total gene deletion in adenovirus vectors dramatically reduces immune response stimulation, thereby enabling prolonged

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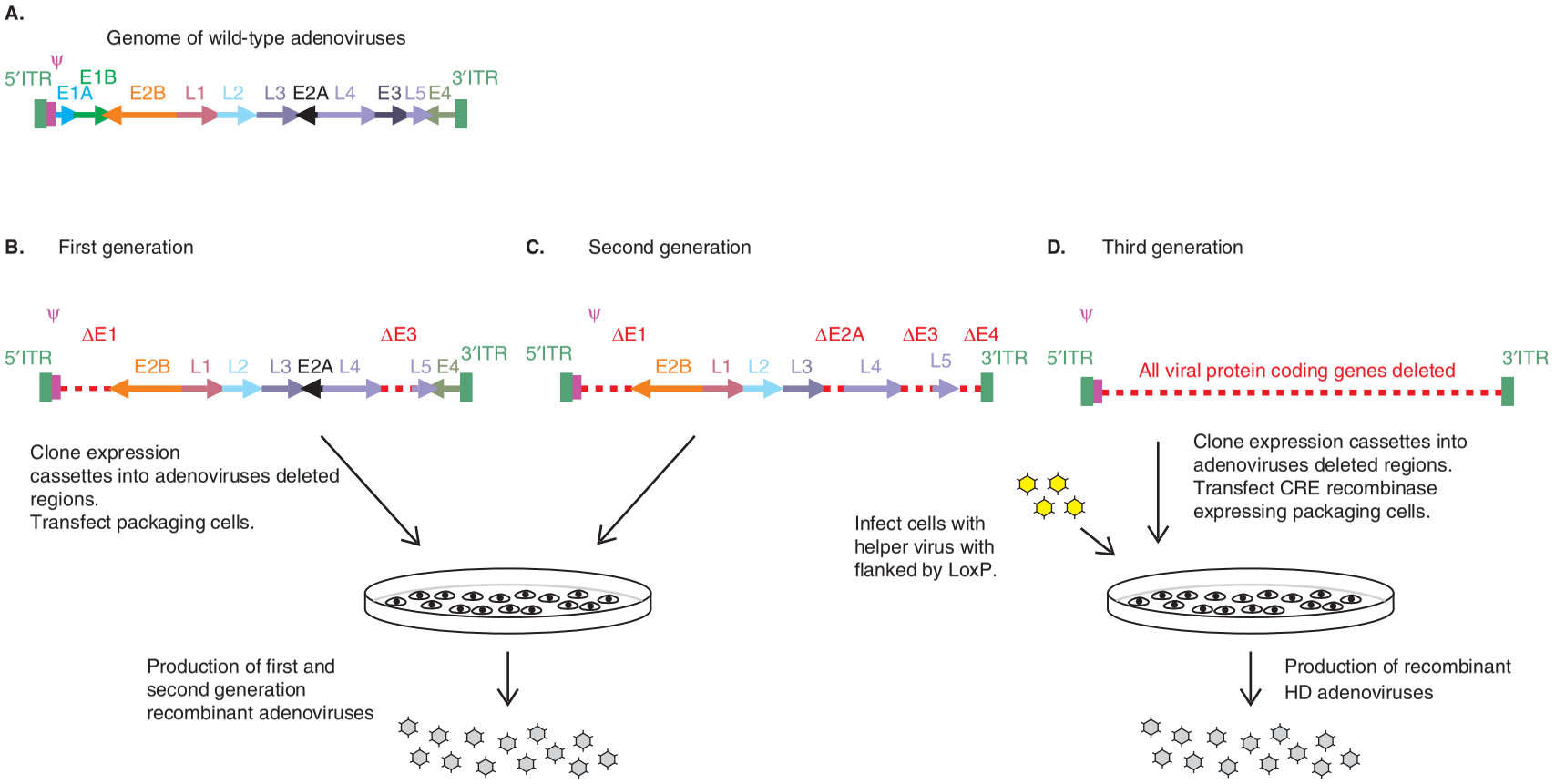


Figure 2. Schematic outline of production of first-, second- and third-generation recombinant adenovirus vectors. A. Linear genome of wild-type adenovirus. **B.** First-generation vectors have E1 and/or E3 deleted. **C.** Second-generation vectors have E1, E2, E4 and/or E3 deleted. Both these categories of recombinant viruses can be produced by introducing recombinant adenovirus DNA containing RNAi expression cassettes into cell lines expressing the missing essential genes. **D.** Third-generation or HD adenoviruses have all the viral sequences deleted, except for the left and right inverted terminal repeats (5′ITR and 3′ITR) and the packaging elements (ψ). To produce HD adenoviruses, a helper virus with its packaging signal flanked by recombinase targets, for example lox P sites, is typically used to complement for deleted adenoviral genes in recombinase-expressing packaging cells. RNAi expression cassettes may be incorporated at sites of Ad gene deletion (dashed lines).

HD: Helper-dependent; RNAi: RNA interference.

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transgene expression with reduced vector toxicity [34,35]. To propagate HD adenoviruses, complementing helper viruses (HVs) produce all of the protein components that are required for the formation of the viral particles. Ingenious methods, particularly the incorporation of LoxP [36] and FRT [37] recombinase recognition sites to flank the packaging signal in HVs, have been devised to remove the contaminating complementing virus. Derivatives of the HEK293 cells that stably express CRE or FLPe recombinases remove the packaging signal from the helper virus, and HD adenovirus DNA is preferentially packaged in the recombinant vector. To overcome problems of HV contamination that may result from homologous recombination between the ψ sequences of helper virus and HD adenovirus, Palmer and Ng reversed the orientation of ψ in the helper virus [38]. This approach successfully reduced HV contamination to 0.01 – 0.1%. Availability of packaging cells that are capable of growing in suspension has also facilitated large-scale production of adenoviruses. Nevertheless, generating adenoviruses in sufficient amounts and purity for clinical application remains difficult and costly. However, protocols are continuously being refined to improve current HD adenovirus preparation methods [39].

3.1 Immunostimulation by adenoviruses

After intravenous administration, adenoviruses are readily taken up by antigen-presenting cells (APCs). Their activation of the innate immune response is mediated by mitogen-activated protein kinases (MAPK). Release of cytokines, such as TNF-α, IL-6 and IL-12, causes local inflammation and toxicity [40-43]. Resulting damage to healthy tissues with decreased transgene expression is thus a significant concern for therapeutic use of recombinant adenoviruses. Importantly, this rapidly occurring effect is not dependent on the expression of viral genes, and unmodified HD adenoviruses also cause stimulation of the innate immune response [40]. Nevertheless, this immunostimulatory effect of HD adenoviruses is more transient than that caused by first-generation unmodified adenoviruses [44,45]. APCs also initiate an adaptive immune response by MHC class I presentation of processed viral antigens to $CD8^+$ T cells. This triggers $CD8^+$ T cells' differentiation into cytotoxic T lymphocytes (CTLs) and adenovirus-specific cytotoxic immunoclearance of transduced cells. In addition, presentation of viral antigens by MHC class II activates $CD4^+$ T cells. This leads to cytokine-mediated destruction of the adenovirus-infected cells as well as B-cell differentiation to induce a humoral immune response. Anti-adenovirus immunity, induced by community-acquired infection, is induced by similar mechanisms and may counter efficiency of adenovirus transgene delivery.

Although essential aspects of the mechanism of adenovirus immunostimulation are generic, vector-induced immune responses are complex, variable and influenced by several factors [46]. These include adenovirus dose, route of administration and tissues that are targeted. Overcoming problems of immunostimulation may be achieved more easily in situations where local and low dose adenovirus administration is adequate for therapeutic benefit. Preventing immunostimulation in situations that require systemic administration of high doses of adenoviruses is more complicated. In such cases, pre-administration of immunosuppressive drugs, such as steroids, has been found to be useful [47]. A major focus of adenovirus vectorology has therefore been on the development of methods to diminish induction of immunostimulation and modification of vector tropism.

