Helper-dependent Adenoviral vectors expressing anti-HBV pri-miR sequences from the liver-specific PEPCK promoter

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of

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DECLARATION

I, Duodané Smit declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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Date Signature

DEDICATION

For all those who encouraged me to fly toward my dreams…

~ All Glory to God ~

CONFERENCE PRESENTATIONS

1. Smit D, Maepa B and Arbuthnot. Production of PEGylated Helper Dependent Adenoviral vectors expressing anti-HBV pri-mir sequences from a liver specific PEPCK promoter. Faculty of Health Sciences Research Day 2016, University of the Witwatersrand, September 2016.
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ABSTRACT

Hepatitis B is a global health problem that kills about 600 000 people annually. It is an infectious disease caused by the Hepatitis B Virus (HBV), which infects the liver and leads to liver inflammation and secondary complications such as cirrhosis and Hepatocellular Carcinoma (HCC). The available therapies are only partially effective and are associated with adverse side effects and viral drug resistance. RNA interference (RNAi) pathway is a gene silencing pathway found in diverse living systems including mammals. Harnessing of this pathway to inhibit HBV replication has shown a lot of promise, with highly effective anti-HBV RNAi activator sequences designed. However, the lack of safe and efficient delivery and expression systems for these sequences is one of the obstacles that need to be overcome before RNAi effectors can reach clinical application. For easy assessment of transduction efficiency, Helper Dependent Adenoviral vectors (HDAd) expressing β-galactosidase (encoded by *lac Z* gene) are commonly used to deliver anti-viral RNAi activators. However, this reporter protein has been blamed for induction of innate immune response and concomitant clearance of the HDAds by the host. For the first time, this study explored the use of *lac Z*-deficient HDAds to deliver anti-HBV RNAi activators expressed under the control of a liver-specific phosphoenolpyruvate carboxykinase (PEPCK) promoter. HDAd expressing *Firefly luciferase* resulted in a significant luminescence detected in cell culture lysates and a sustained bioluminescence in mice. HDAds expressing anti-HBV sequences transduced the liver efficiently and did not induce a pronounced inflammatory response or liver toxicity in mice. However, this did not translate into maximal anti-HBV sequence expression and HBV replication inhibition *in vitro* and *in vivo.* This suggests that the PEPCK promoter is inadequate for RNAi activator expression. This study highlights the importance of careful RNAi activator regulatory elements selection and presents the therapeutic potential and utility of HDAd vectors for hepatotropic delivery of antiviral sequences with markedly attenuated immune response stimulation and toxicity. For improved anti-HBV RNAi activator expression and HBV knockdown, a different liver specific promoter mouse transthyretin receptor (MTTR) promoter is currently being investigated in our lab.

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LIST OF ABBREVIATIONS

Aa	amino acid
AAV	adeno-associated virus
Ab	Antibody
Ads	Adenoviruses
AdV	Adenoviral vectors
AGTRU	Antiviral Gene Therapy Research Unit
ALT	Alanine transferase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
Bafp	α-fetoprotein
APC-A	Allophycocyanin
apoE	Apolipoprotein E
Bp	base pair
CAR	Coxsackie-adenovirus receptor
cDNA	Complementary DNA
cccDNA	covalently closed circular DNA
CMV	Cytomegalovirus
C	Core
DAB	Diaminobenzidine
DBD	DNA binding domain
DGCR8	Di George Critical Region 8
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsAAV	double stranded Adeno-associated vectors
dsRNA	double-stranded RNA
dsDNA	double stranded DNA
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
ELISA	enzyme linked immunosorbent assay
EMEM	Eagle's Minimum Essential Medium
Epo	Erythropoietin
FCS	foetal calf serum
FBS	Fetal Bovine serum
FGAd	First generation adenoviral vectors
Fluc	*Firefly luciferase*
hAAT	human α-1-antitrypsin
HBsAg	hepatitis B virus surface antigen
HBV	hepatitis B virus
HBx	hepatitis B virus X protein
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDAd	Helper Dependent Adenoviral vector
HEK293	human embryonic kidney cell line

HIV	human immmunodeficiency virus
hOTC	human ornithine transcarbamylase
HRP	horseradish peroxidase
hTERT	human telomerase reverse transcriptase
Huh7	human hepatoma cell line
HV	helper virus
IFN-α	interferon-α
IL-12	interleukin-12
IPTG	isopropyl-beta-D-thiogalactopyranoside
ITR	inverted terminal repeats
LA	Luria Bertani agar
LB	Luria Bertani medium
LTR	long-terminal repeat
LV	Lentiviral vectors
miR	microRNA
MLP	major late promoter
MOI	multiplicity of infection
mRNA	messenger RNA
NF-κB	nuclear factor-kappaB
NPC	nuclear pore complex
Nt	Nucleotide
NVV	non-viral vector
OD	optical density
ORF	open reading frame
P	Polymerase ORF
PBS	phosphate-buffered saline
PC	pre-core
PCR	Polymerase Chain Reaction
PE-A	Phycoerythrin conjugate
PEG	polyethylene glycol
PEPCK	Phosphoenolpyruvate carboxykinase
pgRNA	pregenomic RNA
PKR	protein kinase receptor
Pol	RNA polymerase
pre-miR	precursor miRNA
pre-mRNA	precursor mRNA
pri-miR	primary microRNA
rAAV	recombinant AAV
rcDNA	relaxed circular DNA
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
Rpm	revolutions per minute
rRNA	ribosomal RNA
S	Surface
SEM	standard error of the mean
shRNA	short hairpin RNA
siRNA	short interfering RNA
SV40pA	simian virus 40 polyadenylation signal

TAE buffer	tris-acetate-EDTA buffer
TCEP	Tris(2-carboxyl)phosphine)
TEM	Transmission Electron Microscopy
TLR	toll-like receptor
TP	terminal protein
TRBP	TAR- double stranded RNA-binding protein
tRNA	transfer RNA
UTR	untranslated region
UV	Ultraviolet
UVP	Ultra-Violet Product
VPEs	viral particle equivalents
VPS	Viral particles
WHO	World Health Organisation
WHV	Woodchuck Hepatitis virus
WPRE	Woodchuck Hepatitis virus posttranscriptional regulatory element
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1

1 INTRODUCTION

1.1 Hepatitis B

Hepatitis B is a global health problem that affects millions of people, with a high incidence found in areas such as East Asia and Sub-Saharan Africa (WHO, 2016). This is an infectious disease caused by the Hepatitis B Virus (HBV), which infects the liver and leads to liver inflammation. HBV is mainly transmitted through infectious blood or other body fluids either percutaneously, sexually or perinatally. Secondary complications of HBV infection include acute and chronic infections, cirrhosis and Hepatocellular Carcinoma (HCC). HBV accounts for the infection of approximately 2 billion individuals worldwide, of which 240 million are chronic cases that lead to an estimated 600 000 individual deaths each year (Mueller *et al*., 2015, Ott *et al*., 2012). The prevention of, and effective therapies against HBV infection are key to reducing the spread of this condition.

There has been a safe and effective vaccine available against HBV infection since 1982. Nevertheless, infant vaccine administration is preferred as soon as possible after birth. During 1991, the World Health Organisation (WHO) suggested the implementation of mass immunization against HBV infection in all countries. This resulted in the decrease in HBV infection occurrence among infants, children and young adults in various countries. However, due to several countries not implementing the suggested mass vaccination program, HBV remains prevalent in a large number of individuals [reviewed in (Lavanchy, 2004, MacLachlan and Cowie, 2015)].

Current licensed treatments of Hepatitis B make use of conventional interferon-α (IFN-α) or pegylated IFN-α, with finite duration that has both antiviral activity and immunomodulatory actions. Furthermore, these treatments involve long duration nucleoside and nucleotide analogues that are inhibitors of viral reverse transcriptase and DNA polymerase. Nucleoside and nucleotide analogues, which include Lamivudine, Adefovir dipivoxil, Entecavir, Telbivudine, or Tenofovir, have been approved for the treatment of chronic HBV infection, with Lamivudine being the most widely administered drug [Reviewed in (Weinberg and Arbuthnot, 2010, Tang *et al*., 2014)]. However,

prolonged treatment with interferon-α (IFN-α) and nucleoside and nucleotide analogues monotherapy can bring about viral drug resistance and adverse side effects such as hepatic damage, effectiveness in only 50 % of patients and is also expensive. Major side effects and drug resistance most frequently occurs in interferon-α and Lamivudine treated individuals, respectively (Perrillo, 2004, Lok, 2008). The emerging viral drug resistance is usually a result of the mutations within the viral polymerase gene. Despite cross-resistance of other agents such as Entecavir, a combination of nucleoside and nucleotide analogs with a high genetic barrier to resistance can prevent or limit the emergence of viral drug resistance [Reviewed in (Weinberg and Arbuthnot, 2010, Keeffe *et al*., 2008)]. IFN-α has a broad antiviral activity and has a different mechanism to that of nucleosides and nucleotides analogues, for it is not associated with the blocking of a specific part in the viral replication process. As a result, IFN-α functions are not influenced by drug resistant mutations. Nevertheless, the available therapies are only partially effective against the virus and therefore do not completely eradicate the virus resulting in the reactivation of HBV (Lok, 2008, Perrillo, 2004). The challenges of current treatments prompted the need for new Hepatitis B therapies.

1.2 RNA interference

The interference with viral gene expression has been proven to be an effective method in controlling viral infections (Peng *et al*., 2005, Jacque *et al*., 2002, Yang *et al*., 2013). RNA interference (RNAi) is a pathway triggered by double-stranded RNA (dsRNA) and functions as a regulatory and self-defense mechanism in eukaryotic cells. The conserved RNAi pathway has been found to occur in a wide range of organisms including animals and plants (Elbashir *et al*., 2001). The first discovery of the RNAi pathway was by Andrew Fire and Craig Mello, whereby induced gene silencing by dsRNA was observed in the *Caenorhabditis elegans* nematode (Fire *et al*., 1998). This discovery provided the first glimpse into controlling the expression of target messenger RNA (mRNA) using the RNAi mechanism. The most studied RNAi pathway is known as the mammalian microRNA (miR) pathway. The first step of this pathway involves the transcription of miR-encoding genes by RNA polymerase II within the nucleus to give rise to a hairpin-structured primary miR (pri-miR). The pri-miR is then processed to produce the 60-80 nucleotides (nt) hairpin structured precursor miR (pre-miR) by the microprocessor

complex comprised of Drosha (RNase III) and DiGeorge Critical Region 8 (DGCR8 - Drosha double stranded RNA binding domain partner). The pre-miR is then exported from the nucleus into the cytoplasm by nuclear karyopherin exportin-5. Pre-miRs are further processed to form mature-miR duplexes of 21-23 nt in length by Dicer (an RNase III protein) and TRBP (TAR - double stranded RNA -binding protein) (Han *et al*., 2009, Zeng *et al*., 2003, Denli *et al*., 2004). This is followed by the integration of the 21-23 nt miR duplexes into the RNA induced silencing complex (RISC) that contains an Argonaute 2 protein. This complex selects and retains one strand as a guide strand and releases the remaining passenger strand. The guide sequence is used for sequence-specific recognition of the target mRNA and through complementary base pairing pairs with the target mRNA (Denli *et al*., 2004, Hammond *et al*., 2001). The guide strand can have a partial complementarity with the sequences of the targeted mRNA 3' untranslated region (UTR), which results in the translation inhibition. On the other hand, should the guide strand have complete complementarity, then the mRNA can be targeted for degradation (Khvorova *et al*., 2003, Zeng and Cullen, 2003, Zeng and Cullen, 2005, Zeng *et al*., 2002, Zeng *et al*., 2003).

The exploitation of the RNAi pathway for the sequence specific silencing of genes is well established as a research tool in gene therapy, especially as a therapeutic method for viral disease treatments (Elbashir *et al*., 2001, McBride *et al*., 2008, McCaffrey *et al*., 2003, Peng *et al*., 2005). A number of studies have shown the successful therapeutic application of the RNAi technology for the treatment of serious viral diseases like Human Immunodeficiency Virus (HIV) and Hepatitis C (HCV) (Gitlin *et al*., 2002, Jacque *et al*., 2002, Lee *et al*., 2002, Wilson *et al*., 2003). This is achieved by introducing mimics of pre-miR [short hairpin RNA (shRNA)], pri-miR [artificial pri-miR (pri-miR)] or mature miR duplexes [short interfering RNA (siRNA)] in the host to target viral mRNA sequences (Dykxhoorn *et al*., 2006, Keck *et al*., 2009).

1.2.1 HBV as a potential target for RNA interference

HBV is an enveloped, noncytopathic DNA virus that belongs to the *Hepadnaviridae* family of hepatotropic viruses and has been known to infect both humans and chimpanzees (Lyons *et al*., 2012). The HBV genome is 3.2 kb in length and contains a relaxed circular and partially double stranded DNA (rcDNA) (Figure 1.1). In infected

hepatocytes, the rcDNA is converted to covalently closed circular DNA (cccDNA). The formed cccDNA acts as the template for the production of pregenomic RNA (pgRNA) used for viral replication and all viral RNAs important for viral protein synthesis and viral assembly. The difficulty associated with eliminating cccDNA from infected hepatocytes is the main reason for the ineffectiveness of many antiviral therapeutics. The HBV genome consists of an evolved organization of conserved protein coding and regulatory regions. It encodes four overlapping open reading frames (ORF). The ORF's include the pre-core (PC) and core (C) ORF, Polymerase ORF (P), the Surface (S) ORF that codes for envelope proteins pre-S1, pre-S2 and S, and lastly the X ORF that encodes for multifunctional X protein (*HBx*). It also contains a promoter and enhancers (regulatory elements), as well as signals required for polyadenylation and encapsidation. The conserved regions can encode for multiple proteins and for the *cis*-elements of HBV required for viral replication (Beck and Nassal, 2007, Araki *et al*., 1989, Tang *et al*., 2014). The HBV genome consist of a single transcription signal, this signal results in the presence of the common 3' end in the HBV transcripts that includes the *HBx*. This arrangement of the HBV genome limits the viral sequences flexibility, thereby making the *HBx* a suitable target for gene silencing and antiviral therapies that are based on nucleic acid hybridisation. It enables the degradation of multiple HBV viral mRNA transcripts with a single anti-HBV RNAi activator, making it an ideal target for RNAi based therapies [(Jia *et al*., 2007, Carmona *et al*., 2006), Figure 1.1].

The use of RNAi-based therapies for the gene silencing and inhibition of HBV gene expression by targeting specific genes of interest has been extensively studied (Wooddell *et al*., 2013, McCaffrey *et al*., 2003). As a result of the cytoplasmic site of action and their small size, short interfering RNA (siRNAs) has distinct advantages. These include easy dose control, production and delivery. In addition, siRNAs can be subjected to chemical modifications to improve stability, specificity and safety by reducing immunostimulatory effects and hepatotoxicity (Wooddell *et al*., 2013, Marimani *et al*., 2013, Marimani *et al*., 2015). Previous studies have shown the ability of siRNAs to effectively inhibit HBV gene expression and replication in both *in vitro* and *in vivo* applications (Giladi *et al*., 2003, Jia *et al*., 2007, McCaffrey *et al*., 2002). Giladi and colleagues showed the successful inhibition of HBV viral gene expression and replication through the targeting of the HBV surface antigen. The inhibition of viral gene expression was observed in both HepG2.215 cells and a HBV mouse model (Giladi *et al*., 2003). However, the inhibition of gene

expression by siRNAs is short-lived and requires repeated administration for a sustained suppression of viral replication (McCaffrey *et al*., 2002, Giladi *et al*., 2003, Marimani *et al*., 2013).

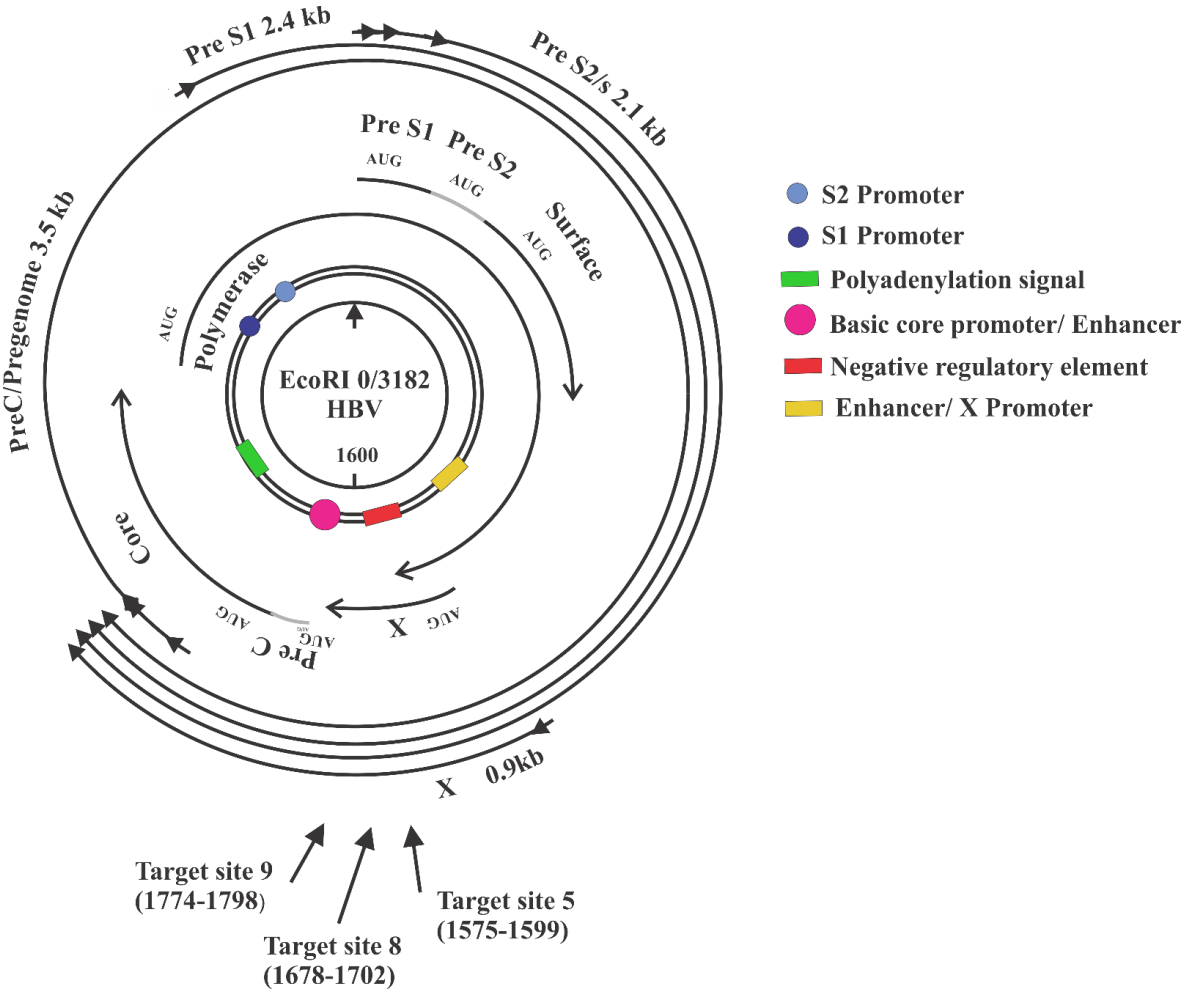


Figure 1.1: HBV genome organisation
The HBV genome is a partially double stranded genome with *cis*-acting elements presented by the circles and squares that regulate HBV transcription. The viral genome co-ordinates are given relative to the single *Eco* RI restriction site. The surrounding arrows indicate the viral open reading frames (with initiation codons) of the surface, core, polymerase and *HBx*, encompassing the entire HBV genome. The four outer arrows indicate the HBV transcripts which have a common 3′ polyadenylation site. As well as, the targeted site 5, 8 and 9 within the *HBx* for the RNAi pri-miR 31/5, pri-miR 31/589 and pri-miR 122/5 activator cassettes [Adapted from (Ivacik *et al*., 2011, Mowa *et al*., 2012)].