3.2 Adenovirus tissue tropism

Targeting RNAi activators to specific tissues is essential to limit unintended effects of adenoviruses. Benefits of specific target tissue tropism include: i) minimizing the required dose of adenoviruses; ii) avoiding cells that mediate immunostimulation; and iii) escape from pre-existing adenovirus immunity [46]. A major focus of adenovirus vector development has therefore been to improve specificity of transduction of intended target cells. Adenovirus tropism is complex and varied. As new receptors are identified and new serotype-specific cellular transduction processes are elucidated, so the repertoire of adenoviruses will increase. This should enable more specific vector targeting and also allow for serotype switching to avoid vectors' interaction with neutralizing antibodies [48].

Much of the knowledge on adenovirus transduction, particularly the binding of CAR by adenovirus serotype 5, comes from studies carried out on cells in culture. Extrapolation to use *in vivo* has not been easy as adenoviruses display a strong liver tropism when injected systemically, despite low expression of CAR in hepatocytes [49]. It was only recently that the central role of FX in liver targeting by adenoviruses was identified (discussed earlier) [28]. Direct binding of FX to the adenovirus serotype 5 hexon is required for liver transduction. This is supported by the reduction in adenovirus serotype 5 liver transduction following inhibition of FX interaction with hexon [28,50], which is not observed after CAR disruption [51].

So far three methods have been used to alter vector tropism [46]: i) genetic modification of the capsid; ii) chemical conjugation of the vector particles with polymers such as polyethylene glycol (PEG); and iii) introduction of a two-component modification that comprises a fiber knob binding domain at one end and a retargeting moiety at the other. Changing sequences of receptor-interacting capsid proteins, particularly the fiber knob domain, have been used successfully to modify vector tropism [52]. However, this approach may be complicated by defective fiber trimerization. PEGylation of adenoviruses is a convenient approach that provides a hydrophilic coat to adenoviruses, which decreases the proteolytic degradation and shields vector epitopes [53]. PEGylated adenoviruses are less immunogenic and their blood clearance rate is fourfold lower than unPEGylated adenoviruses. The increased circulation time in blood also facilitates transduction of non-hepatic tissue [54]. Liver tropism can

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also be altered by using PEG of different molecular masses. Adenovirus serotype 5 conjugated to PEG_{5000} (PEG with molecular mass of 5000 Da) maintains normal liver transduction, but PEG_{20000} and PEG_{35000} reduce hepatocyte transduction [55]. Attaching targeting ligands to the end of the PEG chain has also enabled tissue-specific targeting. For example, integrin-binding RGD (Arg-Gly-Asp) peptides attached to a PEG chain (RGD-PEG-adenovirus) demonstrated a 200-fold increased transduction in both CAR^+ and CAR^- negative cells [56].

4. HD adenovirus vectors are attractive for achieving sustained RNAi-based therapeutic gene silencing

As indicated earlier, HD adenoviruses have the dual advantages of a high capacity (~ 36 kb) for accommodating transgene elements and a lack of sequences encoding viral proteins. Although typical RNAi expression sequences are small, inclusion of extra cassettes in HD adenoviruses may be useful to make vectors more functional. The feasibility of such an approach was demonstrated in a study that generated an adenovirus vector that encoded both transforming growth factor-β3 and a shRNA targeted to type I collagen [57]. This particular application to treating articular cartilage degeneration facilitated both chondrocyte growth stimulation and inhibition of type I collagen production. The resulting formation of cartilage that contains type II collagen and proteoglycans has greater mechanical strength. Extension of this method to incorporate immunomodulatory transgenic elements may be useful to modulate toxicity of HD adenovirus vectors. Proof of principle of such an approach was provided by a study showing that silencing the Cys-X3-Cys chemokine ligand could be used to prevent acute liver injury caused by adenoviruses [58]. In another impressive study showing the utility of HD adenoviruses for delivery of RNAi expression cassettes, shRNAs targeting the insulin-responsive *SREBP-1* gene resulted in sustained 90% *SREBP-1* knockdown [59]. Therapeutic benefit in a type 2 diabetes mouse model was manifested as reduced body weight gain, which was observable at week 3 after HD adenovirus administration. Similarly, a recent study explored using HD adenoviruses to express shRNA against mutated huntington protein (htt)-encoding mRNA as a possible Huntington's disease therapeutic strategy [60]. This study reported 90% reduction in formation of htt aggregates at 4 weeks after vector administration. These silencing effects of HD adenoviruses are typically more sustained than what can be achieved with first-generation adenoviruses [61-63]. Interestingly, unlike the disruption of the endogeneous RNAi pathway and liver or neuronal degeneration caused by shRNAs overexpressed from an AAV [13,15], high levels of shRNA from HD adenoviruses did not appear to affect the exportin-5 pathway or to induce liver toxicity in mice [16]. Together, these observations showing prolonged transgene expression and good safety support the notion that HD adenovirus vectors are useful for RNAi-based therapeutic application.

5. Viral infections as targets for adenovirus vector-based RNAi therapy

The natural targeting of hepatocytes by adenoviruses following systemic administration *in vivo* makes them well suited to delivery of RNAi expression cassettes that target liver-specific virus infections. Globally, chronic infections with either HCV or HBV are the chief causes of hepatocellular carcinoma (HCC) and cirrhosis [1,64]. Despite the similarities in the sequelae of chronic infection by these viruses, they differ in genetic structure and life cycles, which necessitates different approaches to developing RNAi therapy.

Several studies have been carried out aimed at utilizing RNAi to treat HBV infection (reviewed in [65,66]). Approximately 6% of the world's population is chronically infected with HBV. Drugs available at present have limited efficacy, and carriers of the virus are at risk of HCC. Despite implementation of vaccination programs in parts of the world where HBV infection is endemic, persistent HBV infection is likely to be a significant global problem for many years, and improved treatment of the infection remains a priority. The virus has a compact genome that comprises partly double-stranded relaxed circular DNA (rcDNA), which is converted to covalently closed circular DNA (cccDNA) in infected hepatocytes. This cccDNA then serves as a template for transcription of viral RNAs from which *core*, *surface*, *polymerase* and *X* overlapping open reading frames (ORFs) are translated.

Several different target sites within all four HBV transcripts have been found to be suitable for RNAi-mediated inhibition of HBV replication [67-73]. As there is no convenient small animal model of HBV infection, use has been made of HBV transgenic mice to simulate the clinical condition. These animals have HBV DNA stably integrated into their genomes, and constitutively produce the virus in a manner that is similar to the HBV carrier state in humans. Initial studies demonstrated that recombinant adenoviruses carrying U6 promoter-driven anti-HBV shRNA cassettes efficiently inhibited HBV replication in transgenic mice [61,74]. Comparison with the efficacy that was achieved after injection of similar mice with siRNA-containing lipoplexes [75] reveals that inhibition of HBV replication was considerably better with recombinant adenoviruses. To address concerns about the immunostimulatory effects, polyethylene glycol modification of anti-HBV shRNA-expressing adenoviruses was tested [62]. Successful attenuation of mouse immune responses to the vector, a better safety profile and improved anti-HBV effects after repeat adenovirus administration were demonstrated. Although adenoviruses encode VA RNAI and RNAII, which act as RSSs [31], these sequences do not appear to compromise silencing efficacy of adenovirus-delivered RNAi activators [33]. This is likely to be a result of VA RNA production during late

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adenovirus infection, which does not occur after replication-defective adenovirus vector infection of cells. Assessment of anti-HBV HD adenoviruses has been carried out in one study reported so far [76]. Modest silencing was observed, and is likely to have resulted from poor antiviral efficacy of the RNAi expression cassette used in this study. Nevertheless, proof of principle of the utility of HD adenoviruses for the treatment of HBV infection was demonstrated in an immunotherapy-based approach. A mifepristone-inducible expression cassette was used to produce IFN-α and IL-12 in murine and woodchuck models of HBV infection. In these studies, prolonged and specific intrahepatic expression of transgenes with sustained antiviral effects was observed [34,77]. Most importantly, when compared with first-generation adenoviruses carrying the same IFN-α cassette, HD adenoviruses resulted in superior liver damage protection in acute infection [77].

The liver tropism of HCV should make infection with this virus amenable to RNAi-based therapy using adenoviruses. As with HBV, HCV infection is a significant cause of global health problems. Approximately 170 million people are chronically infected with HCV [78]. The virus is typically transmitted percutaneously and infection persists in 60 – 80% of cases, which places individuals at high risk of cirrhosis and HCC. Efficacy of licensed HCV therapies, which include PEG IFN-α and ribovirin, is variable and ranges from 45 to 80% [79].

HCV is a member of the Flaviviridae family and has a single sense strand uncapped RNA genome of 9.6 kb [80]. An internal ribosomal entry site (IRES) located within the 5′ non-translated region (5′NTR) is responsible for initiating translation of the one HCV ORF. The large precursor polyprotein is cleaved by host cellular and viral proteases to form individual viral proteins. The entirely cytoplasmic life cycle of this RNA virus makes it a good candidate for RNAi-based treatment. A difficulty with the development of RNAi-based anti-HCV treatment has been the paucity of suitable animal models of the infection. Subgenomic replicons have been used commonly to study efficacy of antiviral therapeutic agents in cell culture, and assessment of antiviral efficacy with HCV-infected cells in culture has not been possible until recently. Available models of HCV infection *in vivo* are limited to chimpanzees [81] and chimeric immunodeficient mice that are grafted with human hepatocytes [82]. The complex nature of these models makes convenient comprehensive long-term studies difficult to undertake. As a result of its important function as an IRES, evolution of this 5′NTR sequence is constrained [80], and despite being highly structured, the IRES is a favored target of RNAi activators [83-87]. As with HIV-1, HCV has a plastic genome and overcoming HCV escape by using combinatorial RNAi approaches has been an active area of research [88,89]. Silencing of host dependency factors [90,91], including endogenous miR-122 [92], may also be useful to counter the emergence of resistant HCV strains. So far, there have been few if any studies that have utilized adenoviruses to deliver HCV silencing sequences. Given the dearth of available models to test anti-HCV efficacy *in vivo*, the slow progress in developing anti-HCV adenoviruses is perhaps not surprising. Nevertheless, lessons learned from studying HBV silencing will no doubt be useful and directly applicable to advancing anti-HCV RNAi-based therapy.

6. Conclusion

Adenoviruses have been used widely in the development of gene therapy and according to current information they are the most commonly used vector in gene therapy trials. Applications have been varied and include use for treatment of cancer and infectious diseases. As expected, adenoviruses are well suited to incorporation of RNAi-activating expression cassettes. Useful properties of adenoviruses that have contributed to the popularity for gene therapy include the following.

- The molecular biology of adenoviruses is understood well, and this has facilitated engineering of vectors to confer specific intended biological properties.
- Adenoviruses are capable of infecting both dividing and non-dividing cells *in vivo*.
- Lack of interference by viral transcriptional regulatory sequences enables rapid unimpeded transgene expression.
- Methods of propagating the vectors are continually being improved.
- The vectors are typically stable and transduced DNA exists episomally, with minimal risk of insertional mutagenesis.
- HD adenoviruses in particular have a high capacity for incorporation of large transgene sequences or multiple cassettes, and are capable of prolonged transgene expression with reduced toxicity at high vector doses.

Successful propagation of HD adenoviruses was a significant step in the improvement of safety and efficacy of this class of viral vector. Lack of expression of viral proteins means that vector antigens are not presented by MHC class I/II antigens and more sustained transgene expression can be achieved. Concomitant modification of HD adenoviruses with polymers, such as PEG, may be used conveniently to attenuate innate immunostimulation. The very large capacity of HD adenoviruses allows for incorporation of up to 36 kb into these vectors. Although this may not be particularly important for propagation of vectors that contain small RNAi expression cassettes, the extra capacity allows for incorporation of more expression cassettes that could be used to modify host immune responses to vectors.

Hepatotropism of adenovirus serotype 5-derived vectors is very useful for treatment of viral infections that occur primarily in the liver. However, transduction of liver tissue is a disadvantage for the treatment of extrahepatic diseases, and creative methods continue to be developed to redirect the

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tropism of adenoviruses. Despite these positive attributes, the potential of adenoviruses for gene silencing applications has not been explored fully, and the reason is their powerful stimulation of host innate and adaptive immune responses. The toxic effects that result may be serious, and the cautious approach to using adenoviruses for systemic administration is justified. Nevertheless, recent developments, particularly in the field of modification of viral capsid proteins to evade cells of the immune system and efficient means of propagating HD adenoviruses, have provided the field with the technology for producing safer vectors that may be applied successfully in the future.

7. Expert opinion

Demonstration that adenovirus vectors are capable of highly efficient gene transfer to cells *in vivo* led logically to their use for delivery of expressed RNAi activators. Using adenoviruses for delivery of RNAi effectors has benefited from the wealth of research on the topic of general applicability of adenoviruses for gene therapy. There are many positive features of adenoviruses, summarized above, which make them appealing for delivery of expressed RNAi activators. For certain applications the highly efficient delivery and expression of transgenes is not surpassed by other vectors. However, harnessing of this very important feature for clinical application has been complicated. Enthusiasm for the use of adenoviruses has been reduced by concerns about toxicity that may result from their immunostimulatory effects, as well as costliness of scalable synthesis of these vectors. Consequently, use of non-viral vectors for delivery of synthetic siRNAs and AAVs or lentiviral vectors to deliver RNAi expression cassettes has been favored by researchers. Nevertheless, recent advances in modifying adenoviruses have succeeded in moderating toxic effects, and it is appropriate that their utility for therapeutic transfer of expressed RNAi activators should be revisited.

In summary, clinical application of adenoviruses to RNAi therapy will depend on improvements that achieve the following.

- Conclusive demonstration that the innate and adaptive immune responses to adenoviruses can be sufficiently attenuated to avoid toxicity and achieve sustained gene silencing.
- Improvement in methods for convenient large-scale preparation of pure adenoviruses that are suitable for clinical use is essential. Current procedures are costly, time-consuming and difficult to implement on a scale that is large enough for human use.
- Development of methods to evade interaction with cells of the reticuloendothelial system and avoidance of pre-existing immunity to wild-type adenoviruses. Infection with adenoviruses is common in general populations, and immunity from community-acquired infections attenuates efficacy of the recombinant viral vectors.
- Comprehensive understanding of the clinical conditions that are best suited to adenovirus-mediated delivery of RNAi activators. It is clear that one vector will not be suitable for all applications within the field of RNAi therapy, and identification of those diseases that are best suited to adenovirus-mediated RNAi activator delivery is important.

The field of RNAi-based treatment of diseases has advanced at a rapid pace over the past 10 years. There is now substantial enthusiasm for the vast potential of this method of therapeutic silencing of gene expression. Limitations of the available delivery methods and lack of information on the long-term effects of RNAi activators' expression *in vivo* are the main obstacles to RNAi-based gene silencing realizing its full potential. It is likely that the considerable momentum that has been gained in the field of RNAi-based gene silencing will enable rapid progress to overcome current difficulties with harnessing RNAi for treatment of viral infections. Adenoviruses, and in particular availability of HD derivatives, have very useful features that should contribute significantly to progress in the exciting field of RNAi-based therapy. Overcoming the obstacles listed above, which is a reasonable expectation, should make adenovirus delivery of anti-HBV and anti-HCV RNAi activators a feasible future therapeutic option.

Declaration of interest

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16

Approaches to Delivering RNAi Therapeutics that Target Hepatitis B Virus

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16.1 Introduction

Despite hepatitis B virus (HBV) vaccination programmes causing a decline in the global incidence of infections with the virus, HBV remains highly prevalent in sub-Saharan Africa, East Asia and South East Asia. Worldwide it is estimated that there are 387 million chronic carriers of the virus [1]. Persistently infected individuals have an increased risk of developing cirrhosis and hepatocellular carcinoma (HCC). Ideally, anti-HBV therapy should stop HBV replication and thereby avert complicating cirrhosis and HCC. Conventional treatments for chronic HBV infection include interferon-α (IFN-α and pegylated IFN-α), which functions as an immunomodulator, and nucleoside or nucleotide analogs (lamivudine, entecovir, adefovir and tenofovir), which inhibit HBV genome replication by targeting the viral reverse transcriptase [2]. These therapies have only limited success and availability of effective new HBV therapeutics is an unmet medical need.