Therefore studies have evaluated the use of expressed shRNAs and artificial pri-miRs *in vitro* and *in vivo* for a sustained inhibition of gene expression in HBV infection models. These expressed activators have the advantage of being more sustainable and compatible

with more efficient viral vector delivery systems (Grimm and Kay, 2006, Grimm *et al*., 2006, Jenke *et al*., 2008, McCaffrey *et al*., 2003, Wooddell *et al*., 2013). Effective inhibition of HBV gene expression was observed by Carmona and colleagues using anti-HBV shRNA cassettes which target specific regions within the *HBx* sequence (Carmona *et al*., 2006). In addition McCaffrey and colleagues showed efficient HBV inhibition *in vitro* and *in vivo* using anti-HBV shRNA cassettes targeting the HBV mRNAs of the pregenominc RNA, the core antigen and polymerase. However, shRNAs can only be effectively expressed from Polymerase III (Pol III) promoters e.g. U6 small nuclear RNA and Human ribonuclease P RNA component H1 (U6 and H1 promoters) (Jenke *et al*., 2008, McCaffrey *et al*., 2003), which have been previously associated with *in vivo* cytotoxicity and tissue damage. These toxic effects were as a result of the saturation of the endogenous RNAi pathway, caused by the overexpression of shRNA by Pol III promoters. Furthermore, Pol III promoters are non-tissue specific and therefore have robust activity across several cell types (Ely *et al*., 2008, Giering *et al*., 2008, Grimm and Kay, 2006, Grimm *et al*., 2006, Grimm *et al*., 2010). Expression of shRNAs from cytomegalovirus (CMV) Polymerase II (Pol II) promoter demonstrated HBV knockdown, but the efficiency of the silencing was found to be unpredictable and variable (Li *et al*., 2007).

To alleviate this problem, antiviral expression cassettes consisting of the Pol II transcription regulatory elements upstream of the pri-miR mimic's encoding sequence were constructed. This strategy has successfully been used against HBV infection and has shown to be less toxic *in vitro* and *in vivo* (Ely *et al*., 2008, Mowa *et al*., 2012, Mowa *et al*., 2014, Ivacik *et al*., 2015). Moreover, Pol II expression allows for tissue specificity and/or can be inducible for precise transgene expression (Maczuga *et al*., 2012, McBride *et al*., 2008). To enhance silencing efficiency and avoid viral escape, pri-miR mimics can be engineered to express multiple (polycistronic) anti-HBV sequences that can target several specific sites within the HBV genome (Ely *et al*., 2009, Ely *et al*., 2008). Zeng and colleagues have previously shown that the substitution of the guide sequence in both naturally occurring pri-miR 30 and pri-miR 21 with a synthetic sequence produced a novel miR sequence that was complementary to the target site. These monocistronic sequences inhibited the expression of specific human genes, by either translation inhibition or mRNA degradation (Zeng and Cullen, 2003, Zeng *et al*., 2002, Zeng *et al*., 2003). Recently, pri-miR 106 and pri-miR 155 polycistronic expression systems have been engineered. Pri-miR 106-cluster demonstrated potent inhibition of HIV-1 replication and pri-miR155-cluster

increased the inhibition of the targeted BIC RNA [non-coding RNA that causes lymphoma by cooperating with c-myc transcription factor] (Aagaard *et al*., 2008, Chung *et al*., 2006).

To further investigate the application of pri-miR mimics in HBV replication inhibition, CMV promoter driven, monomeric and trimeric mimics of naturally occurring pri-miR 31 and pri-miR 122 were constructed within the Antiviral Gene Therapy Research Unit (AGTRU). The backbone of pri-miR 122 was selected due to its dominant expression in the liver (Chang *et al*., 2004). Pri-miR 31 backbone is also well suited for the RNAi therapeutics design as it is efficiently processed by Drosha (Zeng and Cullen, 2005). The anti-HBV pri-miRs were generated by replacing the guide and complementary sequence with the anti-*HBx* sequences in the structure of pri-miR 31 to generate monocistronic pri-miR 31/5 and polycistronic pri-miR 31/589 or pri-miR 122 to generate monocistronic pri-miR 122/5 (Figure 1.1). The use of these cassettes demonstrated efficient knockdown of HBV gene expression and were found to be safer in mice. The CMV promoter is one of the strongest promoters and has been widely applied in *in vivo* expression of therapeutic genes in various tissues including the liver of mice. Even though the results obtained indicated efficient HBV silencing, the CMV promoter is not liver specific. Hence, it is capable of driving high levels of transgene expression in non-hepatic tissue (Ely *et al*., 2009, Ely *et al*., 2008, Ivacik *et al*., 2015). In addition, the expression and activation of the CMV promoter in hepatocytes are Nuclear factor-kappaB (NF-κB) dependent. However, NF-κB is absent in mouse hepatocytes. Hence, strong short-term expression (1 week after delivery) of transgenes was observed, followed by reduced gene silencing (barely detectable) after 1-6 weeks (Loser *et al*., 1998, Mowa *et al*., 2014). Therefore, the CMV promoter would not be suitable for therapeutic application against chronic Hepatitis B.

Alternatively, studies have investigated the use of vectors bearing Phosphoenolpyruvate carboxykinase (PEPCK) promoter which are predominately active within the liver. Tissue-specific activity of the PEPCK promoter was analysed by Song *et al.*, (2006) *in vitro* using plasmids expressing *Firefly luciferase* (*Fluc*) from a PEPCK promoter. The PEPCK promoter resulted in a significant *Fluc* expression in different liver cell lines. Song and colleagues further explored the use of the PEPCK promoter to improve liver specific cancer therapy (hepatocellular carcinoma). The experiment entailed the design of a liver specific expression cassette with a human telomerase reverse transcriptase (hTERT) RNA-targeting specific *trans*-splicing ribozyme. The results showed liver specific anti-cancer

gene activity by effectively inducing cytotoxicity in the liver cancer cells and decreased activity in non-liver cancer cells (Song and Lee, 2006).

On the other hand, studies have investigated the use of vectors bearing the PEPCK promoter for long-term transgene expression (Brunetti-Pierri *et al*., 2007, Mian *et al*., 2004). Main and colleagues investigated the design of vectors expressing human ornithine transcarbamylase (hOTC) from a PEPCK promoter in the presence or absence of a Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) downstream of the hOTC Complementary DNA (cDNA), to enable long-term correction of OTC deficiency in mice. A long-term rectification of orotic aciduria was observed for 12 months (length of experiment) with considerable liver OTC activity (expression) in the absence of chronic liver toxicity (Mian *et al*., 2004). Brunetti-Pierri and co-workers persued the investigation of the expression of α-fetoprotein (bAFP) from a PEPCK promoter in non-human primates. The results indicated long-term (> 1 year) and stable hepatic expression of the bAFP transgenes in non-human primates in the absence of chronic hepatotoxicity (Brunetti-Pierri *et al*., 2007, Brunetti-Pierri *et al*., 2006). This long-term transgene expression provoked the investigation towards the application of this PEPCK promoter for HBV treatment. Hence, this study aimed to design PEPCK promoter driven pri-miR 31 mimics to achieve liver specific expression and HBV replication knockdown.

In addition to finding the most efficient regulatory elements for anti-HBV RNAi activator expression, the lack of an effective and safe *in vivo* delivery system for these highly effective sequences remains one of the biggest challenges in anti-HBV gene therapy (Aagaard and Rossi, 2007). Hence, this study also focuses on finding an efficient and safer delivery system for anti-HBV RNAi activators.

1.3 Delivery systems for antiviral RNAi activators

For successful gene therapy, suitable amounts of therapeutic sequences need to be transported to the targeted tissue without significant toxicity. For RNAi activator sequences to be effectively transported to the liver, vectors are required to deliver the anti-HBV effector sequences directly to the hepatocytes after administration. A sustained HBV

silencing that can be achieved with a single administration is the optimal target for vector based therapeutics. Hence, finding a suitable and safe delivery system remains one of the biggest obstacles (Lundstrom and Boulikas, 2003).

Physical, viral and chemical/non-viral methods can be used to deliver therapeutic sequences. Physical delivery is performed by applying a force that allows the permeation of the cell membrane for intracellular nucleic acid translocation. This can be applied through needle injection, electroporation, hydrodynamic delivery or ultrasound. These methods have been used successfully in pre-clinical studies, however most are less efficient and unsafe in a clinical setting [reviewed in (Kaneda and Tabata, 2006, Villemejane and Mir, 2009)]. The second approach is non-viral (NVVs) methods, whereby the desired transgenes are transported into the cell as a complex with synthetically engineered or naturally occurring compounds (Deng *et al*., 2009). The use of these vectors for HBV treatment for delivery of RNAi activators to the liver has proven to be very useful (Carmona *et al*., 2009, Marimani *et al*., 2013, Marimani *et al*., 2015, Morrissey *et al*., 2005). However, the NVV's are less efficient in transfecting cells and are more compatible with small synthetic siRNAs than expressed RNAi activators. To improve the *in vitro* and *in vivo* transduction efficiency, further studies were performed using a third approach, namely viral vectors. Experiments conducted have found viral vectors such as Adeno-associated viral vectors (AAV), Lentiviral vectors (LV) and Adenoviral vectors (AdV) to be more efficient and can be targeted to desired tissue (Tomanin and Scarpa, 2004, Uchida *et al*., 2002, Breyer *et al*., 2001).

1.3.1 Viral vectors

1.3.1.1 Lentiviral vectors

Lentiviral vectors (LV's) are recombinant vectors that integrate into the host genome, thereby providing a means of long-term gene expression. LV's have been derived from human immunodeficiency virus type 1 (HIV-1) and have been applied in the RNAi-based HBV therapy. They have a larger transgene cloning capacity compared to that of AAV's and can allow efficient transgene delivery. These vectors are capable of transducing a wide range of proliferating and non-proliferating cells, which enables long-term

expression of transgenes in hepatocytes *in vitro* and *in vivo* (Ivacik *et al*., 2015, Nguyen *et al*., 2005, Nguyen *et al*., 2002).

The ability of LV's to transduce a variety of tissues, especially non-dividing cells has prompted new possibilities and applications of LV's in gene therapy. The first demonstrations of the long-term therapeutic potential of Lentiviral vectors was performed on many central nervous system cell types through the injection of these vectors into animal models. This led to the idea that LV's could represent a possible tool for liver studies, as the liver is composed of non-dividing, active and highly differentiated cells. Further studies demonstrated the efficient transduction in murine models using LV's for numerous hereditary metabolic liver diseases (Nguyen *et al*., 2002, Park *et al*., 2000, Pfeifer *et al*., 2001, Rittelmeyer *et al*., 2013, Kubo and Mitani, 2003). In Hepatitis B and C virus infection cases, the liver cells transduced with the LV carrying transgenes can suppress the replication of HBV and HCV. Ivacik and colleagues presented the first efficient silencing of HBV gene expression using RNAi-activating anti-HBV sequences delivered with LV's in transgenic HBV mice (Ivacik *et al*., 2015). However, *in vivo* testing in murine models indicated that hepatocyte LV transduction efficiency is less than that of neurons. As a result, *ex vivo* methodologies that yielded high transduction efficiency and hepatocyte survival are considered for the gene therapy based treatment of liver disease using LVs. But *in vitro* and *ex vivo* gene delivery is still limited by the low titers produced during viral production (Kubo and Mitani, 2003, Nguyen *et al*., 2002). Although performing viral production with transient transfection is capable of increasing the titers, the method itself is burdensome and scaling up is difficult [reviewed in (Matrai *et al*., 2010)].

Another concern is the clinical safety of LVs, which focuses on potential oncogene activation and/or tumor suppressor genes as a result of the LV integration into the host genome. As well as, the pathogenicity that may be caused by the recombination and regeneration of the wild type virus, HIV-1 from which LV's have been derived, in patients with an infectious retrovirus such as HIV-1 or HIV-2 (Matrai *et al*., 2010, Manjunath *et al*., 2009).

1.3.1.2 Adeno-associated viral vectors

As a result of the lack of pathogenicity of AAV's, these viruses were seen as having no medical significance. Hence, lack of AAV biology understanding prevented AAV's broad use as delivery vectors. But after recent extensive research, promising results were obtained, showing stable tissue transduction and minimal immune stimulation by AAVs. The non-pathogenicity, viral persistence, non-significant immune response induction and existence of a large number of serotypes provoked further studies into using these vectors as a delivery system for gene therapy (Wang *et al*., 2010, Wang *et al*., 2004).

Adeno-associated virus is an icosahedral, non-enveloped, single-stranded DNA virus that is small in size (genome size of ± 4.7 kb) with a capsid diameter of 18-25 nm and a genome of either a plus or minus polarity. This virus is a non-pathogenic parvovirus that requires a co-infection with a helper virus, either adenovirus or herpesvirus to replicate and spread. In the AAV vector, 96 % of the viral coding genes (± 4.4 kb) have been deleted, leaving both ends of the AAV with the inverted terminal repeats (ITR's). The ITR's contain the *cis*-element that is required for replication and genome packaging. The AAV vector therefore contains only the gene of interest and ITR's. In turn, this further reduced the possibility of immune simulation by the viral proteins (Kaplitt *et al*., 1994, Srivastava *et al*., 1983, Xiao *et al*., 1996).

Thirteen AAV serotypes have been identified in humans (AAV2, -3, -5 and -6) and non-human primates (AAV1, -4, and -7 to -13). Furthermore, more than 100 new and unique AAV variants have been isolated in human and non-human primates. However, serotype 2 (AAV-2) has been more extensively studied and therefore serves as the model for the AAV family (Gao *et al*., 2004, Rayaprolu *et al*., 2013, Venkatakrishnan *et al*., 2013, Schmidt *et al*., 2004). Recent studies have shown that each serotype has different structural and functional characteristic and has efficient transduction with long-term gene expression in a number of different cell types. These cell types include liver, muscle, lung and pancreases. The first long-term transgene expression using recombinant AAV vectors with a *lac Z* reporter gene (rAAV-*lac*Z) *in vitro* (mice muscles cells) and *in vivo* without immune stimulation was observed by Xiao and colleagues (Xiao *et al*., 1996, Wang *et al*., 2004). Furthermore, studies have shown a great reduction in HBV serum titers and mRNA present in the liver of transgenic mice by anti-HBV shRNA delivered by three different

double stranded Adeno-associated vectors (dsAAV); dsAAV8, dsAAV7 and dsAAV9 (Chen *et al*., 2007, Chen *et al*., 2009). However, despite AAV vectors achieving some level of success in highlighting their potential as vectors for clinical application, a number of hurdles still need to be overcome. These hurdles include the extremely high viral dosages required for efficient transduction and the antibody neutralization of AAV's due to the high prevalence of AAV-2 specific antibodies in humans. Limited knowledge behind the mechanism of AAV-host interaction hinders the efficient use of AAV as a vector for gene delivery. Furthermore, the smaller size of the AAV genome limits its transgene capacity (Nakai *et al*., 2005, dos Santos Coura, 2008).

1.3.1.3 Adenoviral vectors

Adenoviruses (Ads) are one of the most extensively studied viruses used for gene therapy and vaccine development. The discovery of Ads has prompted the interest in broadening the knowledge on the Ads genome and their application as delivery vectors. The easy production of high viral titers and high, long-term hepatocyte transduction efficiency and gene expression (*in vivo)* without integrating into the host genome, the large DNA insert accommodation and ability to transfer genes to a variety of dividing and non-dividing cells has drawn great attention to Ads (Croyle *et al*., 2005, Croyle *et al*., 2000). Adenoviruses are useful delivery vectors for anti-HBV effecter sequences, due to their natural tropism for the liver and efficient targeting into hepatocytes (Crowther *et al*., 2008, Jozkowicz and Dulak, 2005). This study therefore investigated the use of Adenoviral vectors for the delivery of anti-HBV pri-miR sequences.

Adenoviruses belong to the *Adenoviridae* family, and are non-enveloped, icosahedral particles that are 70-90 nm in diameter. The Ad is surrounded by an outer protein capsid that is composed of capsomeres that consist of 252 subunits and are made up of 3 major proteins. These proteins include hexon proteins (timers of hexon proteins pIIIa, pVI, pVIII and pIX), penton base proteins and fiber proteins along with a number of minor proteins that covers an inner nucleoprotein core (pV, pVI, mu and the histone like pVII proteins) [reviewed in (McConnell and Imperiale, 2004, Rosewell *et al*., 2011)]. The capsomeres form 12 capsid vertices, where each of the vertices have 5 hexon proteins that surround a penton base protein that serves as an anchor for fiber proteins. From each of the penton

base proteins, a fiber protein protrudes and aids in the binding of the vector to the host receptor [(Majhen and Ambriovic-Ristov, 2006, Reynolds and Curiel, 2002), Figure 1.2].