The HBV is the prototype member of the hepadnavirus family. The DNA genome has a partly double stranded and relaxed circular structure (rcDNA). After infection of hepatocytes, rcDNA is converted to a 3.2 kb covalently closed circular DNA (cccDNA). The cccDNA serves as a template for production of HBV transcripts with open reading frames

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(ORFs) encoding the core, polymerase, surface and X proteins [3]. HBV pregenomic RNA (pgRNA) is also formed from the cccDNA. HBV cccDNA is a stable replication intermediate, which has proven difficult to eliminate with currently available therapies. pgRNA is packaged into viral capsids then reverse transcribed to form virion rcDNA. Encapsulation of the capsids within an envelope, which is derived from the endoplasmic reticulum and has embedded surface proteins, results in formation of the intact hepatitis B virion. The compact nature of the HBV genome restricts its plasticity and the virus has limited ability to evade the silencing effects of hybridizing nucleic acids without compromising its own fitness. This feature and the essential requirement for the pgRNA replication intermediate indicate that HBV should be a good target for RNAi-based gene therapy.

16.1.1 RNAi Therapeutics

There is compelling evidence that exogenous activators can be used to exploit the endogenous RNAi machinery and achieve specific and potent silencing of genes of interest. The mechanism provides the means of studying gene function and has potential application to silencing of pathology-causing genes. The RNAi pathway may be activated by synthetic or expressed exogenous RNAi activators. Features of the expressed and synthetic RNAi activators that have been used to counter HBV replication are described in Figure 16.1. Chemically synthesized short interfering RNAs (siRNAs) typically resemble mature

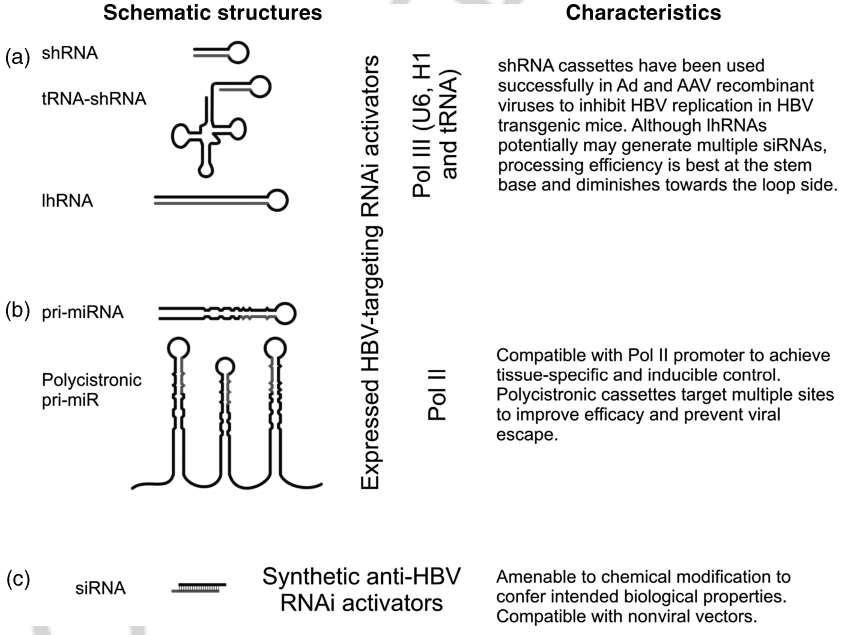


Figure 16.1 *Types of RNAi activators that have been used to silence HBV replication. Expressed sequences, generated from either from Pol III (a) or Pol II (b) promoters, are compatible with incorporation into viral vectors. (c). Synthetic anti-HBV siRNAs are used in NVV formulations. Characteristics of the different types of RNAi activator are briefly summarized. For each type of RNAi activator, the mature guide is illustrated in grey.*

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endogenous micro RNAs (miRNAs), and DNA expression templates encode artificial mimics of upstream miRNA intermediates of the RNAi pathway. These exogenous sequences reprogramme the RNAi pathway to silence intended gene targets. The negative charge, sensitivity to nucleases, hydrophilicity and immunostimulatory effects of siRNAs have led to investigating use of chemical modifications to confer better drug-like properties. Chemical modifications have been aimed at improving delivery efficiency, reducing clearance, increasing stability, enhancing target specificity and minimizing immunostimulatory effects [4–6]. Synthetic siRNAs have a smaller size than RNAi-activating expression cassettes. This feature together with cytoplasmic site of action makes siRNA delivery and dose control easier to achieve than it is with expressed RNAi activators.

The more sustained silencing that may be achieved with expression cassettes is useful for the treatment of chronic infection caused by HBV [7,8]. These cassettes may be propagated conveniently using standard techniques of molecular biology. They are also stable and compatible with incorporation into highly efficient viral vectors (VVs). Expressed short hairpin RNAs (shRNAs), which mimic pre-miRNAs, have been widely used to activate the RNAi pathway. Typically, their expression has been placed under control of RNA polymerase (Pol) III promoters and the powerful and constitutively active U6 small nuclear RNA promoter has been commonly used [9]. Although effective silencing is achieved, saturation of the endogenous RNAi pathway may occur that can lead to fatal toxicity [10]. To overcome these problems and improve transcriptional control of RNAi activators, pri-miRNA mimics that are compatible with expression from Pol II promoters have been developed [11–18]. These artificial expression cassettes are amenable to multimerization to simulate natural polycistronic miRNAs. Using such a combinatorial RNAi approach enables simultaneous targeting of various regions of a viral sequence. This is a useful strategy to improve silencing efficacy and prevent viral escape [11,12,17,19]. Production of multiple antiviral siRNAs from long hairpin RNAs (lhRNAs) has also been described, but the efficiency with which the individual siRNAs are generated from these templates is variable [20–22].

16.1.2 Hepatitis B Virus as a Target for RNAi-based Gene Silencing

Although evidence exists that viruses have evolved mechanisms to evade cellular RNAi silencing mechanisms [23,24], HBV-encoded factors that are capable of suppressing RNAi have not been described. In support of this, several investigations carried out *in vitro* and *in vivo* demonstrated that the virus is indeed susceptible to RNAi-based inhibition of replication [25–30]. Synthetic and expressed RNAi activators have been used successfully to target different sites of the viral genome. In addition to typical Pol III expression cassettes, Pol II artificial mono- and polycistronic anti-HBV pri-miRNAs have been used successfully to knockdown HBV replication [11,12,30].

16.2 Vectors Suitable for Hepatic Delivery of HBV Gene Silencers

Since HBV is hepatotropic, efficient delivery of RNAi activators to the liver hepatocytes is critically important to have therapeutic utility. This is challenging as vectors should ideally take antiviral sequences to their intended sites of action within hepatocytes after

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systemic administration. Optimally, only a single administration should need to be given to achieve a sustained inhibition of viral replication. If this is not possible vectors should then be amenable to readministration and retain efficiency of inhibition of HBV replication. To gain access to hepatocytes, vectors need to traverse the endothelial fenestrated barrier between the blood and hepatocytes. VVs and nonviral vectors (NVVs) should therefore ideally have a uniform size of approximately 100 nm to enable them to cross the fenestrated barrier and come into contact with hepatocytes (reviewed in [12]). Nonviral vectors have commonly been used to deliver synthetic siRNAs, and recombinant VVs engineered to deliver artificial RNAi expression cassettes. Characteristics of the types of vectors that have been used to silence HBV replication are summarized in Table 16.1. As synthetic formulations, NVVs are amenable to large-scale preparation, which is important for clinical application. These vectors are capable of efficient delivery of synthetic siRNAs to their cytoplasmic site of action but are generally inadequate for delivery of anti-HBV DNA expression cassettes to hepatocyte nuclei. Developments in use of VVs and NVVs for delivery of HBV-targeting sequences, as well as for other hepatic therapeutic applications, are discussed below.

16.2.1 Viral Vectors

Adenoviruses (Ads) and adeno-associated viruss (AAVs) are both capable of effective hepatocyte transduction and are able to achieve long-term transgene expression in the liver. Lentiviral vectors (LVs) transduce hepatocytes stably, but efficiency of transgene delivery to these cells following systemic administration in adult animals is generally inadequate for treating chronic HBV infection. Nevertheless, LVs have potential therapeutic utility using *ex vivo* approaches (discussed below).