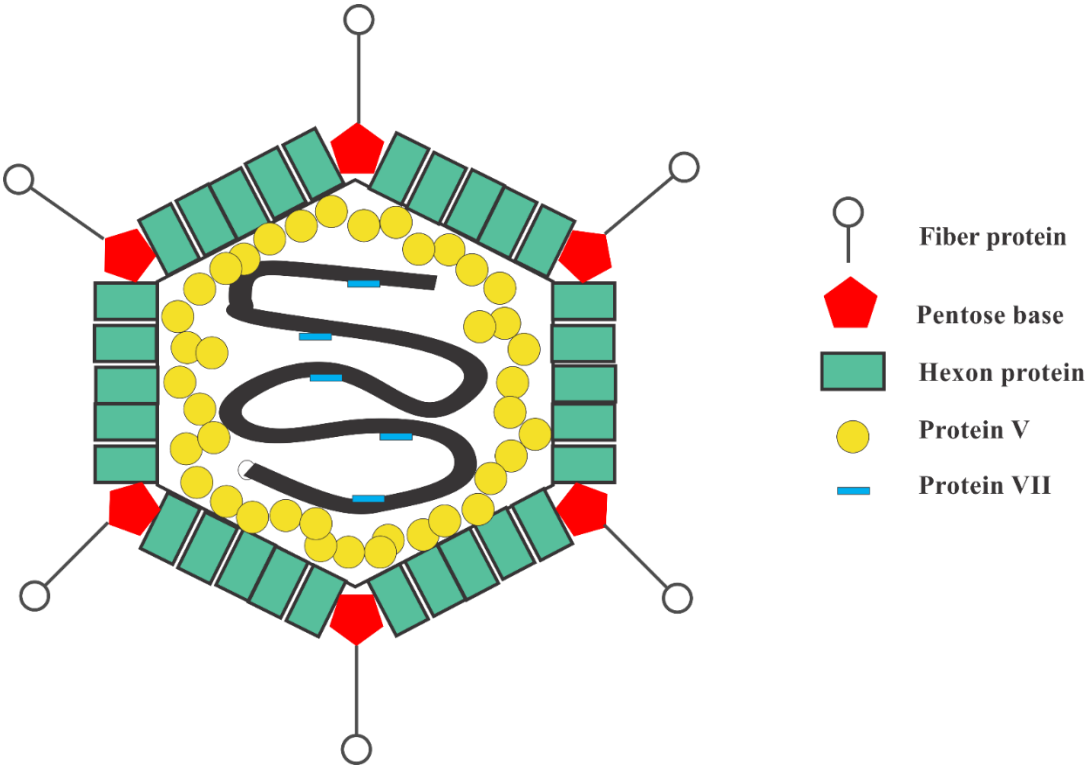


Figure 1.2: Adenovirus particle structure
Schematic presentation of the Adenovirus that is made up of 3 major proteins. The Fiber proteins, Pentose base and Hexon proteins, as well as the inner nucleoprotein core (Protein V and VII). The capsomeres form 12 capsid vertices where each of the vertices have 5 hexon proteins that surround a penton base protein that serves as an anchor for fiber proteins. Adapted from (McConnell and Imperiale, 2004).

The human Adenovirus has ~50 different serotypes and is classified into 6 groups (A-F). Of these serotypes, type 2 (Ad2) and type 5 (Ad5) of group C have been the most characterized and shown the best potential as recombinant adenoviral vector delivery systems for gene therapy [reviewed in (Kay *et al.*, 2001, McConnell and Imperiale, 2004)]. The Ad5 genome (Figure 1.3) is composed of 36 kb linear double stranded-DNA with *cis*-acting ITR's at both ends. It also has a 5' terminal protein (TP), which serves as a primer for viral replication initiation and a packaging signal (ψ) adjacent the left ITR (Liu *et al.*, 2003, Majhen and Ambriovic-Ristov, 2006). Both the DNA strands serves as templates for transcription. The genome is divided in to three major transcription units

based mainly on the stage at which they are transcribed during viral replication. These are: the early transcription unit, mainly involved in early transcription activation, DNA replication and immune suppression (encoding E1A, E1B, E2, E3 and E4 transcripts); the delayed early transcription unit (encoding transcripts IX, IVa, VA RNA I and VA RNA II) mainly important for late transcription activation and capsid assembly; and late transcription unit encoding L1-L5 transcripts, from which the viral structural proteins are translated (Breyer *et al*., 2001). The E1 genes can be divided into the E1A and E1B transcription units. The E1A proteins regulate transcription and the S phase entrance of the host cells and the E1B aids in the transport of the viral mRNA and preventing the transport of the host mRNA. The E2A and E2B makes up the E2 region, where the DNA-binding protein is encoded by E2A and the viral polymerase and terminal protein precursor is encoded by the E2B region. The E3 region encodes for the proteins that provides the immunomodulatory functions. The remaining E4 region provides the proteins that are required for DNA replication, the reduction in host proteins synthesis and the enhancement of late gene expression. The late genes (L1-L5) are transcribed from a single promoter known as the major late promoter (MPL), to form one pre-mRNA, which is later processed by alternative splicing to produce multiple mRNAs. These late mRNAs encode for the structural Hexon, Pentose and Fiber proteins required for assembly of the viral particle [Reviewed in (McConnell and Imperiale, 2004, Vetrini and Ng, 2010)].

Adenoviruses uses a two stage process to infect the host cells. The efficiency of host cell infection by Ads is dependent on the binding and entry, which in turn is dependent on the primary and secondary receptors present on the host cell surface. The first stage entails the Ad fiber protein binding to the most commonly used Coxsackie-adenovirus receptor (CAR) on the cell surface. This is then followed by the initiation of receptor-mediated endocytosis that occurs through the clathrin-coated pits. Endocytosis is activated by the interaction between the arginine-glycine-aspartic acid (RGD) motif found on the pentose base of the Ad virion and the host cell surface integrins (αvβ3 and αvβ5). After the host cell entry by Ads and prior to lysosome formation, the virion escapes into the cytosol from the early endosome. The virion is then disassembled during trafficking through the microtubule network towards the nucleus, where the virion then binds to the nuclear pore complex (NPC) allowing for the release of the viral genome (DNA) into the nucleus. Once the viral DNA is within the nucleus, viral transcription process is initiated.

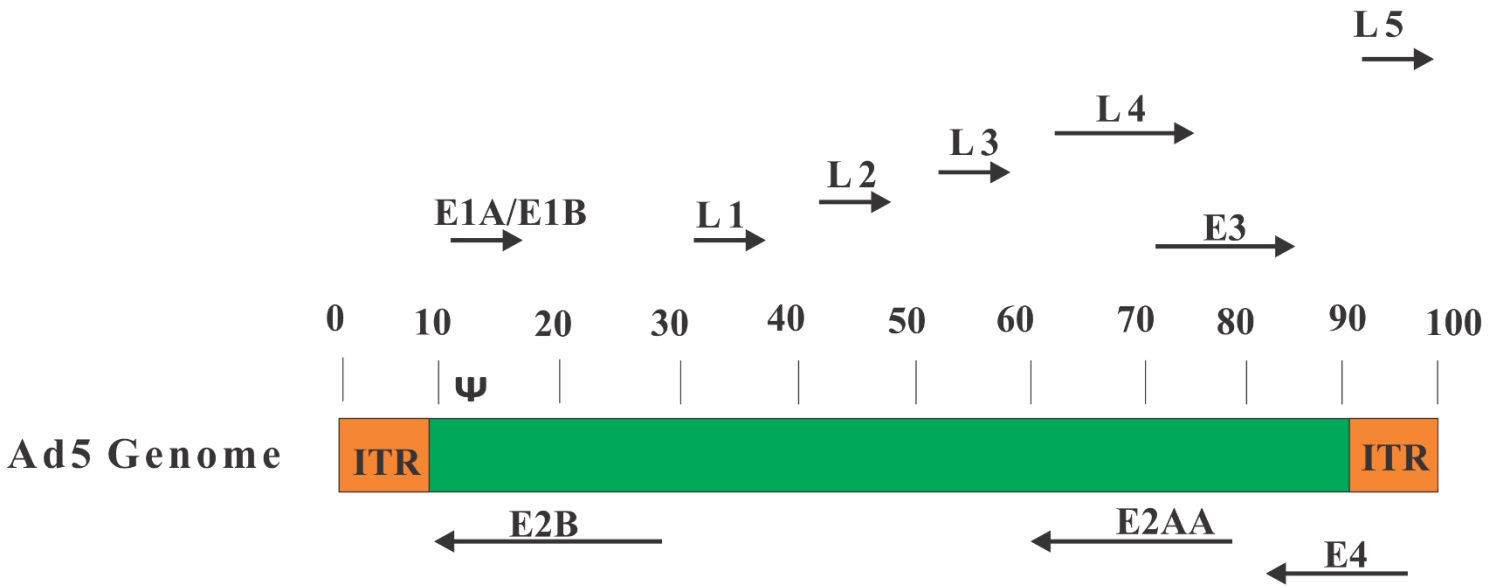


Figure 1.3: Map of Adenoviral genome 5 and transcription units.
Ad 5 genome is a linear double stranded DNA genome of 36 kb in size, with inverted terminal repeats (ITR) represented by the orange boxes on each end and the Ψ indicating the position of the packaging signal. The genome is divided into the early transcription unit (E1-E4), the late transcription unit (L1-L5) that is expressed from a major late promoter (MLP) and the delayed early transcription unit encoding proteins IX (pIX), pIVa, as well as VA RNA I and II. Adapted from (McConnell and Imperiale, 2004, Rosewell *et al*., 2011).

The viral DNA replication begins 6-8 hours post-infection, followed by the formation of progeny virion. The life cycle occurs within 24-36 hours and can produce 10^4 viral particles per infected cell [reviewed in (McConnell and Imperiale, 2004, Vetrini and Ng, 2010, Reynolds and Curiel, 2002)].

Prior studies showed that recombinant adenoviruses could be used to deliver anti-HBV sequences *in vivo*, but with a short duration of transgene expression. The use of these vectors has been limited by the immune stimulation that induces an innate and adaptive immune response (Crowther *et al*., 2008, Crowther *et al*., 2014, Zhao *et al*., 2008). However, progress has been made to increase the duration of the transgene expression and to make Ad safer and more efficient. This was achieved by the deletion of multiple adenovirus genes, which also allow the insertion of transgenes, resulting in three generations of Ad vectors: first generation, second generation and third generation Helper Dependent Adenoviral vectors (Ehrhardt and Kay, 2002).

1.3.1.3.1 First and second generation vectors

First generation adenoviral vectors (FGAd) have been constructed by the substitution of the E1 region of the Ad5 with a transgene (Figure 1.4). Hence, FGAd are E1 region (early region) deficient, which is required for the stimulation of the viral gene expression. The removal of this region results in a replication impaired vector with impaired early and late gene expression. E1 deficient Ad vectors propagation is performed in a HEK293 cell line (human embryonic kidney derived) that express E1 genes of Ad to provide the E1 function in *trans* (Amalfitano *et al*., 1998, Imperiale *et al*., 1984, Morral *et al*., 1997). Despite the removal of E1, expression of the viral genes is not completely blocked, resulting in the continuing expression of remaining viral genes. In turn, the viral gene expression results in the stimulation of the immune response (Holkers *et al*., 2014, Graham, 2000). Furthermore, another shortcoming associated with FGAd, is the short-term (3 weeks – 5 weeks) transgene expression after administration. Studies have suggested that the short-term transgene expression is due to the activation of the cellular immune response, specifically the $CD8^+$ T lymphocytes activation by viral proteins (Holkers *et al*., 2014, Morral *et al*., 1997).

These shortcomings have prompted the development and construction of second generation vectors by further deletion of genes E2 and E4. In addition, the E3 region can also be removed allowing up to 8.2 kb space for insertion of foreign DNA or gene (Figure 1.4). The E3 region can be removed due to the non-essential function that the E3 region has in viral production and growth during *in vitro* culturing (Amalfitano *et al*., 1998, Morral *et al*., 1997, Ilan *et al*., 1997). This produces less immunogenic vectors and increases the transgene capacity. However, viral gene expression still occurs and an immune response against vira1 proteins persists, which lead to the destruction or clearance of the transgene expressing vectors (Ehrhardt and Kay, 2002).

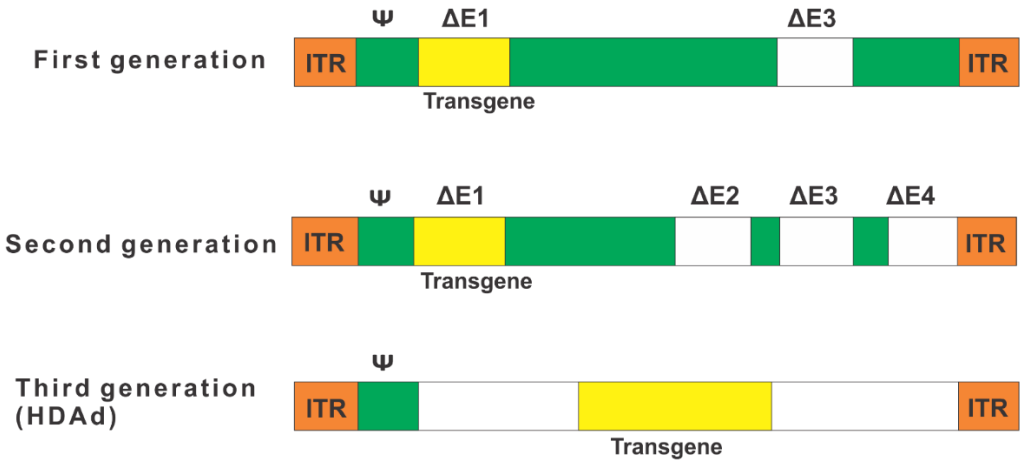


Figure 1.4: Genome structure of First-, Second- and Helper dependent Adenoviral vectors.
Adenoviral vector genome organization of First-, Second- and Third generation Helper Dependent Ad vectors (HDAd) with the inverted terminal repeats (ITR) represented by the orange boxes and the Ψ indicates the position of the packaging signal. The white boxes indicated the Ad genes that have been deleted to generate the different Ad vectors, the transgene (yellow box) has replaced the ΔE1 region in first- and second Ad vectors, HDAd vector yellow box indicates site of transgene insert. Adapted from (McConnell and Imperiale, 2004).

1.3.1.3.2 Helper-dependent Adenoviral vectors

To avoid the liver toxicity and the immune stimulation associated with the early generations of Adenoviral vectors, Helper Dependent Adenoviral vectors (HDAd) have been constructed. All the viral-coding genes are deleted in HDAds, thereby supporting a large cloning capacity that can accommodate transgene sequences of up to 37 kb.

Consequently, leaving only the two ITRs required for replication and the packaging signal (Figure 1.4) that is necessary for encapsidation of the viral genome (Brunetti-Pierri *et al*., 2006, Croyle *et al*., 2005, Ng *et al*., 2002). As a result of the removal of all the viral genes and the fact that expression of all the required adenoviral genes in a cell line is toxic, a helper virus (HV) is used to aid in the propagation of HDAds. The HV used should be able to express all the viral proteins required for the packaging and propagation of the HDAd genome. However, for a pure HDAd to be produced, the packaging of HV genome needs to be inhibited [reviewed in (McConnell and Imperiale, 2004, Rosewell *et al*., 2011)].

Previous techniques used a HV with a mutated packaging signal and an HDAd vector that is smaller in size compared to the HV. The size difference was used with the hope to minimize the HV contamination by separating the two viral particles based on size and density. However, the HDAd preparation remained contaminated with substantial levels of HV after the purification stage. The unresolved HV contamination issue was approached by the Cre/loxP technique that was engineered by Graham, Parks and co-workers (Parks *et al*., 1996, Parks and Graham, 1997). This strategy has shown to be most efficient and widely used for the large production of HDAd according to the production method developed by Palmer and Ng (Palmer and Ng, 2011).

1.3.1.3.2.1 Cre/loxP system for HDAd production and propagation

Cre/loxP system (Figure 1.5) makes use of HEK293 kidney derived cells that expresses Cre recombinase (Cre protein, 116 cells). These 116 cells are transfected with the linearized HDAd genome bearing plasmid and infected with a helper virus (HV) that is an FGAd that bears loxP sites flanking the packaging signal (AdNG163). In the presence of Cre recombinase the HV packaging signal is excised through site-specific recombination between the direct repeats of loxP, thereby rendering the HV genome unpackageable. This allows for selective replication and packaging of the HDAd genomes. However, the HV still retains its functional abilities to complement in *trans* for the adenoviral genes required for replication and packaging of the HDAd genome in *trans* (Ng *et al*., 2002, Parks *et al*., 1999, Parks *et al*., 1996, Parks and Graham, 1997).