16.2.1.1 Adeno-associated Virus Vectors

AAVs are nonenveloped viruses that belong to the *Parvoviridae* family. They are small ($\pm$ 20 nm) and have a single-stranded DNA genome of 4.8 kb. Recombinant AAVs can carry an insert of up to 4.6 kb, which is adequate for accommodating typical RNAi expression cassettes [31]. An important advance in AAV vector design was the development of second-generation double-stranded or self-complementary AAV vectors (scAAVs). Transgene expression from these vectors is more efficient and allows for administration of lower vector doses [32]. There are 81 clinical trials in progress that use AAVs (http://www.abedia.com/wiley/vectors.php, accessed 13 January 2013). These vectors are suitable for use in humans because they are nonpathogenic, do not replicate without Ad co-infection, have low immunogenicity and high titers of the vectors may be produced conveniently [33]. Although AAV safety is an advantage, a recent study showed that AAVs may cause liver inflammation by activating a TLR-2-mediated responses in hepatocytes [34]. An additional concern is that there is a high prevalence of neutralizing antibodies (NAb) to AAV-2 in human populations [35]. Some of the NAbs also cross-react with other AAV serotypes, which may limit their use as vectors in a clinical setting. Recombinant AAVs lack the viral Rep protein, which restricts integration into the host genome, and contributes further to vector safety [36]. The first AAV gene therapy vectors were based on AAV-2, which is capable of transducing many different cell types [37]. There are currently more than 100 known AAV serotypes and it is possible to package the

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Table 16.1 *Advantages, disadvantages and significant articles describing vectors used for delivery of nucleic acids to the liver.*

	Ads	AAVs	Lentiviruses	NVVs
Advantages	Hepatotropic	Serotype-dependent liver tropism	Broad cell tropism	Targeting moieties facilitate liver tropism (e.g. galactose)
	Efficient transduction of RNAi-activating RNA sequences	Efficient transduction of RNAi-activating RNA sequences	Efficient transduction of RNAi-activating RNA sequences	Amenable to chemical modification to confer biological properties
	Sustained therapeutic effect using HD Ads	Limited toxicity	Limited immunogenicity	Feasible scale up of synthesis
		Sustained therapeutic effect for up to 8 weeks	Stable integration and long-term therapeutic effect offset need for repeat administration	Suitable for delivery of synthetic RNAi sequences
				Repeat administration possible
Disadvantages	Large-scale production is costly and labour intensive	Labour-intensive large-scale production	Labour intensive large-scale production	Poor delivery of expressed RNAi activators *in vivo*
	Immunostimulatory and potentially toxic	Immunostimulation limits repeat administration	Poor transduction of liver cells following systemic administration	Variable immunostimulation
	Pre-existing immunity attenuates vector efficacy	Repeated administration requires serotype switching	Low hepatotropism	Transient therapeutic effect
	Repeat administration may be necessary			

(*continued*)

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Table 16.1 (*Continued*)

	Ads	AAVs	Lentiviruses	NVVs
Significant publications relevant to HBV therapeutics	Uprichard *et al.* [72]	Grimm *et al.* [7]		Morrissey *et al.* [27]
	Use of Ad to target HBV in transgenic mouse model	Utility of scAAV-8 vectors for targeting HBV in transgenic mouse model	Successful liver transduction of LVs using partial hepatectomy	Liposome delivery of siRNAs to HBV mouse model
	Carmona *et al.* [28]	McCaffrey *et al.* [33]	Pichard *et al.* [95]	Zimmerman *et al.* [101]
	Use of Ad to target HBV in transgenic mouse model	Highlighted the safety of AAVs for gene therapy	Pharmacological priming of hepatocytes to improve LV-mediated transduction	Using SNALPs for hepatotropic nucleic acid delivery in nonhuman promates
	Crowther *et al.* [29]	Chen *et al.* [41]		
	Polymer modification to improve anti-HBV efficacy of Ads	Repeated administration of AAVs possible with serotype switching		
	Rauschhuber *et al.* [75]			
	Use of HD Ad to target HBV in transgenic mouse model			

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AAV-2 genome with the capsid of any of these serotypes (pseudotyping). This feature is useful to change vector tropism and evade host immune responses. AAV-8 and AAV-9 have a high affinity for hepatocytes (3–4 times higher than AAV-2) and have consequently been used for hepatotropic delivery of RNAi effecters targeting HBV [38,39]. Recently, DNA shuffling has been employed to generate libraries with variations in the exposed loops of AAV capsid proteins [40]. Subsequent positive or negative selection enables purification of vectors with defined properties, such as specific tissue tropism and attenuated NAb interaction. This method is likely to be very useful for future application in AAV vectorology.

Grimm *et al.* were the first to demonstrate the utility of scAAV-8 vectors for RNAi-mediated HBV silencing [10]. However, although HBV replication was efficiently inhibited in the transgenic mice, there was an associated high mortality. This prompted subsequent studies which established that high concentrations of exogenous shRNAs compete with the natural miRNA machinery to prevent processing of essential endogenous miRNAs. Compromised function of hepatocyte miRNAs resulted in the death of the mice. Other studies have subsequently employed AAVs to deliver HBV-targeting RNAi expression cassettes. A dsAAV-2/8 vector, a dsAAV-2 genome pseudotyped with an AAV-8 capsid, was successfully used to inhibit HBV replication in a transgenic mouse model [41,42]. Significant HBV inhibition was maintained for 22 weeks. Reduction in appearance of complicating liver adenomas in HBV transgenic mice was also demonstrated after AAV delivery of anti-HBV expression cassettes [43,44]. To overcome problems of neutralizing antibodies to the AAV-8 capsid, a vector expressing the same anti-HBV sequence, but pseudotyped with AAV-9, was then administered. This dsAAV-2/9 vector silenced HBV effectively and evaded the AAV-8 NAbs. Thus, for successful repeated administration of anti-HBV AAVs, the serotype of the capsid protein may be changed for each administration to avoid neutralizing effects of antibodies [45].

An important recent advance in AAV vectorology has been demonstration of clinical utility of AAVs that effect hepatic blood clotting factor IX gene expression in livers of haemophiliac patients [46]. This clinical study reported that peripheral vein infusion of the vectors resulted in improvement in the bleeding phenotype, and four of the six treated patients did not require further factor-IX prophylaxis. The authors suggest that concerns about toxicity and immune-mediated elimination of the vector may be countered by treatment with a short course of immunosuppressive glucocorticoids. This successful clinical study represents a significant milestone and paves the way for use of AAVs for other applications such as in RNAi-based HBV therapy.

16.2.1.2 Adenovirus Vectors

Ads belong to the *Adenoviridae* family and according to available data are used in 24% of current gene therapy clinical trials, which makes them the most widely used vectors (http://www.abedia.com/wiley/vectors.php, accessed 13 January 2013). Ads are nonenveloped and have a double-stranded linear DNA genome of approximately 35 kb. There are 55 known Ad serotypes, with derivatives of human serotypes 5 (Ad5) and 2 (Ad2) being most commonly used as gene therapy vectors [47]. Ad vectors have several advantages: they (i) efficiently transduce a broad range of dividing and nondividing cell types; (ii) can be produced in high titers relatively easily; (iii) are capable of carrying large

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transgene inserts; and (iv) their molecular biology is well understood [48]. A significant advantage of Ads for use in the delivery of anti-HBV RNAi activators is that they are efficiently hepatotropic. Ad particles have a diameter of approximately 120 nm and are able to traverse fenestrations in liver sinusoidal endothelial cells. This gives them access to the microvillus surface of hepatocytes to enable receptor-mediated internalization into these cells [49,50]. Interestingly, binding of blood clotting factor X to the Ad hexon protein confers hepatoropism on the vectors [51].

Following systemic administration of Ads to mice, the vectors are efficiently transported to the liver. However, once they reach hepatic tissue, up to 98% of the virus particles are sequestrated by the reticuloendothelial system, and in particular the Kupffer cells [52]. These antigen presenting macrophages express a scavenger receptor A (SR-A), which binds negatively charged regions on the hypervariable region 1 (HVR1) of Ad5 hexon protein [53]. Ads are destroyed in the phagocytic Kupffer cells, which themselves undergo dose-dependent necrosis within 10 minutes of systemic delivery of the vector [52,53]. Activation of the reticuloendothelial system by Ads also stimulates an innate inflammatory response. This effect is characterized by a rapid release of inflammatory cytokines and may result in acute toxicity. Overcoming unintended effects that result from immunostimulation following Ad administration, as well as evading pre-existing immunity, have therefore been a priority of research involving use of Ads for gene therapy.