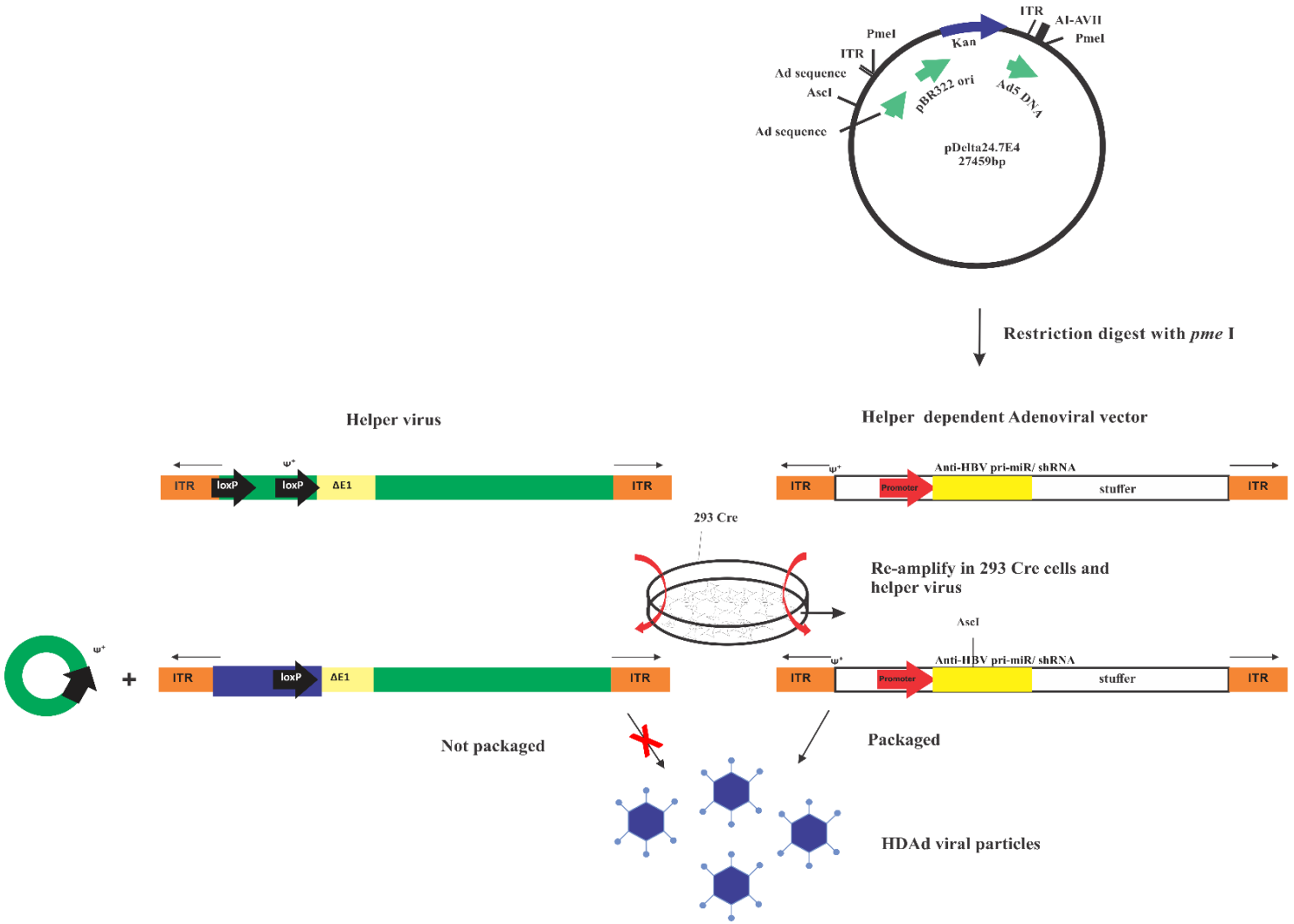


Figure 1.5: Cre/loxP Helper dependent viral production system.
HDAd genome is released through Pme I restriction digest from HDAd genome bearing plasmid. The HDAd genome is made up of cis-acting sequences, with ITRs and packaging signal (Ψ), the promoter driving the transgene expression, with the remainder stuffer DNA. The HDAd

genome is transfected into Cre expressing HEK293 cells. Cells are infected with helper virus with a packaging signal flanked by loxP sites. Cre expression renders the helper virus unpackagable, but still provides the factors necessary for HDAd propagation. Adapted from (Palmer and Ng, 2008b).

1.3.1.3.2.2 HDAd for liver directed gene therapy and HBV treatment

Adenoviruses have a tropism for a wide range of tissues, but are highly efficient hepatotropic vectors. Majority of *in vivo* liver directed studies have been performed with Ads as delivery vectors. Numerous studies have shown the potential use of Helper Dependent Adenoviral vectors for liver directed gene therapy in the absence of chronic toxicity with long-term transgene expression in both mice and non-human primates after systemic administration. Liver directed HDAd vectors were constructed that expresses the human α-1-antitrypsin (hAAT) gene that was administered to three baboons. Sustained transgene expression was obtained for more than one year with insignificant liver toxicity (Morral *et al*., 1999).

Further studies performed by Kim and colleagues (Kim *et al*., 2001) with Apolipoprotein E (apoE) HDAd vectors showed a stable, long-term and high transgene expression of apoE after a single intravenous administration. The high apoE expression resulted in complete long-term (2-2.5 years, mice died of natural causes) correction of hypercholesterolemia in apoE deficient mice (Kim *et al*., 2001).

HBVs ability to infect almost 100 % of liver hepatocytes, prompted the use of Ad vectors as a viable treatment for HBV. The durable transgene expression observed with using HDAd vectors, made these vectors appropriate for the treatment of chronic viral infections such as HBV (Mowa *et al*., 2012, Mowa *et al*., 2014, Ng *et al*., 2002). Numerous studies have shown the use of HDAd in liver directed gene therapy for HBV treatment both *in vitro* and *in vivo* (Crowther *et al*., 2008, Crowther *et al*., 2014, Mowa *et al*., 2012, Mowa *et al*., 2014, Ng *et al*., 2002). Different approaches for HBV treatment were investigated, one of these approaches focuses on immunotherapy treatments that are based on IFN-α antiviral ability to suppress *hepadnavirus* replication. Efficient reduction in the replication of Woodchuck Hepatitis virus (WHV) had been demonstrated using HDAd vectors (HD-AdwIFN) that express Woodchuck cytokine interferon (IFN) cytokines. As a result, the development of *hepadnavirus* infection into chronic infection and HCC could be

prevented (Fiedler *et al*., 2004). Aurisicchio and colleagues further investigated the utility of IFN expressing HDAd vectors in transgenic mice. HDAd vectors expressing murine specific IFN-α (mIFNa2) from a liver specific transthyretin promoter were constructed. These HDAd vectors resulted in an antiviral intrahepatic IFN-α expression, which produced a higher degree of liver protection in mice with acute hepatitis compared to FGAd (Aurisicchio *et al*., 2000). Both these studies present liver-restricted expression of IFN as being a potential gene therapy treatment for chronic hepatitis.

A different approach for HDAd based HBV treatment is the application of RNAi-based therapy. One of the first studies using HDAd vectors expressing shRNA from U6 promoter against HBV was performed. However, a modest level of HBV replication inhibition in the transgenic HBV mouse model was demonstrated (Rauschhuber *et al*., 2008). The inadequate level of HBV replication inhibition is speculated to be due to lack of potent silencing by the designed anti-HBV shRNA. Hence, the design of the anti-HBV sequences is of great importance to achieve potent and efficient HBV replication silencing. Another study performed addressed the use of the HDAd vectors expressing anti-HBV shRNA that target the *HBx* sequence. During this study the HDAd shRNA transduced 80-90 % of hepatocytes and generated the desired HBV targeted guides successfully. As a result, 95 % of HBV replication was inhibited with sustained HBV silencing of up to 8 weeks (Crowther *et al*., 2014).

However, to further improve the safety, sustainability and efficiency of HBV silencing, recombinant HDAds expressing previously designed artificial anti-HBV pri-miRs (Ely *et al*., 2009, Ely *et al*., 2008) were generated within the Antiviral Gene Therapy Research Unit. Mowa and colleagues performed one of the first experimental studies using this approach by constructing anti-HBV HDAds expressing pri-miR from a cytomegalovirus (CMV) promoter. These vectors transduced 90 % of hepatocytes and inhibited HBV replication efficiently *in vivo* without toxic side effects (Mowa *et al*., 2012). To advance this approach, further studies were conducted by Mowa and colleagues expressing both mono and trimeric anti-HBV pri-miRs in HDAds from a liver specific modified mouse transthyretin (MTTR) promoter. It was demonstrated that the expression of these anti-HBV pri-miRs by HDAd was more sustained (3 weeks for the monomeric pri-miR cassettes and after 5 weeks for the trimeric pri-miR cassettes) as compared to the CMV promoter driven pri-miRs and a significant inhibition of HBV replication *in vivo* was

achieved. Moreover, hepatotoxicity that have been found to occur with shRNA was not observed with antiviral pri-miR (Mowa *et al*., 2014). The results obtained showed the therapeutic potential and effective utility of HDAd for HBV gene silencing and hepatotropic delivery of antiviral sequences. Nevertheless, one of the main obstacles that prevent HDAd as clinical application for liver-directed gene therapy is the triggering of the host innate inflammatory response against the capsid proteins and reporter genes that are usually carried by the vectors. However, efforts have been made in elucidating the factors triggering the host response and various strategies have been investigated to improve the therapeutic potential of HDAd (Brunetti-Pierri and Ng, 2011).

1.3.1.4 Suppression of Ads immunostimulatory effects

Despite the progress that has been made by manipulating the viral genome of the vectors, Ad vectors still have to overcome the host innate response that limits the efficient delivery and safe application of these vectors *in vivo.* This is as a result to the immune response that causes acute toxicity, as well as influence and limit transgene delivery and expression (Crowther *et al*., 2008, Crowther *et al*., 2014, Croyle *et al*., 2005).

The host immune response caused by Ad vectors occurs in phases. Phase one, also known as acute phase follows 1 hour to 4 days post administration. The acute phase is associated with the activation of Kupffer cells present in the liver, as well as macrophages and dendritic cells found within the spleen and peritoneum. These cells produce and release numerous cytokines (IL-1, IL-6, TNF-α) and chemokines (MCP-1, MIP-1, IP-10) into circulation. The released chemokines and cytokines sets an acute innate inflammatory response and toxicity in progress. Phase two occurs 5-7 days post administration and targets Ad viral particle transduced cells. This entails the activation of major histocompatibility complex (MHC) class I and II that is specific for Adenovirus and results in the production of anti-adenovirus neutralizing antibodies. MHC class I $CD8^+$ T cells are directed towards the transgene products and cells expressing the viral genes. MHC class II $CD4^+$ T cells are activated by the viral particles capsid proteins. As a result, rapid Ad viral clearance from the blood circulation occurs, influencing the hepatocyte transduction efficiency and preventing transgene expression (Lieber *et al*., 1997, Muruve *et al*., 1999, Schiedner *et al*., 2003, Zhang *et al*., 2001). Phase two is followed by the chronic host immune response (late phase) that continues for several weeks to months post

administration. During this time all transgene products and transduced cells are eliminated by cytotoxic T cells (Croyle *et al*., 2005, Yang *et al*., 1996, Yang *et al*., 1995).

First generation adenoviral (FGAd) vectors have fallen short as an ideal gene delivery vectors due to the activation of the innate immune response and toxicity following administration. Consequently, HDAd vectors were generated to minimize the immune response and toxicity that have been associated with FGAd vectors by the removal of the ORFs (all viral genes), this reduces long-term humoral and cell-mediate adaptive immunity (Crowther *et al*., 2008, Crowther *et al*., 2014, McCaffrey *et al*., 2008, Muruve *et al*., 2004). However, the formation of Ad specific neutralising antibodies generated against the Adenoviral capsid proteins limits transgene expression and prevents the re-administration of the same Ad serotype (O'Riordan *et al*., 1999, Yang *et al*., 1995). Given the initiation of the host response by the capsid proteins, methods have been developed to diminish the host innate response by modifying the capsid proteins of Ad vectors using polymers such as polyethylene glycol (PEG), PEG derivatives and poly-N-(2-hydroxypropyl) methacrylamide (Croyle *et al*., 2001, Kreppel *et al*., 2005, Mok *et al*., 2005, Eto *et al*., 2004, Prill *et al*., 2011, Croyle *et al*., 2005).

1.3.1.4.1 PEGylation of delivery vectors

PEGylation has been introduced by pharmaceutical companies since the 1970s to protect a variety of therapeutic proteins and transgenes. This method decreases the non-specific uptake by macrophages while maintaining the uptake of Ad vectors into target cells *in vivo* (Mok *et al*., 2005, Croyle *et al*., 2001, Kreppel *et al*., 2005). PEG is an uncharged, non-immunogenic and hydrophillic molecule with low toxicity and reduces the protein-protein interaction that occurs between proteins, therapeutics and cells. The chemical modification is achieved through the covalent attachment between Adenovirus vector and PEG that forms a hydrophillic coat. Attachment of PEG to lysine's amine residues that are present on the Ad capsid has been the most commonly used method of PEGylation. Previous studies have shown that PEG protects the viral particles against neutralizing antibodies. This increased viral particle circulation time, decreased the blood clearance of PEG-FGAd and -HDAd vectors by 2-5 fold and demonstrated a reduction in cytotoxicity and helper T lymphocyte innate immune response (Croyle *et al*., 2005, Eto *et al*., 2008, Kreppel and Kochanek, 2008, Hofherr *et al*., 2008, Mok *et al*., 2005, Croyle *et al*., 2000). These

promising results prompted the use of PEGylated Ad vectors for HBV treatment. During recent studies performed by Crowther and colleagues, FGAd and HDAd vector surface chemical modification was performed using amine reactive PEG. The anti-HBV FGAd and -HDAd PEGylated vectors were shown to improve hepatocyte transduction and silencing of HBV replication in HBV transgenic mice (Crowther *et al*., 2008, Crowther *et al*., 2014).

However, evidence have also shown that using amine reactive PEG is non-specific and can interfere with the vector particles intracellular trafficking to targeted tissue (Croyle *et al*., 2005, Hofherr *et al*., 2008). Hence, a more effective approach was investigated by applying genetic and chemical capsid modification. The genetic technique is used to introduce a specific genetic mutation by the substitution or addition of desired amino acids in the capsid proteins. This introduced a specific chemical reactivity with a specific PEG at a desired site on the viral capsids. The use of site-specific PEGylation has found to reduce the attachment of PEG to undesired protein surface, reduce biological activity loss, as well as minimized the amount of PEG conjugates required for masking the desired capsid epitopes (Kreppel *et al*., 2005, Prill *et al*., 2011). Prill and colleagues applied this method to improve the delivery of Adenoviral vectors to the liver by using thiol reactive PEG, which reacts with thiol groups in cysteine residues that was introduced into the hypervariable region 5 (HVR 5) of the hexon protein (Figure 1.6). This was done by generating a helper virus (E1 deficient Ad vector); AdNG163Cys with same ratio of Amide (A) to Cysteine (C) mutations into the hexons hypervariable region 5 (HVR 5) of AdNG163 helper virus. This approach showed an increase in hepatocyte transduction, higher particle uniformity by allowing precise shielding at the desired sites, and a reduction in the amount of polymers required. Furthermore, this strategy also showed that the introduction of a specific point mutation at a single site of a hexon protein can alter hepatocyte tropism by either reducing or increasing hepatocyte transduction depending on the chemical moieties that covalently binds to the genetically modified hexon (Prill *et al*., 2011). A similar approach was applied whereby peptides with cysteine residues were genetically introduced into a solvent-exposed fiber protein H1-loop of Ad5 E1 deleted Adenovirus vector. This genetic introduction enable the detargeting and retargeting of Adenoviral vectors to specific tissue sites (Kreppel *et al*., 2005).

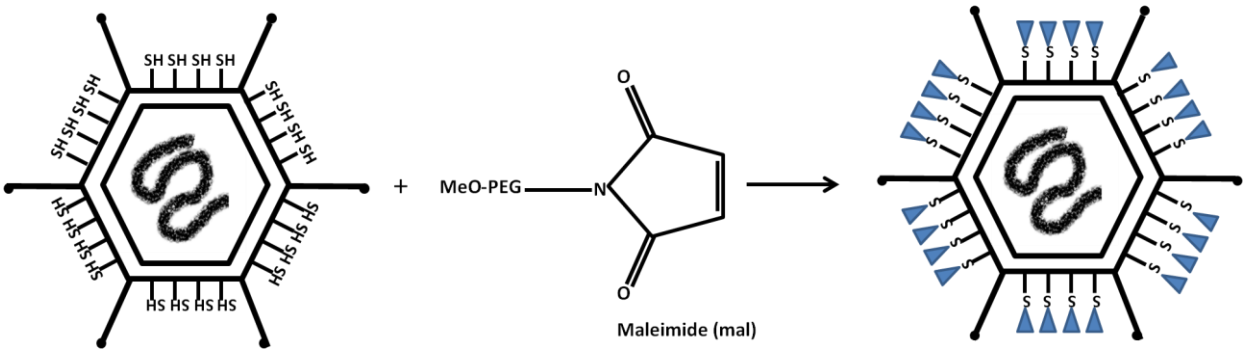


Figure 1.6: Chemical modification of adenovirus capsid proteins.
Chemical modification of the genetically introduced cysteine residues in the HVR5 of hexon, with thiol-reactive maleimide-activated PEG molecules. , indicates the reaction between maleimide and thiol groups. Adapted from (Prill *et al*., 2011, Kreppel and Kochanek, 2008).

1.3.1.4.2 Immune stimulation by transgene products

In addition to the capsid specific immune stimulation, other studies reported that reporter gene products can activate an immune response. *Lac* Z is a reporter gene that encodes *E. coli* β-galactosidase and has mainly been used for the visualization and detection of cells infected with vectors carrying the desired transgenes (Mian *et al*., 2005, Sullivan *et al*., 1997). The presence of β-galactosidase in cells can be recognized by the catalyses of X-gal (5-bromo-4-chloro-3-indoyl-b-D-galactopyranoside) substrate, to produce a blue colour (Juers *et al*., 2012). However, despite the great use of *lac* Z as a model gene marker in both animal and human pre-clinical studies, an induction of a host immune response by β-galactosidase has been observed. A loss in viral gene expression is observed as a result of the stimulation of the adaptive immune response and/or cellular immune response that is characterized by $CD4^+$ and $CD8^+$ cytotoxic T lymphocytes which results in the destruction of transduced cells (Mian *et al*., 2005, Morral *et al*., 1997). Studies performed indicated the induction of the inflammatory response ($CD4^+$ and $CD8^+$ cell) by the expression of β-galactosidase in non-transgenic mice (Chen *et al*., 1997). Further studies performed by Sullivan and colleagues showed the activation of an immune response that induced antigen T-cell proliferation and neutralising antibody production against the β-galactosidase expressed in Adenoviral vectors (Sullivan *et al*., 1997). Experimental studies performed within AGTRU used the *lac Z* reporter gene to enable visualization of cells infected with shRNA or pri-miR expressing HDAd vectors respectively. An activation of the adaptive

immune response of the host was observed through the increased antibody production against β-galactosidase using ELISA and ELISpot assays (Crowther *et al*., 2014, Mowa *et al*., 2014). Although the use of a reporter gene is convenient for the detection of *in vivo* hepatocyte infection, viral vector clearance and short-term HBV gene silencing was observed.

Hence, this study focused on liver specific expression and HBV silencing by using anti-HBV HDAd vectors lacking *lac* Z, making them more efficient and more relevant for chronic HBV infection treatment. The elimination of the *lac Z* cassette of the vectors may rid the system of short-term HBV replication inhibition problem. On the other hand, incorporation of the PEPCK promoter in anti-HBV HDAds lacking the *lac* Z gene may aid in prolonged HBV gene silencing.