A study published in 2008 used microarray analysis to assess the murine host responses to first generation Ad, HD Ad and AAV vectors [33]. Mice were injected with equivalent amounts of the different VVs containing human factor IX (hIX) expression cassettes. RNA was extracted from livers at 1 hour, 6 hours, 72 hours and four weeks after injection. The gene expression patterns following Ad and HD Ad administration were very similar. Both profiles were compatible with expression of genes that are involved in the type I IFN response observed six hours after infection. AAVs elicited a more modest immune response and highlighted the better safety profile of AAV vectors.

Various methods have been employed to avoid Ad sequestration by Kupffer cells. These include administering chemicals, such as clodronate liposomes [54], which are specifically toxic to Kupffer cells. Alternatively high doses of Ad5 have been used to cause Kupffer cell death [55]. This approach has been employed in Ad5 'predosing' regimens to deplete the Kupffer cell populations. Administration of the therapeutic viral vector soon thereafter results in greater efficiency of liver cell transduction [56]. Another commonly used and more clinically relevant approach to evade detection by Kupffer cells is polymer modification of Ads [57]. Conjugation of viral capsids with polyethylene glycol (PEG) shields the negative charges on Ad5 hexon to diminish vector interaction with Kupffer cells. Hepatocyte transduction has successfully been achieved using this approach [58–60], but gene delivery to primate hepatocytes using PEG-modified vectors may be less efficient [61–63].

Gene-deletion strategies have also been employed to improve safety, efficiency and transgene capacity. Initially, E1 and E3 genes were removed to render first-generation Ad vector replication deficient and able to accommodate transgenes. These vectors, however, elicit a strong acute innate and later cell mediated and humeral adaptive immune responses [64]. The resulting effects may cause toxicity and also reduce the duration of transgene expression. To overcome immune-stimulatory effects, additional viral genes

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have been removed from the Ads. As well as deleting E1 and E3 genes, E2 and E4 have been removed to form second-generation vectors [48]. Immunostimulation has been further attenuated by development of helper-dependent (HD) or gutless Ad vectors. These third-generation Ads have all viral protein coding genes deleted. The only remaining sequences that do not encode viral proteins are the packaging signal and flanking inverted terminal repeat (ITR) sequences. Deletion of all of the viral genes in HD Ads limits induction of a cytotoxic T-cell mediated response and has an additional advantage of prolonging transgene expression [48,65].

The innate immune response occurs one to six hours after intravenous injection of Ads and is dose-dependent [66]. In mice the innate response is followed by a secondary release of pro-inflammatory cytokines and chemokines that occurs five to seven days after infection. This effect is thought to result from an adaptive immune response to expressed viral proteins [67]. Activation of the innate response causes release of various chemokines and proinflammatory cytokines such as tumour necrosis factor-α, interleukins (IL-6 and IL-1β) and interferon-γ (IFN-γ). Toll-like receptors (TLRs) and MyD88, which is a TLR adaptor gene, have been implicated in mediating the response Ads *in vivo* [68]. IFN-α and -β production, which also contributes to the toxic effects of Ads [66,69–71], occurs in splenic cells (myeloid dendritic cells) by a mechanism that is independent of TLRs and cytosolic receptors of RNA and DNA. The effect is however dependent on endosomal viral escape which activates MAP kinase and SAPK/JNK-signalling pathways.

In 2005 Uprichard and colleagues showed that HBV replication was inhibited by a recombinant first generation Ad vector that delivered a HBV-targeting shRNA expression cassette [72]. The effect lasted for at least 26 days in a HBV transgenic mouse model following systemic administration of the vector. In a similar study published in 2006, first generation Ad vectors carrying an RNAi effecter targeting the *X* ORF of HBV resulted in inhibition of HBV replication [28]. Following on from this study, the silencing efficacy of the shRNA Ad vectors was improved by chemical modification with PEG [29]. Importantly, polymer modification enabled HBV inhibition after repeat administration of the vector. This effect was associated with attenuated release of proinflammatory cytokines, adaptive immunostimulation and hepatotoxicity, which was not observed after administration of the unmodified Ads.

The sustained hepatic transgene expression and attenuated immune stimulation that may be achieved with HD Ad vectors are useful for delivery of HBV-silencing RNAi therapeutics [73,74]. Nevertheless, as the structure of the HD Ad virions is the same as that of the first-generation vectors, HD Ads remain capable of inducing an acute, dose-dependent innate immune response [9,33]. The usefulness of RNAi-activating anti-HBV HD Ad vectors has been assessed in one study reported to date [75]. Although potentially effective, the specificity of the silencing effect could not be confirmed. In a recent study undertaken by our group, intravenous administration of 5×10^9 recombinant HD Ads to HBV transgenic mice transduced 80–90% of hepatocytes. HBV replication was decreased by approximately 95% in animals receiving the HD Ads and this effect was sustained for eight weeks without any apparent adverse effects. This inhibition of viral replication was significantly more sustained than that achieved by first-generation Ad vectors targeting the same region of the HBV genome (unpublished data).

Assessing efficacy of HD Ads for RNAi-based treatment of other diseases has provided insights that are relevant to using these vectors for delivering HBV-silencing sequences.

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In a study aimed at inhibiting an endogenous hepatic gene, HD Ads successfully delivered a shRNA expression cassette targeting the gene encoding the sterol regulatory element-binding protein-1c (SREBP-1) [76]. This transcription factor is an important mediator of insulin effects on lipid and carbohydrate metabolism in the liver. Following systemic administration of 2×10^{11} HD Ad particles to mice that model type 2 diabetes, 90% knockdown of the target gene was observed in the liver after one week and the effect was sustained for 21 days. An interesting observation was that there appeared to be a limit to the level of gene silencing, and administration of higher vector doses did not augment knockdown but increased immunostimulatory effects [76,77].

Although studies on the use of HD Ads in large animal models of HBV infection have not yet been reported, results from investigations in other disease models are relevant. Successful long term-expression of transgenes delivered with HD Ad vectors has been achieved in non-human primates [78,79]. However, when administered in high doses ($>1 \times 10^{13}$ viral particles) acute and sometimes fatal toxicity was reported to occur [9,74]. There is some preliminary data available from a study that used HD Ads to deliver a blood-clotting factor VIII sequence to the liver of a patient with haemophilia A. The patient apparently developed liver toxicity which lasted 19 days, and factor VIII was, unfortunately, not expressed [80]. Some of the strategies that have been used to attenuate Ad toxicity caused by immunostimulation have been discussed above. As well as polymer modification of immunostimulatory epitopes, administration of dexamethasone, which is an anti-inflammatory glucocorticoid, and transient pharmacological suppression of B and T cells have been used [29,57,61,81–84]. Another factor that diminishes efficiency of Ads in a clinical setting is vector sequestration by the Coxsackie Adenovirus Receptor (CAR) and Complement 1 receptor on human erythrocytes [86]. These receptors are not present on mouse erythrocytes, which emphasizes limitations of murine models in predicting clinical utility of Ad vectors [85,86]. To overcome this sequestration problem it may be possible to modify vectors with polymers or to isolate the liver circulation and deliver the Ad vector directly to the liver by using an intravenous catheter [87,88].

16.2.1.3 Lentiviral Vectors

Lentiviral vectors comprise a subgroup of retroviruses that transduce both dividing and nondividing cells. An important feature of the vectors is that stable integration of their proviruses enables long-term and potentially indefinite expression of transgenes, which may be up to 7.5 kb in length [45,89]. This is useful to achieve sustained expression of anti-HBV sequences and render infected cells resistant to HBV. Although provirus integration into host genomes is potentially mutagenic, targeting to heterochromatin should improve the vectors' safety profile [90]. Interestingly, it has recently been demonstrated that LVs were less likely to integrate into transcriptionally active sites in nondividing cells than in dividing cells [91].

To date, LVs have been used in 40 clinical trials (http://www.abedia.com/wiley/vectors.php, accessed 13 January 2013). Most trials involve *ex vivo* modification of hematopoietic stem cells and T-lymphocytes for the treatment of HIV-1 and monogenic diseases. Although *ex vivo* modification of hepatocytes to render them resistant to HBV infection offers interesting therapeutic possibilities, the methods required to employ this approach are yet to be established. Transduction of autologous hepatocytes derived from induced

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pluripotent stem cells followed by hepatic infusion may become a feasible method of populating the liver with HBV-resistant cells [92].

As for their utility for treating chronic HBV after systemic administration, a limitation is that LVs transduce only a small proportion of adult murine hepatocytes. Liver cell transduction can be improved if cell proliferation is occurring at the time of LV administration. In support of this, injection of vectors into young and newborn animals [93] or following partial hepatectomy in adults [94] achieves greater hepatocyte transduction efficiency. Priming hepatocytes for LV infection by pretreating animals with cholic acid and phenobarbital has recently been investigated as a clinically relevant alternative to improving LV transduction of hepatocytes [95]. Interestingly phenobarbital has a weak stimulatory effect on cell proliferation, but cholic acid has no direct effect on the cell cycle. Without increasing markers of cell proliferation, both compounds were shown to improve transduction of hepatocytes *in vivo* following systemic administered LVs by a factor of 6 to 9-fold. This priming strategy is easy to implement but may not enable transduction of adequate numbers of adult hepatocytes to be of use in RNAi-based HBV therapy with LV vectors.

16.2.2 Nonviral Vectors

As gene-delivery vehicles, NVVs offer a number of advantages over VVs. These include low immunogenicity, ability to accommodate large nucleic acids, modular assembly and potential for large-scale synthesis. The recent announcement by Alnylam Pharmaceuticals Incorporated, together with Tekmira Pharmaceuticals Corporation, of a successful Phase I clinical trial testing a siRNA formulated within a NVV has been an important milestone in advancing these nucleic acid delivery vehicles (http://alturl.com/aadcn, accessed 13 January 2013). The data, presented at the International Symposium on Familial Amyloidotic Polyneuropathy, demonstrated safety and tolerability of an antitransthyretin (TTR) siRNA formulated within Tekmira's lipid nanoparticle (LNP) vectors. Furthermore, a rapid and dose-dependent decrease in serum TTR protein concentrations was observed in patients with amyloidosis of TTR aetiology. Tekmira's LNP technology is formulated to target hepatocytes specifically, which is the major site of TTR synthesis. Although appealing, the applicability of this technology to other diseases of the liver, such as chronic HBV infection, remains to be tested.

Lipid nanoparticles may be categorized within the cationic liposome class of NVVs. Lipid-mediated DNA-transfection (lipofection) was first described in 1987 [96]. The methodology aims to form nucleic acid/lipid complexes to neutralize the inherent negative charge of nucleic acids and thereby facilitate transfer across lipid-rich and negatively charged plasma membranes. In the first study aimed at testing this approach, Felgner and colleagues assessed the utility of the synthetic cationic lipid DOTMA (*N*-[1-(2, 3-dioleyloxy) propyl]-*N*, *N*, *N*-trimethyl ammonium chloride) as a DNA-binding cationic lipid carrier. It was elegantly demonstrated that cationic lipids spontaneously form liposomes and complex with DNA to form lipoplexes. Neutralization of the negative charge and condensation of the nucleic acids to form lipoplex particles enabled DNA delivery to cells. Since this first description of lipofection, numerous advances in the field have taken place, and these vectors have emerged as being suitable for *in vitro* and *in vivo* application (reviewed in [97]).

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An important development in advancing lipoplex vectors was the incorporation of neutral lipids such as DOPE (dioleoylphosphatidylethanolamine) and DOPC (dioleoylphosphatidylcholine) into the formulations. DOPE improves lipofection by aiding release of NVVs from endosomes [98]. The modular way in which liposome formulations may be assembled has enabled evaluation of many combinations of cationic lipids, neutral lipids, targeting and 'stealth' components. This has been particularly useful to adjust the composition of lipoplexes to influence biological properties. In one of the first studies aimed at exploring therapeutic utility of anti-HBV siRNAs, Morrissey and colleagues demonstrated efficient liposome-mediated delivery of siRNAs in a mouse model of HBV replication [99]. Potent silencing of viral gene expression was observed for up to seven days after siRNA administration and the therapeutic effect was maintained after administration of repeat doses. The vectors used in this study, termed stable nucleic acid lipid nanoparticles or SNALPs, have since been used for other hepatic gene-silencing applications. These include nonhuman primate studies that demonstrated inhibition of Ebola virus replication [100] and silencing of endogenous ApoB expression [101]. Interestingly, SNALPs were the forerunners for Tekmira's LNP technology, which has been used in the Phase I clinical trial for the treatment of familial amyloidosis (discussed above).

Cationic polymers comprise the second major class of NVVs [102]. This group of compounds, as with cationic lipids, binds nucleic acids to neutralize negative charges through the formation of polyplexes. Condensation of nucleic acids enables generation of highly compact nanoparticles, which may be taken up by cells through endocytosis (reviewed in [103]). Bioconjugation of the 5′ or 3′ end of the sense or antisense strands of siRNAs with lipids, proteins, peptides and inorganic molecules has also been explored as a means of targeted delivery (reviewed in [104]). More recently, Zhu and Mahato successfully conjugated galactose-bound PEG (Gal-PEG) to the 3′ end of the sense strand of siRNAs and demonstrated silencing of target sequences in hepatocytes ([105]). The silencing achieved with the siRNAs conjugated to Gal-PEG was, however, improved when encapsulated within a cationic liposome. Further characterization of the siRNA conjugates *in vitro* and *in vivo* should provide insights into the therapeutic utility of the technology.

In addition to lipoplex and polyplex NVVs, several other nonviral delivery strategies have been developed. Novel NVVs that have specifically been developed for siRNA delivery include carbon nanotubes [106], lipidoids [107], membrane translocation peptides [108], universal base derivatives [109], and modified arginine peptides [110].

Although NVVs can be complexed with both DNA and RNA, these delivery vehicles have primarily been used *in vivo* to deliver siRNAs to target cells. An important reason for this derives from the fact that delivery of siRNAs to their site of action faces fewer hurdles than delivery of RNAi expression cassettes that comprise DNA. siRNAs function in the cytoplasm and unlike DNA expression cassettes do not have to traverse the nuclear membrane to be functional. Nevertheless, delivery of siRNAs to the cytoplasm of target cells in sufficient quantities to have a desirable effect remains challenging. Difficulties include ensuring NVV stability, specificity of cell targeting, facilitating cellular uptake and cytoplasmic release of siRNAs. As HBV infection occurs in hepatocytes, efficient delivery of anti-HBV complexes to these cells should ideally be achievable after systemic

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administration. To access hepatocytes and traverse hepatic fenestrations, the NVV formulations should also be of uniform small size of approximately less than 120 nm in diameter. Since HBV infection is chronic, repeated NVV administrations may be required and formulations should not be toxic or immunogenic.