1.4 Aim and objectives

This study focuses on finding efficient, tissue specific expression and a safer delivery system for anti-HBV RNAi activators. The specific aim of this study therefore was to produce recombinant HDAd that are *lac* Z deficient and express anti-HBV pri-miR sequences from a PEPCK liver-specific promoter. To achieve this aim, the following objectives were set:

Objectives:

1. Construction of recombinant Helper Dependent Adenoviral vector (HDAd) backbones expressing anti-HBV sequences from a liver-specific PEPCK promoter (pPEPCKHBVHDAds).
2. Production and propagation of anti-HBV recombinant Helper Dependent Adenoviral vectors (HBVHDAds) using a helper virus with or without cysteine modification in the Hypervariable region.
3. Validation of PEPCK promoter liver specific activity by producing HDAd expressing *Firefly luciferase* and characterization *in vitro* and *in vivo*.
4. Assessment of *in vitro* and *in vivo* expression and processing of anti-HBV sequences using Northern blotting.
5. Assessment of silencing of HBV gene expression in cell culture and mice using Dual Luciferase reporter assay and/or ELISA.
6. Determination of immune stimulation by anti-HBV Helper Dependent Adenoviral vectors using cytometric bead array.
7. Measurement of liver enzymes levels as markers of liver toxicity using kinetic assay on an automated photomeric analyser.

CHAPTER 2

2 MATERIAL AND METHODS

2.1 Bacterial culturing and manipulations

2.1.1 Culturing of *Escherichia coli* strains

XL1-Blue and DH5-α *E. coli* strains were cultured in Luria Bertani medium (LB, Appendix 6.1) or grown on Luria Bertani agar plates (LA, Appendix 6.1) at 37℃ overnight. The XL1-Blue strains carrying ampicillin resistance marker were cultured in LB or LA supplemented with 100 µg/ml ampicillin (Appendix 6.1). The DH5α strains that are kanamycin resistant were cultured in LB or LA supplemented with 50 µg/ml kanamycin (Appendix 6.1). Blue white screening was performed by evenly spreading 40 µl of 20 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Appendix 6.1) on LA plates containing the relevant antibiotic. All liquid bacterial cultures were shaken at 150 - 250 rpm.

2.1.2 Preparation of chemically competent XL1-Blue *E. coli* cells

A single colony or 5 µl of freezer stock XL1-Blue cells were inoculated in 10 ml LB. The cultures were incubated overnight at 37℃ shaking at 150-250 rpm. The pre-cultures were diluted 100 × in 100 ml of LB medium and incubated at 37℃ shaking at 150 - 250 rpm until the absorbance reading (optical density, OD) measured 0.4 - 0.6 at 600 nm. The cultures were then centrifuged at 2500 rpm for 20 min at 4℃. The pellets were resuspended in 20 ml of transformation buffer (Appendix 6.1) and incubated on ice for 20 - 30 minutes. The cells were centrifuged at 2500 rpm for 20 minutes at 4℃ and resuspended in 1 ml of transformation buffer (Appendix 6.1). Aliquots were made by transferring 100 µl of competent cells in to sterile microcentrifuge tubes and stored at -80℃.

2.1.3 Transformation of *E. coli* competent cells

2.1.3.1 Transformation of chemically competent XL1-Blue *E. coli* cells by heat shock

Pre-chilled DNA was added to 100 µl of competent cells. The mixture was incubated on ice for 10 minutes followed by heat shock at 42℃ for 90 seconds using a heating block. Immediately after heat shock, 500 µl of LB pre-warmed at 37℃ was added to the cells and incubated for an hour at 37℃ to allow selection marker expression. The whole transformation mixture was then plated onto LA media containing X-gal and/or the relevant antibiotic and incubated overnight at 37℃.

2.1.3.2 Electroporation of DH5-Alpha *E. coli* cells

DNA was added to 50 µl of commercially obtained DH5-Alpha electro-competent cells (New England Biolabs, Inqaba Biotec, Gauteng, South Africa) and incubated on ice for 10 minutes. After incubation, the mixtures were transferred to a pre-chilled 2 mm electroporation cuvettes. Cells were then electroporated using the Biorad Gene Pulsar Xcell^{TM} Electroporation system under the following conditions: 2000 V (voltage), 25 µF (capacitance) and 200 Ω (resistance). Immediately after electroporation, 1 ml of rescue C-medium (Thermo Fisher Scientific, MA, USA) pre-warmed at 37℃ was added to the cells. Cells were then transferred into a sterile microcentrifuge tube and incubated at 37℃ shaking at 150 - 250 rpm for 1 hour for phenotypic expression. After incubation, the transformed cells were plated onto LA plates supplemented with desired antibiotic and incubated overnight at 37℃.

2.2 Isolation and purification of DNA

2.2.1 Small Scale plasmid DNA isolation by alkaline lysis (mini prep)

A single colony was inoculated into 1 ml of LB supplemented with the relevant antibiotic and incubated overnight in a 37℃ incubator shaking at 150 - 250 rpm. The bacterial cultures were transferred to 1 ml microcentrifuge tubes. The cultures were centrifuged for

1 min at maximum speed at room temperature. The pellets were resuspended in 180 µl of Buffer P1 with RNase A (resuspension buffer, Appendix 6.2). The cells were then lysed by adding 160 µl of Buffer P2 (lysis buffer, Appendix 6.2). The solution was mixed thoroughly by inverting and incubated at room temperature for 5 minutes. After incubation, 120 µl of Buffer P3 (neutralization buffer, Appendix 6.2) was added to the solution and mixed thoroughly by flicking. The solution was incubated on ice for 5 minutes. The bacterial lysate was then centrifuged at maximum speed for 15 minutes at 4℃. The supernatant was transferred to a new sterile tube to which 600 µl of warm 100 % isopropanol was added to precipitate the plasmid DNA. The mixture was mixed by inverting and incubated at room temperature for 2 minutes, followed by centrifugation at maximum speed for 25 minutes at room temperature. The supernatant was discarded and the pellet was washed with 150 µl of chilled 70 % ethanol through centrifugation at maximum speed for 5 minutes at 4℃. The supernatant was carefully removed as completely as possible and the pellet was left to air-dry for 10 minutes. The DNA was re-dissolved in 50 µl of distilled water and incubated at room temperature for 30 minutes. The DNA concentration was determined using Nanodrop® Spectrophotometer (Thermo Fisher Scientific, MA, USA).

2.2.2 Large scale plasmid DNA isolation (maxi prep)

Plasmid DNA required in large quantities were isolated using QIAGEN Plasmid Maxi Kit (Qiagen, MD, USA) or Genopure Plasmid Maxi Kit (Roche Holding AG, South Africa) according to the manufacturer's instructions with minor changes. Briefly, a single colony was inoculated in 100 - 200 ml of LB medium supplemented with the appropriate antibiotic and incubated overnight at 37℃, shaking at 150 rpm. The bacterial cells were harvested by centrifugation at 4000 rpm for 30 minutes at 4℃. The pellets were resuspended in 12 - 20 ml of chilled resuspension buffer with RNase A. To lyse the cells, 12 - 20 ml of lysis buffer was subsequently added and mixed by inverting the tube 6 - 8 times. The mixture was incubated at room temperature for 5 minutes. To neutralize the lysis buffer, 12 - 20 ml of chilled neutralising buffer was added and the samples were mixed by inverting 6 - 8 times and incubated on ice for 5 - 20 minutes. The lysates were cleared by centrifugation at 4000 rpm for 1 hour at 4℃. During centrifugation step, the columns were mounted onto a collection tube and equilibrated by applying 12 - 20 ml of equilibration buffer and allowed to empty by gravity flow. Each column had a filter paper

to which the cleared lysate was loaded and allowed to flow through and enter the resin by gravity flow, followed by washing twice with 16 - 30 ml of wash buffer. The columns were transferred to new collection tubes and the plasmid DNA was eluted with 15 ml of Elution buffer. The DNA was precipitated by adding 10.5 - 11 ml of room temperature isopropanol. The precipitated solution was mixed and incubated at -20℃ for 30 minutes. The plasmid DNA was collected by centrifugation at 4000 rpm for 45 minutes at 4℃. The supernatant was carefully discarded, the pellet was washed with 4 - 5 ml of chilled 70 % ethanol and centrifuged at 4000 rpm for 10 minutes at 4℃. The ethanol was carefully discarded and the pellets were air-dried for 10 minutes. The plasmid DNA pellet was re-dissolved in 500 µl of sterile distilled water (dH_2O). DNA concentration was determined by using the Nanodrop® Spectrophotometer (Thermo Fisher Scientific, MA, USA).

2.2.3 Viral DNA isolation and purification

Viral DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, MD, USA) according to the manufacturer's instructions. All centrifugation steps were carried out at room temperature. For tissue lysis, 200 µl of homogenates and tissue lysis buffer (ATL) were added into a 1.5 ml micro-centrifuge tube containing 20 µl of Proteinase K and incubated at 56℃ until tissue was completely lysed (1 - 3 hours). For viral lysis a total of 200 µl of sample was added to the tube containing 200 µl of buffer AL and mixed my vortexing for 15 seconds. Samples were incubated at 56℃ or 70℃ for 10 minutes, followed by a brief centrifugation. Absolute ethanol (200 µl) was added to the samples, mixed by vortexing for 15 seconds and briefly centrifuged. The samples were then applied to the QIAamp mini spin columns and centrifuged at 8000 rpm for 1 minute. The spin columns were transferred to new 2 ml collection tubes, 500 µl of Buffer AW1 was added and centrifuged at 8000 rpm for 1 minute. The columns were transferred to new 2 ml collection tubes, 500 µl of Buffer AW2 was added and centrifuged at maximum speed for 3 minutes. The columns were again transferred into new collection tubes and centrifuged at maximum speed for 1 minute and then transferred into a new 1.5 ml micro centrifuge tubes. To elute the DNA, 50 - 200 µl of Buffer AE was added and incubated at room temperature for 1 min. The columns were then centrifuged at 8000 rpm's for 1 minute. For tissue samples the final elution and centrifugation step was repeated.

2.2.4 DNA analysis and purification by agarose gel electrophoresis

A 1 % agarose gel was prepared by adding 1 g of powdered agarose to 100 ml 1× TAE (Tris-Acetate-EDTA, Appendix 6.2) running buffer and mixed by swirling. The mixture was melted in the microwave for 3 minutes. Distilled water was used to adjust the volume to 100 ml after heating to compensate for the evaporated water. The agarose was left to cool and 10 µl of 10 mg/ml Ethidium bromide (Sigma-Aldrich, MO, USA) was added to the melted agarose to enable visualisation of DNA. The gel was poured into the casting tray and allowed to solidify at room temperature. Orange DNA loading dye (1×, Thermo Fisher Scientific, MA, USA) was added to the samples and loaded into the gel wells. The O' GeneRuler DNA Ladder Mix (Thermo Fisher Scientific, MA, USA) was added to a well alongside the samples to determine the relative size of the fragments. The samples were separated through electrophoresis at 100 V. The agarose gel was visualised under UV light and pictures were taken using the Syngene G:BOX Gel Documentation and Analysis system (Syngene, UK).

To purify DNA from the gel, a DNA fragment of interest was excised from the gel using a sharp, clean scalpel. The extraction of the DNA was further completed using QIAquick Gel Extraction Kit (Quigen, MD, USA) according to manufacturer's instructions. All centrifugation steps were performed at room temperature. The excised gel piece was transferred into a 2 ml eppendorf tube and weighed. Three volumes of buffer QG was added to 1 volume of gel (0.1g ~ 100 µl) and incubated at 50℃, vortexing the tube every 2 - 3 minutes. Once the gel slice was entirely dissolved, one volume of isopropanol (0.1g ~ 100 µl) was added to the sample and mixed by inverting. The QIAquick spin column was placed into the provided 2 ml collection tube. The sample was applied to the column to bind the DNA and centrifuged for 1 minute at maximum speed. The flow through was discarded and column was placed back into the collection tube. The binding of DNA was continued until all the sample had been applied to the spin column. To remove all traces of agarose, 500 µl of Buffer QG was applied to the spin column and centrifuged for 1 minute. Flow through was discarded. To wash, 750 µl of Buffer PE was added to the spin column and centrifuged for 1 minute. The residual ethanol from Buffer PE was removed by discarding the flow through and centrifuging the QIAquick spin column for 1 minute at

maximum speed and transferred into a clean 1.5 ml eppendorf tube. The DNA was eluted by adding 30 µl of sterile distilled water (dH_2O) and incubating at room temperature for 1 minute. The column was then centrifuged at maximum speed for 1 minute. To ensure maximum elution of DNA, the eluted sample was added back to the column and centrifuged at maximum speed for 1 minute. The DNA concentration was determined using the Nanodrop® Spectrophotometer.

2.3. DNA manipulations

2.3.1 Restriction digestion of plasmid DNA

The restriction digest reactions were carried out according to the manufacturer's instructions in recommended restriction buffer (Thermo Fisher Scientific, MA, USA or New England Biolabs, MA, USA). Briefly, 1-11 µg of DNA, 1-11 U of enzyme and 1× reaction buffer was added into a 1.5 ml Eppendorf tube and distilled water was used to scale up the reaction to the required volume of 10 - 50 µl. The restriction digests were performed at 37℃ for 2 hours to overnight.

2.3.2 Plasmid DNA dephosphorylation

Plasmid dephosphorylation was performed to prevent the religation of the linearised plasmid by removing the 5' phosphate groups at the ends of the plasmid. A total of 1 - 5 U Antarctic Phosphatase (New England Biolabs, MA, USA) was added to 1 - 5 µg of linearised DNA in a 1× restriction buffer. The reactions were incubated at 37℃ for 1 hour and heat inactivated at 65℃ for 5 minutes.

2.3.3 Polymerase Chain Reaction (PCR)

KAPA HiFi HotStart ReadyMix kit (KAPA Biosystems, Cape Town, South Africa), KAPA Taq Polymerase ReadyMix PCR kit (KAPA Biosystems, Cape Town, South Africa) or Thermo Scientific High Fidelity PCR kit (Thermo Scientific, MA, USA) were used for DNA amplifications according to manufacturer's instructions. Each KAPA reaction contained 5-10 ng of template DNA, 0.2 - 0.4 µM of forward and reverse primer, 1× KAPA HiFi HotStart ReadyMix (containing 2.5 mM Mg^{3+}) or 1× KAPA Taq

ReadyMix (containing 1.5 mM $MgCl_2$) and PCR grade (nuclease free) water to a final volume of 25 - 50 µl. For colony PCR, a single colony was suspended in 10 µl of dH_2O, and 1 µl per reaction used as the template. The reaction for Thermo Scientific High Fidelity PCR kit was set up with 1× High Fidelity PCR buffer with 15 mM $MgCl_2$, 0.2 mM dNTP mix, 1 µM forward and reverse primer, 5 ng of template DNA, 12.5 - 25 µl 2× High Fidelity PCR enzyme mix and nuclease free water up to a final volume of 50 µl. Primers used are shown in Table 2.1 and PCR cycling conditions were as outlined in Table 2.2.

Table 2.1: Oligonucleotides (primers) used for amplification during PCR and qPCR

Primer Name	Primer sequence	Description	Source/ Reference
*Eco*RVF	5'GATC**GATATC**C AGGTGTCCACTCC CAGTTC3'	Forward primer with engineered *Eco*RV sites used for *Firefly luciferase* sequence amplification	Unpublished
*Eco*RVR	5'GATC**GATATC**C CTCACTAAAGGG AAGCGGC3'	Reverse primer with engineered *Eco*RV sites used for *Firefly luciferase* sequence amplification	
Helper Virus F (HVF)	5'TGGGCGTGGTG CCTAAAA'3	Forward primer specific for HV amplification	(Palmer and Ng, 2003)
Helper Virus R (HVR)	5'GCCTGCCCCTGG CAAT'3	Reverse primer specific for HV amplification	
HDAdF	5'GAAAAAACACA CTGGCTTGAAACA '3	Forward primers specific for HDAd amplification	(Palmer and Ng, 2003)
HDAdR	5'TGCCACCTCGTA TTTCACCTCTA'3	Reverse primer specific for HDAd amplification	

The *Eco* RV restriction sites indicated in blue.

Table 2.2: PCR cycling conditions

Step	Temperature	Time	Number of cycles
Initial denaturation	95℃	2 min	1
Denaturation	98℃	30 sec	×34
Primer annealing	60℃	30 sec	
Extension	72℃	30 sec – 2 min	
Final extension	72℃	5 min – 10 min	1
Cooling	4℃	∞	1

Quantitative PCR was performed with FastStart Essential Green Master mix (Roche Holding AG, South Africa) according to manufacturer's instructions. Each reaction was set up with 1× SYBR Green Master mix, 1 µM forward and reverse primer, 1 - 100 ng of template and nuclease free water up to a final volume of 20 µl. Primers used are shown on Table 2.1. PCR cycling conditions were set up as mentioned in Table 2.3.

Table 2.3: qPCR cycling conditions

Step	Temperature	Time	Number of cycles
Initial denaturation (hotstart)	95℃	10 min	1
Denaturation	95℃	15 sec	×40
Primer annealing	60℃	15 sec	
Extension	72℃	15 sec	
Melt curve	65℃ - 95℃	5 sec - 50 sec	1
Cooling	4℃	∞	1

2.3.5 DNA ligation

The ligation was performed with InsTAclone PCR Cloning Kit (Thermo Fisher Scientific, MA, USA), T4 DNA ligase kit (Thermo Scientific, MA, USA) or Fast-Linked DNA Ligation Kit (Epicentre Biotechnologies, WI, USA) according to manufacturer's instructions. Each reaction contained 1× ligation buffer, 1µl T4 DNA ligase or 1 µl Fast-Link DNA Ligase, 50 - 55 ng of plasmid and different ratios of insert to plasmid. The

reactions were made up to a final volume of 15 - 30 µl with nuclease free water. For Fast-Linked DNA Ligations, ATP was included to a final concentration of 0.1 - 0.2 mM in the reactions. The ligation reactions were incubated overnight at 4℃. Following the incubation, the ligation reactions were heat inactivated at 70℃ for 15 minutes. The entire mixtures were briefly spun down and transformed into suitable *E. coli* cells.

2.3 Construction of HDAd plasmid backbones with the PEPCK promoter expression cassette

The cloning was performed as illustrated in Figure 2.1 using standard procedures described above. To generate an intermediary plasmid expressing *Firefly Luciferase* from PEPCK promoter, a PCR based technique was used. The *Firefly Luciferase* sequence was PCR amplified from previously described plasmid pCI-neoCMV*Fluc* [(Passman *et al*., 2000), Table 2.4] using primer *Eco*RVF and *Eco*RVR (Table 2.1). The amplicon with the *Eco* RV engineered sites was then cloned into a PCR cloning vector, pTZ57R/T (Thermo Fisher Scientific, MA, USA) to generate pTZ*Fluc*. Sequencing of the positive clone to assess for PCR amplification errors was performed by Inqaba biotec- Africa's Genomics Company (Inqaba Biotec, Gauteng, South Africa). A plasmid bearing PEPCK promoter (pLPBL-pepck-wl-*Swa*-I-*Spe* I), was kindly donated by Phillip Ng, Houston Baylor College of Medicine. The *Firefly Luciferase* sequence was digested with *Eco* RV, purified and ligated downstream of PEPCK promoter into the *Swa* I site of the pLPBL-pepck-wl-*Swa*-I-*Spe* I plasmid (pLPBL), to construct pLPBL*Fluc*. The PEPCK promoter cassette was extracted by digestion with *Asc* I and sub-cloned into the HDAd genome bearing plasmid, pΔ24.7E4 (Table 2.4, kindly donated by Phillip Ng, Houston Baylor College of Medicine) to generate pPEPCK*Fluc*HDAd.

The anti-HBV sequences pri-miRNA 31/5 and pri-miRNA 31/589 sequences have previously been cloned into the *Swa* I site of pLPBL plasmid downstream of PEPCK promoter to construct pLPBL31/5 and pLPBL31/589 respectively (unpublished). The PEPCK promoter cassettes were isolated from pLPBL31/5 and pLPBL31/589 through restriction digests with *Asc* I. The fragments were excised and sub-cloned through ligation into the pΔ24.7E4 (kindly donated by Phillip Ng, Houston Baylor College of Medicine) *Asc* I site to generate pPEPCK31/5HDAd or pPEPCK31/589HDAd.

Table 2.4: Plasmids used and constructed during this study

Plasmid	Description	Reference/Source
pCI-neoCMV*Fluc*	Mammalian expression vector (pCI-neo) carrying the *Firefly luciferase* gene	(Passman *et al*., 2000)
pTZ57R/T	TA cloning vector	Thermo Fisher Scientific, MA, USA
pTZ*Fluc*	pTZ vector carrying the carrying the *Firefly luciferase* gene	This study
pLPBL-pepck-wl-*Swa*-I-*Spe* I (pLPBL)	Plasmid carrying a PEPCK promoter	Kindly donated by Phillip Ng
pLPBL*Fluc*	pLPBL-pepck-wl-*Swa*-I-*Spe* I plasmid expressing *Firefly luciferase* gene from a PEPCK promoter	This study
pLPBL31/5	pLPBL plasmid expressing monocistronic anti-HBV pri-miR31/5 sequence from a PEPCK promoter	This study
pLPBL31/589	pLPBL plasmid expressing polycistronic anti-HBV pri-miR31/589 sequence from a PEPCK promoter	This study
pΔ24.7E4	A plasmid carrying the recombinant Helper Dependent Adenoviral vector (HDAd) genome	Kindly donated by Phillip Ng
pPEPCK*Fluc*HDAd	pΔ24.7E4 expressing *Firefly luciferase* gene from a PEPCK promoter	This study
pPEPCK31/5HDAd	pΔ24.7E4 expressing monocistronic anti-HBV pri-miR31/5 sequences from a PEPCK promoter	This study
pPEPCK31/589HDAd	pΔ24.7E4 expressing polycistronic anti-HBV pri-miR31/589 sequences from a PEPCK promoter	This study
pΔ28E4CMV*lac*Z	Helper Dependent Adenoviral vector genome bearing plasmid expressing *lac*Z reporter gene from a CMV promoter	(Palmer and Ng, 2008a)
pNG150D	Plasmid containing segments of both Helper virus and Adenoviral genome sequences	Kindly donated by Phillip Ng
pCH-9/3091	HBV replication-competent plasmid that contains a greater than genome length HBV sequence	(Nassal, 1992)
psiCHECK-*HBx*	Reporter plasmid with *HBx* sequence downstream of the Renilla luciferase ORF	(Weinberg *et al*., 2007)
pCI-neo eGFP	pCI-neo plasmids expressing enhanced green fluorescence protein (eGFP)	(Passman *et al*., 2000)

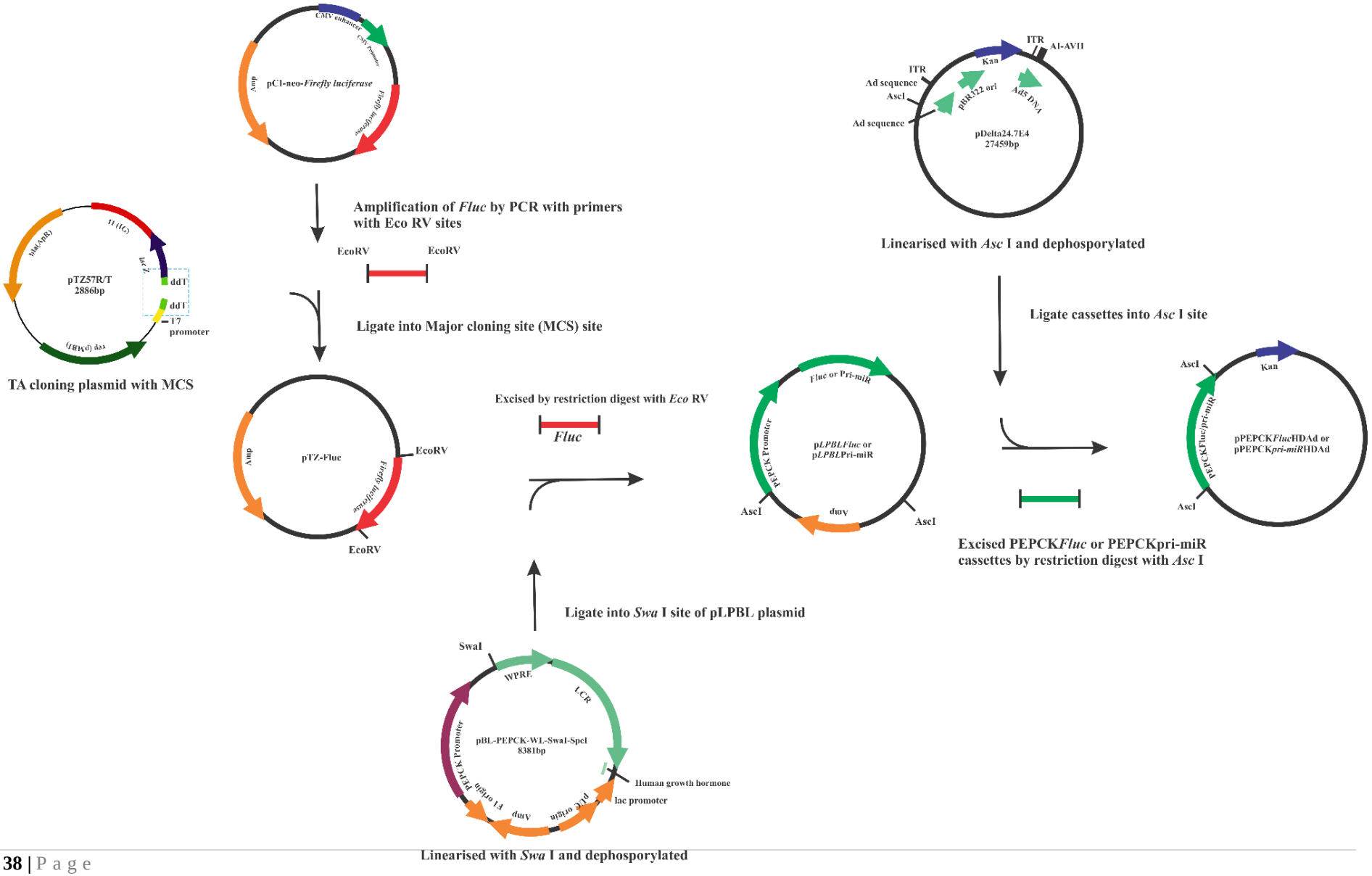

pC1-neo-Firefly luciferase
Amplification of Fluc by PCR with primers with Eco RV sites
EcoRV
EcoRV
Ligate into Major cloning site (MCS) site
pTZ57R/T
2886bp
TA cloning plasmid with MCS
pTZ-Fluc
Excised by restriction digest with Eco RV
Fluc
pLPBLFluc or pLPBLPri-miR
Linearised with Asc I and dephosporylated
pDelta24.7E4
27459bp
Ligate cassettes into Asc I site
pPEPCKFlucHDAd or pPEPCKpri-miRHDAd
Excised PEPCKFluc or PEPCKpri-miR cassettes by restriction digest with Asc I
Ligate into Swa I site of pLPBL plasmid
pBL-PEPCK-WL-SwaI-SpeI
8381bp
Linearised with Swa I and dephosporylated

Figure 2.1: Construction of the anti-HBV and control HDAd genome bearing plasmids.
To construct *Fluc* PEPCK promoter cassette and clone into pΔ24.7E4, the *Firefly luciferase* (*Fluc*) orf was first amplified from the pCI-neoCMV*Fluc* and ligated into pTZ57R/T major cloning site (indicated by blue block) to generate pTZ-*Fluc*. The *Fluc* was excised and subcloned downstream of PEPCK promoter in to pLPBL plasmids *Swa* I site to generate pLPBL*Fluc*. The *Fluc* cassette and the previously constructed PEPCK cassettes with pri-miR 31/5 or pri-miR 31/589 sequence were excised with *Asc* I from pLPBL-pepck-wl-*Swa*-I-*Spe* I (pLPBL) and subcloned in to the *Asc* I site of Helper Dependent Adenoviral vector genome bearing plasmid (pΔ24.7E4) to generate pPEPCK*Fluc*HDAd or pPEPCK31/5HDAd or pPEPCK31/589HDAd.

2.4 Tissue culture methods

2.4.1 Cell-line culturing and maintenance

HEK293T (kidney derived), 116 (HEK293 derived) and Huh 7 (human liver derived) cells were grown at optimum conditions in a humidified incubator at 37℃ and 5 % CO_2. HEK293T and Huh 7 cells were grown in Dulbecco's modified Eagles medium [DMEM, (Appendix 6.3)]. Eagle's Minimum Essential Medium [EMEM, (Appendix 6.3)] was used for the growth of 116 cells. The medium was supplemented with 5 % or 10 % Fetal Bovine serum (FBS) (Life Technologies, CA, USA) and the antibiotic combination of Penicillin (100 000 U/ml) and Streptomycin (100 µg/ml) (Sigma-Aldrich, MO, USA). Suspension 116 cells were grown in Joklik Eagle's Minimum Essential Medium [JEMEM, (Appendix 6.3)] supplemented with 5 % FBS, and the antibiotic combination of Penicillin (100 000 U/ml) and Streptomycin (100 µg/ml) and Hygromycin (0.1 mg/ml).

To grow cells from a liquid nitrogen freezer stock, cells were first thawed by placing the cryovial containing the cells at 37℃. The cells were added to 10 ml of 10 % FBS containing medium in a 15 ml flacon tube and spun at 800 rpm for 3 minutes. The pellet was resuspended in 2 ml of 10 % FBS containing medium and added to a 25 cm^2 culture flask containing 3 ml of 10 % FBS containing medium. The cultures were incubated until the cells were 90-100 % confluent. HEK293T and 116 cells were detached by incubation with 3 ml of Saline-EDTA solution. However, Huh 7 requires trypsinisation, therefore the Saline-EDTA was substituted with 500 µl of 0.5× Triple express (Life Technologies, CA, USA). The cells were incubated at 37℃ and 5 % CO_2 for 3 minutes. The flask was tapped lightly to ensure all cells were detached. To inactivate the 0.5× Triple express solution

(Appendix 6.3), 3 ml of 10 % FBS containing medium was added to the flask. The clumps in the suspended detached cells were broken by gently pipetting up and down. The suspended cells were transferred to a 75 cm^2 culture flask and made up to a total of 10 ml with 10 % FBS containing medium. The cultures were incubated until the cells were 90-100 % confluent. The similar procedure was used for the passaging into 175 cm^2 culture flask, whereby 5 ml Saline-EDTA solution or 1 ml of 0.5× Triple express solution was used. The suspended cells were transferred to a 175 cm^2 culture flask and made up to a final volume of 20 ml with 10 % FBS containing medium. Splitting of cells followed the similar procedure, but the final detached suspension cells were divided either between two or three flasks, by transferring either half or a third of the volume to each of the flasks. The volume was adjusted to 10 ml for 75 cm^2 flask or 20 ml for 175 cm^2 flask with 10 % FBS containing medium. Passaging was continued until required number of flasks/cells have been obtained. The medium was replaced after every 48 hours.

2.4.2 Transfection of eukaryotic cell lines

Transfection of Huh 7 cells was carried out with Lipofectamine™ 3000 reagent (Invitrogen, Thermo Fisher Scientific, MA, USA) according to manufacturer's instructions. Huh 7 cells were grown to 100 % confluency in a 75 cm^2 flask and seeded in 10 % DMEM into a 24 well plate at a density of 2.9×10^5 cells per well. Cells were incubated at 37℃, 5 % CO_2 overnight. One hour prior to transfection the media was replaced with 10 % FCS antibiotic-free DMEM. Each well was co-transfected with the DNA transfection mixture containing 100 ng of pCH-9/3091 (Nassal, 1992) or psiCHECK-*HBx* plasmid (Table 2.4), 100 ng of pCI-neo eGFP plasmid (Table 2.4), 0.2 µl P3000 Reagent (1 µl/µg DNA) and 50 µl of Opti-MEM medium. pCI-neo eGFP was added to the DNA transfection mixture to enable the determination of the transfection efficiency using fluorescence microscopy. Firstly, DNA mix was prepared by mixing 200 ng DNA with 50 µl of Opti-MEM and a lipofectamine mix by mixing 0.2 µl of Lipofectamine™ 3000 reagent with 50 µl of Opti-MEM (Lipofectamine mixture). The two solutions were incubated at room temperature for 5 minutes followed by combining and further incubation for 15 minutes. A 100 µl of the transfection mixture was added to each well, and incubated for 5 hours at 37℃.

Transfection with Polyethylenimine "Max' (PEI max) entailed adding 30 µg of PEI max (Fluke®, Sigma-Aldrich, MO, USA) to 500 µl of Opti-MEM and 10 µg of linearized plasmid DNA to 500 µl of Opti-MEM (Life Technologies, CA, USA) separately. The solutions were incubated at room temperature for 10 minutes. The solutions were then mixed together, incubated at room temperature for 20 minutes and added dropwise to the 116 cells in a 60 mm plate.

2.4.3 AdNG163 and Ad163Cys Helper virus amplification

HEK293T cells were grown to 100 % confluency in a 175 cm^2 flask. The HEK293T cells were counted and plated to 1.5×10^5 cells/ml in a total volume of 15 ml in a 143 cm^2 plate. The cells were immediately infected with a previously produced helper virus at a MOI (multiplicity of infection) of 0.1. Medium change was performed every 48 hours for 10 days, whereby the medium was removed and centrifuged at 1000 rpm for 3 minutes. The supernatant was discarded, the pellet was then resuspended in 15 ml of 5 % FBS DMEM and added back to the plate. On the 10^{th} day the cells were harvested into the supernatant and 1.5 ml of 40 % sucrose was added. The helper virus stocks were subjected to 3× freeze thaw cycles and stored at -80℃.

2.4.4 Immunostaining for Ad helper virus infectious units determination

HEK293T cells were grown to 90-100 % confluency with 10 % FBS DMEM in a 75 cm^2 culture flask. The HEK293T cells were seeded into a 24 well plate at ~ 50 % confluency with 500 µl of 10 % FBS DMEM per well. Cultures were incubated overnight (to reach confluency ~ 90 %) and the medium was removed the following day. Cells were infected with serial dilutions of the AdNG163 or AdNG163Cys helper virus (10^{-1} to 10^{-5}) by adding 100 µl per well of each dilution in duplicate. The infected cells were incubated at 37℃ and 5 % CO_2 for 1 hour rocking the plate every 10 minutes. The remainder of 400 µl of 5 % FBS DMEM was added to the wells. The cells were then incubated for 48 hours at 37℃ and 5 % CO_2.

Immunostaining was performed using the Ultra-Sensitive ABC Peroxidase Mouse IgG staining kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's

instructions with minor adjustments. Medium was removed and cells were left to air dry for 5 minutes. Cells were fixed with 300 µl methanol for 20 minutes at -20℃. Cells were washed 3× with phosphate-buffered saline [PBS, (Sigma-Aldrich, MO, USA)] + 0.5 % bovine serum albumin [BSA, (Roche Holding AG, South Africa)]. Five drops of blocking buffer (3 drops blocking serum + PBS) was added to each well and incubated at room temperature for 5 minutes. Blocking solution was removed and cells were washed once with PBS + 0.5 % BSA. Permeabilization of cells was performed by adding 300 µl of permeabilization buffer (PBS + 0.5 % BSA + 0.25 % Triton-X) and incubating cells at room temperature for 5-10 minutes. Permeabilization solution was removed and cells were washed once with PBS + 0.5 % BSA. Cells were then incubated with 300 µl of mouse anti-Ad fiber Primary antibody (Biocom Biotech, Gauteng, South Africa, 1000× dilution in blocking serum) for 1 hour at room temperature. The mouse anti-Ad fiber Antibody (Ab) was removed and cells were washed 3× with PBS + 0.5 % BSA. Five drops of Biotinylated secondary Ab was added to the cells and incubated for 30 minutes at room temperature. Cells were then washed 1× with PBS + 0.5 % BSA. Five drops of Detector ABC reagent [horseradish peroxidise (HRP) complexed with avidin] was added to the cells and incubated at room temperature for 30 minutes. ABC reagent was removed and cells were washed 1× with PBS + 0.5 % BSA. The metal enhanced substrate, 3,3'-Diaminobenzidine (DAB) chromagen diluted solution [1× DAB+ hydrogen peroxide (H_2O_2,) Roche Holding AG, South Africa] was added to the cells and incubated for 10 minutes at room temperature in a dark area. The Ad infected cells produced a dark brown colour. The positively stained cells were viewed and counted under a light microscope. The infectious units (ifu)/ml of the Ad helper virus was determined with the following formula and fields per well as indicated on Table 2.5:

$$number\ of\ ifu/ml: \frac{(infected\ cells\ per\ field) \times (fields\ per\ well)}{volume\ of\ virus\ (ml) \times dilution\ factor}$$

Table 2.5: Derivation of Area counted in fields/wells

Objective Lenses	Fields/wells		
	12 well plate area = 3.8 cm^2	24 well plate area = 2.0 cm^2	96 well plate area = 0.32 cm^2
4×	19	10	1.6
5×	30	16	2.6
10×	150	79	12.6
20×	594	313	50

2.4.5 Transmission Electron Microscopy (TEM)

TEM of HV was performed at The National Institute for Communicable Diseases at the NHLS according to a published protocol (Goldsmith and Miller, 2009) with adaptions. Briefly, the HV containing samples were placed onto 0.25 % formvar-coated 300 mesh copper grids and incubated for 5 minutes. The grids coated with the samples were then stained with 4 % aqueous uranyl acetate for 5 minutes and dried overnight at room temperature. The samples were viewed at 80kV on a FEI BioTwin Spirit TEM. Imaging was performed with Olympus Quemesa CCD camera.