16.2.2.1 Using NVVs to Deliver anti-HBVsiRNAs to the Liver

Within the liver, HBV replicates exclusively in hepatocytes and as a consequence NVVs designed to deliver antiviral siRNAs are targeted to these cells. Some sequelae related to the viral infection, for example fibrosis, are associated with secondary effects on other hepatic cells such as hepatic stellate cells. NVV-mediated delivery to these cells requires different targeting strategies (reviewed in [111]). To achieve hepatocyte-specific delivery, numerous cationic lipid formulations have been evaluated. Generally, vectors carry their payloads to hepatocytes passively or by a receptor-mediated process. The various ligands incorporated into NVVs and the cognate hepatocyte receptors they target have been extensively reviewed elsewhere [111]. Examples of receptor and target pairings include interaction of the hepatocyte asialogycoprotein receptor [112] with galactose-containing NVVs and binding of apolipoprotein A-1 (Apo A-1) [113] in NVVs with the hepatocyte high density lipoprotein (HDL) receptor. Hepatocytes exclusively and abundantly express the asialoglycoprotein receptor, which interacts specifically with galactose moieties [112]. This fact has often been exploited to direct NVVs to the liver [114]. A novel galactose-modified DOPE derivative (1,2-dioleoyl-*sn*-glycerol-3-phosphatidyl-*N*-(1-deoxylactito-1-yl)etanolamine or GDOPE) has been used to prepare liposome formulations that achieve improved hepatocyte delivery of siRNAs [115]. In addition to the galactose-modified DOPE, the formulation also included a cationic lipid, a PEG-lipid, and a helper lipid. The liposome-siRNA complex exhibited low toxicity in cell culture and efficiently delivered siRNAs to cells. Transmission electron microscopy indicated that the diameter of the liposomes ranged between 100 and 140 nm and had a multilammelar structure. The lipoplexes delivered siRNAs to hepatocytes *in vivo* but delivery to other tissues was not comprehensively evaluated. Although promising as a hepatocyte-specific delivery vehicle, this technology requires further refinement. Apo A-1 interaction with HDL receptors on liver cells has also been exploited to confer hepatotropism on siRNA-carrying lipoplex formulations [113]. Apo A-1 is a component of HDL and consequently is involved in the hepatocyte uptake of cholesteryl esters. Apo A-1-conjugated liposomes were capable of delivering anti-HBV siRNAs to the livers of mice in a transient HBV replication model. Subsequent studies assessed efficacy of improved Apo A-1 conjugated liposomes carrying siRNAs targeting the hepatitis C virus [116,117]. These NVVs demonstrated better liver-specific targeting *in vivo*, more efficient target knockdown and minimal toxicity [117]. A novel cationic lipid DODAG (*N*′,*N*′-dioctadecyl-N-4,8-diaza-10-aminodecanoylglycine amide) was recently shown to encapsulate anti-HBV siRNAs and mediate efficient hepatocyte delivery in a mouse model of virus replication [118]. DODAG-siRNAs, formulated without a neutral helper lipid, efficiently knocked down viral DNA and antigen markers of replication. Other lipoplex formulations have also been employed to deliver anti-HBV siRNAs *in vivo* [119–121]. These included polyamine-conjugated cholesterol [121] or aminoxy cholesterol lipids that facilitate 'stealth' polymer incorporations [119]. These passively hepatotropic vectors were capable of silencing viral replication in HBV transgenic mice over a period of a few weeks.

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16.2.2.2 Off-target Effects

Studies that comprehensively characterize potential toxic side effects of many of the reported NVVs are incomplete. Toxic induction of the innate immune response was shown when using a formulation comprising the cationic lipid CLinDMA, cholesterol and PEG-dimyristoylglycerol [122]. The siRNA within the liposome contributed to IFN response induction but most of the effect was attributed to the lipids making up the lipoplex. In addition to causing unwanted side effects, the immune response may also reduce the duration of siRNA silencing. By administering dexamethasone prior to lipoplex administration, the innate immune response was effectively attenuated but did not have a significant effect on silencing activity of the siRNA. Dexamethasone treatment therefore offers a useful strategy for reducing undesired immune-mediated side effects. This drug has similar utility for the reduction of immunostimulation by hepatotropic Ad vectors (discussed above). Potential toxic side effects may also arise as a consequence of unintended NVV-mediated delivery of siRNAs to untargeted cells. In general, there is a paucity of comprehensive analysis of the biodistribution of siRNAs delivered with NVVs [123–125]. Similarly, there is little information on the subcellular localization of siRNAs after NVV-mediated delivery to target cells [125,126].

16.2.2.3 Recent Advances in Use of NVVs for Hepatotropic siRNA Delivery

Development of NVVs for delivery of nucleic acids is a very active field of research, which has been the subject of excellent reviews [127–129]. Some selected recent studies using synthetic vectors that target the liver, and which may be used for delivery of anti-HBV therapeutics, are discussed below. In a recent study, use of protamine sulfate and sonication was investigated as a means of facilitating production of nanosize (~100 nm) lipoplexes [130]. siRNAs were complexed with protamine sulfate, then mixed with cholesterol and DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) and sonicated. Liposomes of approximately 100 nm in diameter were consistently obtained and shown to be effective NVVs for delivery of siRNAs to liver cells. Importantly the study showed that siRNAs, upon entering the target cell, were taken up by endosomes and efficiently released into the cytosol. Data from *in vivo* analysis demonstrated that anti-GAPDH siRNAs delivered with these vectors were capable of knocking down the target protein in livers of mice and there was little evidence of toxicity. Intraperitoneal administration of lipoplex formulations also resulted in highly specific delivery of siRNAs to the mouse liver. Using a novel approach, Adami *et al.* recently demonstrated utility of an amino acid-based liposomal delivery system [131]. The study described generation of dialkylated amino acid ($DiLA^2$) compounds. These molecules comprise two hydrocarbon chains linked to the α-carbon and α-amino groups of arginine. This effectively created a lipid-like compound with a hydrophilic head and a hydrophobic tail. The guanidinium head group of arginine has two intended functions: (i) it binds negatively charged proteoglycans to facilitate cellular uptake of this carrier molecule; and (ii) interaction with phosphate groups of nucleic acids enables formation of the NVV complexes. To facilitate liposome formation by the $DiLA^2$ compounds, cholesteryl hemisuccinate (CHEMS) was used as a helper lipid. Transmission electron microscopy indicated that diameters of liposome formulations ranged from 100 nm to 125 nm. The NVVs delivered siRNA to the livers of mice specifically and expression of an endogenous gene, anti-ApoB, was reduced by 80% within 2 days of administration. Thereafter, silencing diminished to 50% at 9 days and 20% at 14 days after administration of the formulations.

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Although non lipid-based NVVs such as functionalized nanotubes [106] and lipidoids [107] have shown promise as siRNA vectors, questions about the specificity of delivery, biocompatibility of these compounds and mechanism of action remain to be answered. Nevertheless NVVs in general show great potential as delivery vehicles of anti-HBV siRNAs. The field is developing rapidly and with positive data from clinical trials providing impetus, NVVs are quickly gaining prominence as hepatotropic delivery vehicles for therapeutic siRNA sequences.

16.3 Conclusions

Effective treatment of people chronically infected with HBV remains a major global challenge. Since 2000 we have witnessed significant advances that demonstrate the feasibility of using RNAi to counter the infection. Studies have shown that powerful inhibition of HBV can be achieved using either synthetic siRNAs or expressed shRNAs. The more sustained silencing that is achieved with expressed shRNA activators makes them well suited to treating chronic HBV infections. To advance gene silencing technology to a stage of clinical applicability, emphasis in the field is now justifiably being placed on improving vectors that deliver RNAi effecters to HBV-infected livers. Invaluable information on the advantages and disadvantages of various VVs and NVVs has been gathered from studying cell culture-based and murine models of HBV. However, refinements of existing vectorology technology are still needed. Investigations carried out on large animal models of HBV, which are currently limited, will be important for better understanding of the properties of the various vectors in a more clinically relevant context. Whether RNAi-based therapeutics eliminate HBV cccDNA remains unclear. It will be interesting to assess whether engineered HBV-targeting sequence-specific nucleases, such as Zinc finger nucleases or transcription activator like effector nucleases (TALENS), are capable of disabling this stable HBV replication intermediate. Determining efficacy of engineered nucleases and currently licensed therapies, when used in conjunction with RNAi-based therapeutics, will be important to determine and may well reveal synergistic actions.

Some of the significant delivery hurdles that need to be overcome before RNAi-based HBV therapy is realized have been highlighted in this review. Importantly, many of the obstacles are also faced by researchers working on other topics within the broader fields of gene therapy and RNAi-based therapeutics. Improvements in gene delivery in general and for treating hepatic diseases specifically should be applicable to RNAi-based HBV therapy. It is also likely that multidisciplinary approaches will be important for making advances in vectorology that is applied to RNAi-based HBV therapy. Progress at the interface between chemistry and molecular biology, for example relating to use of polymers and lipids for nucleic acid delivery, is likely to be particularly significant. Also, improved understanding of RNAi and HBV molecular biology will provide better understanding of how the pathway can be harnessed to silence the virus. Although the disinvestment from RNAi therapy programmes by some large pharmaceutical companies recently had a negative impact in the field [132], a high level of enthusiasm for the potential of the technology remains. Advances in gene therapy, and how these will assist in achieving the goal of RNAi-based HBV treatment, are awaited with interest.

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