2.4.6 HDAd production and purification

HDAd production and amplification

The HDAd production was performed according to a published protocol with slight adjustments (Palmer and Ng, 2008b). Briefly, HDAd genome bearing plasmid was digested with *Pme* I (New England Biolabs, MA, USA), thereby liberating the HDAd genome from the bacterial sequence. The 116 cells were seeded into a 60 mm dish and grown to 70 % confluency. The cells were then transfected overnight with the linearized HDAd plasmid using a 3:1 ratio of PEI max:DNA (section 2.4.2, Fluke®, Sigma-Aldrich, MO, USA) to plasmid. After overnight incubation, media was removed from transfected cells and cells were washed once with media supplemented with 5 % FCS. Helper virus at a MOI of 5 in 500 µl of 5 % FCS containing media was added to the cells and incubated

at 37 ^{0}C and 5 % CO_2 for 1 hour rocking the plate every 10 minutes, The media was then topped up to 2.5 ml and incubated for 48 hours to allow selective HDAd production. Cells were harvested, supplemented with sucrose to 4 % and lysed by 3 cycles of freeze thawing.

HDAd amplification was performed by sequential co-infections of 116 cells in a 60 mm dish with HV at MOI of 2 and 400 µl of HDAd containing lysate from the previous passage until optimum HDAd titers were reached in a 60 mm plate. About 70 % confluent 116 cells in a 150 mm dish were co-infected with 500 µl HDAd containing lysate from the lowest passage with the highest titers and helper virus at a MOI of 2 as described above using 2 ml infection volume, which was topped up to 15 ml with 5 % FCS containing media. The co-infected cells were harvested after 48-72 hours [100 % Cytopathic effect (CPE)]. The harvested cells were centrifuged at 7500 rpm for 5 minutes, supernatant was discarded and the pellet was resuspended in 1 ml of 100 mM Tris-HCl (pH 8) and continued with large scale production. Due to the HDAd vectors being *lac* Z deficient, a HDAd vector expressing *lac* Z (HDAd*LacZ*) was amplified in parallel to aid as a amplification control, as well as to provide a mean of quantifying the number of infectious units after each amplification step of the HDAd vectors.

2.4.7 X-gal staining for HDAd infectious particles determination

The 116 or HEK293 cells were grown to 90-100 % confluency with 10 % FBS EMEM in a 75 cm^2 culture flask. The 116 cells were seeded into a 24 well plate at 50 % confluency with 500 µl of 10 % FBS EMEM per well. Plates were incubated overnight (to reach confluency ~ 90 %) and the medium was removed the following day. The cells were then infected with undiluted or serial dilutions (10^0-10^{-6}) of HDAd*LacZ* as described in section 2.4.4. The plate was incubated for 48 hours at 37℃ and 5 % CO_2.

After 48 hours the medium was removed, cells were left to air dry for 5 minutes at room temperature. A fixative solution (1 % formaldehyde; 0.5 % glutaraldehyde in PBS) was then added (300 µl) gently and incubated at room temperature for 10 minutes to fix the cells to the plate. The fixative was removed and cells were washed twice with 300 µl of 1× PBS. The X-gal staining solution [(300 µl), 4 mM potassium ferricyanide; 4 mM

potassium ferrocyanide; 2 mM $MgCl_2$; 0.4 mg/ml X-gal, Appendix 6.3] was added to the cells and incubated at 37℃ and 5 % CO_2 for 3-16 hours. The X-gal positive cells produced a blue colour and was counted to determine the infectious units. The number of ifu/ml was determined using the formula and Table 2.5 as indicated in section 2.4.4.

Large scale HDAd production, purification and quantification

Large scale production was performed with 116 cells in suspension. Suspension cells were prepared by growing 8 large flask of 116 cells to 100 % confluency. The cells were detached with 5 ml of Saline-EDTA and incubation for 3 minutes at 37℃. The cells were transferred into 50 ml falcon (2 flasks/falcon) and centrifuged for 3 minutes at 1000 rpm. The pellets were resuspended in 5 ml of 5 % JEMEM (Appendix 6.3) and transferred to two 1.5 L spinner flasks (4 flasks per spinner flask). Fresh 5 % JEMEM was added to each spinner flask to a final volume of 500 ml. The spinner flasks were incubated overnight at 37℃ spinning at 60 rpm. The following day the cells were counted and 250 ml of 5 % JEMEM was added to each spinner flask and incubated overnight at 37℃ spinning at 60 rpm. This was repeated after 48 hours. On the 3rd day the medium was made up to a final volume of 1.5 L. In between each feeding the cells were counted, when cells were at a density of 2×10^5 to 4×10^5 cells/ml in 1.5 L, coinfection was performed. Cell count was performed by transferring 2 ml of the cells into a 15 ml falcon tube. Two milliliters of 2× citric saline pre-heated at 37℃ was added and cells were votexed for 10 seconds. The cell mixture was incubated at 37℃ for 10 minutes, followed by votexing for 10 seconds. Fifty microliters of cells were added to 50 µl of Trypan blue. Cells were counted and multiplied by the dilution factor of 4 to obtain the total number of cells/ml. To monitor cell health and growth, after each cell count and addition of media, 100 µl of cells were added to 500 µl of 5 % JEMEM in a 24 well plate.

Co-infection was performed by harvesting the suspension cells by centrifugation at 7500 rpm for 5 minutes at room temperature. The pellet was resuspended in 100 ml of spent media and transferred to a 250 or 500 ml spinner flask. The resuspended cells were then co-infected with helper virus at a MOI of 2 and the 1 ml of HDAd obtained from the 150 mm plate. The cells were incubated for 2 hours at 37℃ spinning at 60 rpm. After 2 hours, 50 ml of co-infected cells were transferred to each of the 1.5 L spinner flasks containing 250 ml spent media and made up to a final volume of 1 L with 5 % JEMEM. A 100 µl of

the co-infected cells were added to a well containing 500 µl of 5 % JEMEM. This was to monitor the health of the cells, whereby 100 % rounding up of cells should be seen after 48 hours. The co-infected cells were incubated for 48 hours at 37℃ spinning at 60 rpm. After 48 hours, co-infected cells were harvested by centrifugation at 7500 rpm for 5 minutes at room temperature. The supernatant was discarded and the pellet was resuspended in 15 ml 100 mM Tris-HCl (pH 8) and transferred into a 15 ml falcon tube.

Two milliliters of 5 % sodium deoxycholate was added to the cells and incubated for 30 minutes at room temperature mixing every 5 minutes. A volume of 170 µl of 2 M $MgCl_2$, 150 µl RNAse (10 mg/ml) and 150 µl DNAse (10 mg/ml) was added to the mixture and incubated for 1 hour at 37℃, mixing every 10 minutes. The mixture was then centrifuged at maximum speed for 10 minute at room temperature using a tabletop centrifuge. The CsCl step gradient was then prepared as follows (2 per virus): 2 ml of 1.35 g/ml CsCl was added slowly to the ultraclear ultracentifuge tube, 3 ml of 1.25 g/ml CsCl was then added drop wise to the side of the tube to ensure that the gradient is not disturbed. The virus containing supernatant was then overlaid carefully by adding dropwise to the side of the tube. The tubes were then centrifuged at 35000 rpm for 1 hour at 4℃. Using a 22 gauge needle, the HDAd virus (lowest band) was extracted and transferred to a new tube. The tube was filled up with 1.35 g/ml CsCl and centrifuged at 35000 rpm overnight at 4℃. Using a 22 gauge needle the HDAd virus band was extracted and transferred to a Slide-A-Lyzer cassette presoaked in 10 mM Tris-HCl (pH 8) dialysis buffer. The virus was dialyzed at 4℃, with dialysis buffer changed twice every 3 hours and then left to dialyze overnight at 4℃. Following dialysis, the virus was extracted from the dialysis cassette using a 22 gauge needle and glycerol was added to a final concentration of 10 % and mixed well. The purified virus was then aliquoted into 50 µl stock and stored at -80 ^{0}C.

To determine the absolute quantities of HDAd and HV in the CsCl purified viral preparations, viral DNA was isolated (section 2.2.3) from 50 µl of purified virus and used for qPCR. Standards were set up with dilutions prepared from a designed pNG150D Ad plasmid containing both HV and HDAd sequences (Donated by Phillip Ng, Houston Baylor College of Medicine). Q-PCR was performed with primer sets HVF and HVR or HDAdF and HDAdR (Table 2.1) under the qPCR cycling conditions as indicated in Table 2.3.

2.4.8 Assessment of *in vitro Firefly luciferase* expression

Firefly luciferase expression in Huh 7 cells were determined using the Dual-Luciferase® Reporter Assay System (Promega, WI, USA) with slight adjustment. Briefly, Huh 7 cells were infected with PEPCK*Fluc*HDAd at a MOI of 2000 and incubated at 37℃ for 72 hours. The media was removed and cells were harvested with 1× Lysis buffer (100 µl per well). Ten microliters of lysate was transferred into a well of a luminometer plate. Fifty microliters of LAR II (Luciferase assay substrate, 50 µl per sample) substrate was added to the well containing the sample, followed by luminescence measuring using the Veritas Microplate Luminometer (Turner BioSystems, CA, USA).

2.4.9 Determination of pri-miR *in vitro* expression

2.4.9.1 Northern blot analysis

Northern blotting was performed according to previously described protocol (Crowther *et al*., 2016). For *in vitro* expression analysis, Huh 7 cells were seeded into a 10 cm dish at a density of 1.05×10^7 cells per dish and infected with empty HDAd24.7E4 or PEPCK31/5HDAd or PEPCK31/589HDAd at a MOI of 0 or 1000. Cells were incubated at 37℃ for 48 hours. The cells were harvested by centrifugation at 800 rpm for 5 minutes and resuspended in 1ml TRIzol reagent and total RNA was extracted.

RNA extraction with Trizol reagent

Lysates were incubated at room temperature for 5 minutes. Chloroform (200 µl per 1 ml of TRIzol reagent, Ambion, Thermo Fisher Scientific, MA, USA) was added to the lysates, shaken vigorously for 15 seconds and incubated at room temperature for 2-3 minutes. The lysates were centrifuged at maximum speed for 15 minutes at 4℃. The top aqueous layer was transferred to a fresh tube and 500 µl of isopropanol was added per 1 ml of TRIzol reagent. Mixture was incubated at room temperature for 10 minutes and centrifuged at maximum speed for 10 minutes at 4℃. Supernatant was removed and pellet was washed with 1 ml of 75 % ethanol (in Sabex or DEPC water). The sample was centrifuged at maximum speed for 5 minutes at 4℃, supernatant was removed and RNA was air-dried for 5-10 minutes. RNA pellets were dissolved in 50-700 µl of Sabex water (RNAse free

water) depending on the RNA pellet size. The RNA concentration was determined using Nanodrop® Spectrophotometer (Thermo Fisher Scientific, MA, USA), aliquoted into 30 µg aliquots and stored at -80℃.

Radioactive labelling of probes

Radioactive probes (Table 2.6) were labelled in a 20 µl reaction. This entailed the addition of 2 µl of 10 µM probe stock, 2 µl of PNK buffer A (Thermo Fisher Scientific, MA, USA), 1 µl of T4 Polynucleotide Kinase (PNK, Thermo Fisher Scientific, MA, USA), 1 µl of radioactive [γ-^{32}P]-ATP (PerkinElmer, MA, USA) and Sabex water to a final volume of 20 µl. Probe mixture was incubated at 37℃ for 45 minutes. Sephadex columns were prepared for the purification of the probe by inserting 1 cm of compact filter fiber into a 1 ml syringe. The syringe was then filled with sephadex (Appendix 6.4) and placed into a 15 ml falcon tube. The column was centrifuged at 2000 rpm for 2 minutes and topped up with sephadex, followed by centrifugation at 2000 rpm for 2 minutes. This was continued until the sephadex reached 0.9 ml. The probe was diluted to 50 µl by adding 30 µl of sabex water and transferred to the sephadex column. The columns were centrifuged at 2000 rpm for 2 minutes and the purified probes were stored at 4℃.

Radioactive labelling of RNA decade marker

The decade marker was labelled by mixing 1 µl of Decade marker (100 ng) , 6 µl Nuclease free water, 1 µl 10× Kinase reaction Buffer A (Ambion, Thermo Fisher Scientific, MA, USA), 1 µl of radioactive ^{32}P [[γ-^{32}P]-ATP, (PerkinElmer, MA, USA)] and 1 µl PNK (Ambion, Thermo Fisher Scientific, MA, USA). The mixture was incubated at 37℃ for 1 hour. After incubation, 8 µl of Nuclease free water and 2 µl of 10× Cleavage reagent (Ambion, Thermo Fisher Scientific, MA, USA) was added to the mixture and incubated at room temperature for 5 minutes. Twenty microliters of Gel loading buffer II (Ambion, Thermo Fisher Scientific, MA, USA) was then added to the ladder mixture and stored at -80℃.

Polyacrylamide gel electrophoresis, blotting and exposure

A 15 % polyacrylamide gel was set the day before by mixing 0.45 g bis-acrylamide (Merck Chemicals (Pty) Ltd, Darmstadt, Germany), 8.55 g acrylamide (Merck Chemicals (Pty) Ltd, Darmstadt, Germany), 28.8 g Urea (Merck Chemicals (Pty) Ltd, Darmstadt, Germany) and 6 ml of 10× TBE buffer. About 20 ml of sabex water was added and the gel mixture was dissolved in warm water stirring. Once dissolved, the final volume was made up to 60 ml with Sabex water and the gel was cooled to room temperature. After adding 300 µl of 1 % APS and 30 µl of TEMED (Sigma, MO, USA), the gel was immediately poured and left overnight to set at room temperature.

Before loading of samples and decade marker, the gel was pre-run with 1× TBE running buffer (Appendix 6.4) at 150 V for 30 minutes. Samples were prepared by adding equal amount of loading dye (Ambion, Thermo Fisher Scientific, MA, USA) to 30 µg of RNA and denatured at 80℃ for 5 minutes. The ladder was prepared by mixing 5 µl of Decade marker, Sabex water to the highest volume of RNA sample and equal amount of loading dye. The ladder was denatured at 95℃ for 5 minutes. Samples and ladder were placed on ice to prevent renaturing. The ladder and samples were loaded and ran at 150 V for 1 hour followed by increasing the voltage to 200 V until front dye has reached the bottom. The gel was stained with Ethidium bromide (10 µl per 100 ml of 1× TBE running buffer) for 5 minutes and viewed using the Syngene G:BOX Gel Documentation and Analysis system (Syngene, England, UK) to verify equal loading. The RNA was blotted onto a Hybond-N^+ positively charged nylon membrane (Amersham, NJ, USA) at 4℃ using the Semi-Dry Electroblotting Unit Z34,050-2 (Sigma-Aldrich, MO, USA) at 3.3 mA/cm^2 (0.77A) for 45 minutes. The membrane was then UV Crosslinked for ~2 minute (20 000 µJ/cm^2) using a Ultra-Violet Products (UVP) UV cross linker (UVP Inc., CA, USA). After UV cross-linking the membranes were pre-hybridized for 20 minutes with 10 ml of pre-heated hybridization buffer at 42℃. The [γ-^{32}P]-ATP labelled probes (Table 2.6) were denatured at 95℃ for 5 minutes and added to the buffer. The membranes were then hybridized overnight at 42℃. Following overnight hybridisation, membranes were washed once with 50 ml of 0.1 % SDS (sodium dodecylsulphate) and 5× SSC (NaCl, Nacitrate, Sigma, MO, USA) at room temperature for 20 min. Subsequently the membranes were then washed twice with 50 ml of 0.1 % SDS and 1× SSC at 42℃ for 15 minutes. The membranes were

exposed to a phosphoimager plate for 7 days and decade marker was exposed overnight at room temperature in the dark. The phosphoimaging was performed using the FUJIFILM FLA-7000 instrument with the FLA-7000IR software (Vacutec, Gauteng, SA).

Table 2.6: Sequences of probes used for detection of 5, 8 and 9 pri-miR guide sequences

Probe type	Sequence- 5'- 3'	Reference
Probe 5	5' CCGTGTGCACTTCGCTTC '3	(Crowther *et al*., 2016)
Probe 8	5' CAATGTCAACGACCGACC '3	
Probe 9	5' TAGGAGGCTGTAGGCATA '3	

2.4.10 Assessment of HBV surface antigen knockdown in Huh 7 cells

2.4.10.1 Assessment of HBV gene silencing using ELISA

After 5 hours of transfection with pCH-9/3091, the medium was removed and cells were infected in quadruplicate with HDAd24.7E4 or PEPCK31/5HDAd or PEPCK31/589HDAd at a MOI of 0, 1000, 2000, 10000 and 20000. The 100 µl infection solution was added dropwise to the allocated wells and incubated at 37℃, rocking every 10 minutes. Each well was then topped up to a final volume of 500 µl with 5 % DMEM and incubated at 37℃. After 48 hours, supernatant was collected from the infected cells. The HBV replication knockdown was assessed by measuring the secreted HBV surface antigen (HBsAg) in the supernatant by performing ELISA using the Monolisa™ HBsAg Ultra Kit (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions. All solutions were prepared as indicated before use. Briefly, 100 µl of sample supernatant was added to a 96 well plate and 50 µl of conjugate was then added to each well and incubated at 37℃ for 1 hour 30 minutes. The wells were aspirated and washed 4 times with washing solution. The plate was blotted to ensure all liquid was removed and 100 µl of developing solution was added to the well, followed by incubation at room temperature for 30 minutes in a dark area. Stopping solution of 100 µl was then added to each well and incubated for 4 minutes at room temperature. Optical density (OD) was measured at 490/655 nm using the iMARK, Microplate Reader (Bio-Rad Laboratories, CA, USA).

2.4.10.2 Assessment of HBV gene silencing using Dual Luciferase assay

The efficiency of pri-miR in HBV knockdown was further determined using the Dual-Luciferase® Reporter Assay System (Promega, WI, USA) according to manufacturer's instructions. After 5 hours of transfection with psiCHECK-*HBx*, medium was removed and cells were infected with empty HDAd24.7E4 or PEPCK31/5HDAd or PEPCK31/589HDAd at a MOI of 0 or 2000 or 10000 or 20000 in quadruplicate. Infected cells were incubated at 37℃ for 48 hours. The medium was removed and cells were lysed with 100 µl of 1× Lysis buffer per well. Ten microliters of lysate was dispensed into a well of a luminometer plate. Both the sample plate, 50 µl/well of LARII and 50 µl/well of 1× Stop and Glo substrate were put in the luminometer and luminescence was measured with the Veritas Microplate Luminometer (Turner BioSystems, USA). The HBV knockdown was measure as a ratio of *Renilla* luciferase: *Firefly luciferase* expression.

2.5 *In vivo* methods

HBV transgenic mice used in this study have a greater than genome length HBV sequences integrated into their genome (Nassal, 1992). All experiments were carried out in accordance with the protocol accepted by the University of the Witwatersrand Animal Ethics Screening Committee. Mice were grouped in either 4, 5 or 6 mice. All sampling, handling, injections and general care of animals was done under the supervision of the WITS Central animal services (CAS) and senior investigators.

Single dose of 1×10^{10} viral particles (vps) for HDAd24.7E4 or PEPCK31/589HDAd or PEPCK*Fluc*HDAd was injected through tail vein injection. Poly (I:C) required during cytokine levels analysis was injected through hydrodynamic injection (10 % of the body weight). Blood sampling was performed with mice subjected to 30 seconds of isofluorane anesthetics prior to retro-orbital puncture. For serum harvesting, blood was incubated at 4℃ for 2 hours and centrifuged at 8000 rpm for 10 minutes at 4℃. The top serum layer was transferred to fresh tubes and stored at -80 ˚C. Mice sacrificed for liver harvesting were euthanized by CO_2 exposure and livers reserved for DNA (in saline) and RNA isolation (in Trizol). For Bioluminescence imaging, mice were anesthetized with isofluoran before imaging.

2.5.1 Assessment of *in vivo* expression of *Firefly luciferase*

To assess the *in vivo* expression of *Firefly luciferase*, mice infected with PEPCK*Fluc*HDAd were injected intraperitoneally with luciferase substrate D-luciferin [0.1 % of the body weight, (PerkinElmer, MA, USA)] after 3 days, 14 days and 28 days. The mice were immediately subjected to anesthesia with isofluorane and imaged using Xenogen IVIS® imaging system (PerkinElmer, MA, USA). Bioluminescence was quantified using the Living Image® software (PerkinElmer, MA, USA).

2.5.2 Assessment of *in vivo* HBV replication inhibition and pri-miR expression

The HBV surface antigen knockdown in mice was measured using 100 µl of 50× diluted serum from mice injected with saline or HDAd24.7E4 or PEPCK31/589HDAd at 1 and 2 weeks post injection. This was performed by ELISA using the Monolisa™ HBsAg Ultra Kit (Bio-Rad Laboratories, CA, USA) as instructed by the manufacturer and summerised in section 2.4.10.1. Plate readings were performed with the iMARK, Microplate Reader (Bio-Rad Laboratories, CA, USA).

To determine pri-miR expression and processing, RNA was isolated from livers collected from mice after 1 week injection with saline or HDAd24.7E4 or PEPCK31/589HDAd. Livers were harvested and homogenized in TRIzol reagent. RNA was the isolated and used for Northern blotting as described in section 2.4.9.

2.5.3 Evaluation of liver transduction

Liver tissue obtained from HDAd infected mice was used for the preparation of viral DNA using QIAamp DNA Mini Kit (Qiagen, MD, USA) as per manufacturer's instructions and summarised in section 2.2.3. Q-PCR was performed as indicated in section 2.3.3 under the cycling conditions in Table 2.3 with 100 ng of the prepared DNA and primers specific for the HDAd vector (Table 2.1).

2.5.4 Quantification of inflammatory cytokines using CBA assay

To determine if an inflammatory response is activated by the administration of HDAds, inflammatory protein markers IL6, IL10, IL-12p70, IFN-γ, MCP-1 and TNF were measured at 0 days, 6 hours and 24 hours post infection in mice serum samples using a BD Cytometric Bead-Based array mouse inflammation kit (BD Biosciences, CA, USA), as instructed by the manufacturer. Briefly, the lyophilized Mouse inflammation standards were resuspended in 2 ml assay diluent, allowed to equilibrate for 15 minutes at room temperature and serial dilutions performed. Capture beads for each cytokine were vortexed and 10 µl/sample of each was mixed into a single tube. The mixed capture beads were vortexed and 50 µl was added to each assay tube. Fifty microliters of the prepared standards and undiluted mice serum samples were added to the appropriately labelled assay tubes containing the beads. Mouse Inflammatory PE Detection Reagent (50 µl) was added to assay tube and incubated for 2 hours at room temperature, protected from light. After incubation, 1 ml of wash buffer was added to each assay tube and centrifuged at 2000 rpm for 5 minutes. The supernatant was carefully removed ensuring not to disrupt or remove the beads (pellet). Wash buffer (300 µl) was then added to the assay tube and vortexed. Cytometric bead array was conducted on the samples with the BD, LSRFortessa^{TM} flow cytometry instrument and the BD CBA Software (BD Biosciences, CA, USA) was used to quantify the levels of inflammatory cytokines.

2.5.5 Serum transaminase assay

The safety of anti-HBV adenoviral vectors *in vivo* was assessed, by obtaining blood from the mice at 0 days (before infection), as well as 72 hours, 1 week and 2 weeks post infection. Alanine transferase (ALT) was assessed as a marker of liver toxicity. The ALT activity assay was carried out with the use of a kinetic assay on an automated photomeric analyser (Roche Diagnostics, Rotkreuz, Switzerland) by the National Health Laboratory Service chemistry Laboratory (NHLS).

2.5.6 Statistical analysis

Data was expressed as the mean ± standard error of the mean (SEM). Statistical difference was calculated and performed using GraphPad Prism software package (GraphPad Software Inc., CA, USA) and Microsoft Excel. Statistical differences was considered to be significant when Student's paired or unpaired Two-tailed T test indicated $P<0.05$.

CHAPTER 3

3 RESULTS

3.1 Construction of Helper Dependent Adenoviral vector plasmids bearing PEPCK promoter expression cassettes

Non-specific tissue expression and immune stimulation by β-galactosidase encoded by a *lac Z* selection marker has been suggested to result in short-term HBV inhibition by RNAi activators expressed from adenoviral vectors (Crowther *et al*., 2014, Mowa *et al*., 2014). To overcome these obstacles and improve HBV silencing, HDAd vector genomes deficient of *lac* Z and expressing previously described anti-HBV pri-miR mimics (targeting specific sites within the *HBx* ORF of HBV) from a liver specific PEPCK promoter were constructed. *Lac* Z deficient HDAd genome plasmid was donated by Phillip Ng (pΔ24.7E4, Baylor college of Education, USA).

Construction of Firefly luciferase expressing HDAd plasmid

To use as a convenient confirmation of PEPCK promoter activity, HDAd plasmid carrying the *Firefly luciferase* (*Fluc*) gene downstream of the PEPCK promoter was constructed. *Fluc* ORF was amplified from previously constructed pCI-neoCMV*Fluc* (Passman *et al*., 2000) by PCR and cloned into plasmid pTZ57R/T (Appendix 6.6) to generate pTZ-*Fluc*. The clones that were positive for the *Fluc* insert (1811 bp) were identified with *Eco* RV restriction digest (not shown). One positive clone was selected, plasmid DNA was prepared in large scale and digested with multiple restriction enzymes. The pTZ-*Fluc* plasmid was digested with *Dra* I and *Sca* I and compared to an empty pTZ57R/T plasmid digested with *Dra* I and *Sca* I (Table 3.1). The larger expected bands were present after digestion, however the 19 base pairs (bp) band was not visible due to its small size (Figure 3.1). This confirmed successful cloning of the *Fluc* gene into the pTZ57R/T plasmid. The positive pTZ-*Fluc* construct was then sent for sequencing. The sequence was aligned with the parental sequence and 100 % alignment was observed (Appendix 6.5), validating that the complete *Fluc* insert was present and that the *Fluc* gene sequence was correct.

Table 3.1: Expected bands sizes for pTZ*Fluc* validation after restriction digest

Plasmid type	Enzyme Type	Expected base pair sizes
pTZ57R/T	*Dra* I	2175 bp, 692 bp and 19 bp
	Sca I	2886 bp
pTZ*Fluc*	*Dra* I	3986 bp, 692 bp and 19 bp
	Sca I	2623 bp, 1853 bp and 221 bp

Fragments in red are too small to visualize

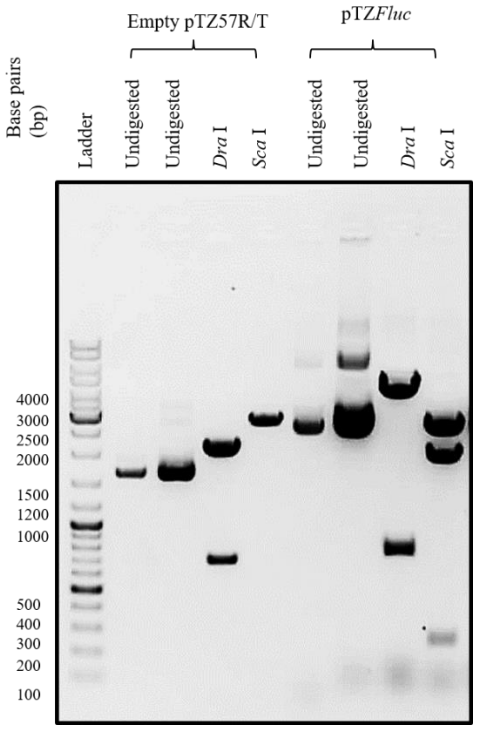


Figure 3.1: Validation of pTZ positive clone for *Fluc* gene insert.
1 % Agarose gel of a positive pTZ*Fluc* clone and empty pTZ57R/T plasmid digested with Dra I and Sca I. Base pair sizes were determined with O' GeneRuler DNA Ladder (Thermo Fisher Scientific, MA, USA).

To clone *Fluc* downstream of the PEPCK promoter, the *Eco* RV excised *Fluc* sequence was ligated into the *Swa* I site of a pLPBL plasmid. Positive clones for pLPBL*Fluc* were identified with *Dra* I restriction digest (not shown). Figure 3.2 represents a positive pLPBL*Fluc* construct digested with *Asc* I, *Bam* HI, *Dra* I and *Nhe* I. The construct digests were compared to an empty pLPBL (Appendix 6.6) plasmid digested with the *Asc* I, *Bam* HI, *Dra* I and *Nhe* I to confirm the presence of the *Fluc* insert. The expected bands shown in Table 3.2 were present with the base pairs in blue migrating close together and could not be differentiated, indicating the presence of the *Fluc* insert. In order for the *Fluc* insert to be expressed from PEPCK promoter, it is required to be in a forward orientation, which

was determined with *Nhe* I digest. The pLPBL*Fluc* construct shown on Figure 3.2 was found to be in a forward orientation (Appendix 6.6) with the banding pattern of 9790 bp and 393 bp, and was therefore used for further cloning.

Table 3.2: Expected bands sizes for pLPBL*Fluc* for validation and orientation confirmation

Plasmid	Enzyme	Expected base pair sizes
pLPBL	*Asc* I	5465 bp and 2916 bp
	Dra I	2887 bp, 1605 bp, 1506 bp, 1335 bp, 692 bp, 337 bp and 19 bp
	Nhe I	8381 bp
	Bam HI	4209 bp, 3229 bp, 661 bp and 282 bp
pLPBL*Fluc*	*Asc* I	7266 bp and 2916 bp
	Dra I	5026 bp, 1605 bp, 1506 bp, 1335 bp, 692 bp and 19 bp
	Nhe I (forward)	9790 bp and 393 bp
	Nhe I (reverse)	8130 bp, 2053 bp
	Bam HI	5031 bp, 4209 bp, 661 bp and 282 bp

Fragments in blue migrate close together and red are too small to visualize

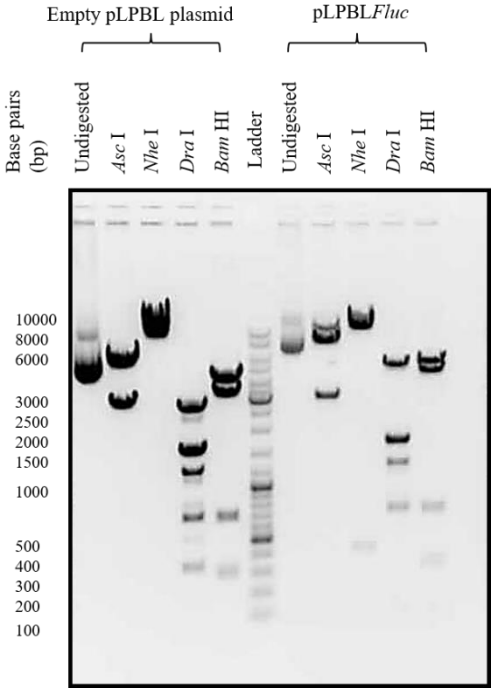


Figure 3.2: Validation of a positive clone containing *Fluc* gene into the pLPBL plasmid.
Agarose gel of the empty pLPBL plasmid and positive clone DNA digested with *Asc* I, *Dra* I, *Bam* HI and *Nhe* I for validation. Base pair sizes were determined using the standard molecular marker, O' GeneRuler DNA Ladder (Thermo Fisher Scientific, MA, USA).

The PEPCK*Fluc* (7267 bp) cassette was excised with *Asc* I restriction digest and ligated into the *Asc* I site of pΔ24.7E4. The positive clones were identified with *Asc* I restriction digest (not shown). Figure 3.3 represents a positive pPEPCK*Fluc*HDAd (Appendix 6.6) construct that was subjected to further validation with restriction digest (Table 3.3) using *Asc I*, *Cla I* and *Pme I* which was compared to an empty pΔ24.7E4. The expected bands were present, indicating that the PEPCK*Fluc* (7267 bp) cassette was successfully cloned into pΔ24.7E4. Although, *Pme I* digest does not differentiate the clone from the empty vector. It was used because it is required to release the bacterial sequence during HDAd production.

Table 3.3: Expected bands sizes for pPEPCK*Fluc*HDAd following restriction digest

Plasmid type	Enzyme Type	Expected base pair sizes
pΔ24.7E4	*Asc* I	27459 bp
	Cla I	20065 bp and 7394 bp
	Pme I	30303 bp and 2949 bp
pPEPCK*Fluc*HDAd	*Asc* I	27465 bp, 7267bp
	Cla I	20450 bp, 7394 bp and 6228 bp
	Pme I	31783 bp and 2949 bp

Fragments in blue migrate close together

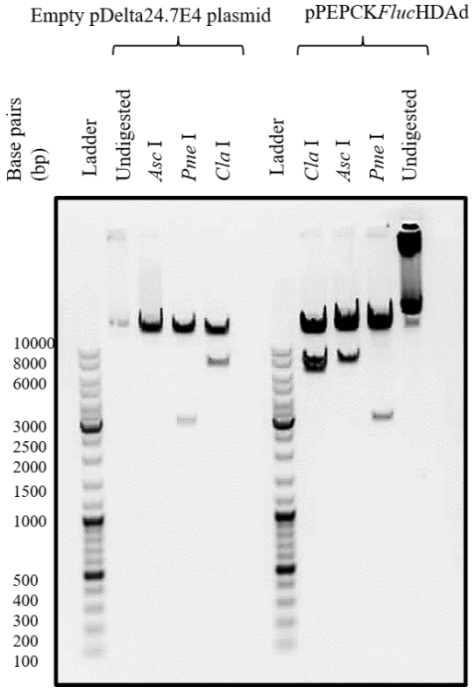


Figure 3.3: Validation of HDAd positive clone carrying the PEPCK*Fluc* cassette. Agarose gel of the positive pPEPCKFlucHDAd clone and empty pΔ24.7E4 plasmid validated with *Asc* I, *Pme* I and *Cla* I digestion, with the O' GeneRuler DNA Ladder (Thermo Fisher Scientific, MA, USA).

Construction of anti-HBV sequence bearing HDAd plasmids

Anti-HBV pri-miR 31/5 and pri-miR 31/589 sequences were previously cloned downstream of the PEPCK promoter in our lab to generate pLPBL31/5 and pLPBL31/589 (Table 2.4, unpublished). HDAd vectors expressing the anti-HBV pri-miR 31/5 and pri-miR 31/589 from a PEPCK promoter were constructed by excising the PEPCKpri-miR31/5 and PEPCKpri-miR31/589 cassettes from pLPBL31/5 and pLPBL31/589 using *Asc* I restriction and cloned into the pΔ24.7E4 *Asc* I site to generate pPEPCK31/5HDAd (Appendix 6.6) and pPEPCK31/589HDAd (Appendix 6.6), respectively. Clones positive for the insert were confirmed with *Asc* I restriction digest (not shown). Figure 3.4 illustrates the confirmation of a pPEPCK31/5HDAd construct with the PEPCKpri-miR31/5 insert. Restriction digests were performed with *Asc* I, *Cla* I and *Pme* I and compared to an empty pΔ24.7E4. The expected bands as illustrated in Table 3.4 were present except for the bands of slight base pair difference due to the bands migrating together (indicated in blue). This confirmed the successful cloning of PEPCKpri-miR31/5 into pΔ24.7E4.

Table 3.4: Expected bands sizes for pPEPCK31/5HDAd after restriction digest

Plasmid	Enzyme	Expected base pair sizes
pΔ24.7E4	*Asc* I	27459 bp
	Cla I	20065 bp and 7394 bp
	Pme I	30303 bp and 2949 bp
pPEPCK31/5HDAd	*Asc* I	27465 bp and 5784bp
	Cla I	18864 bp, 7394 bp, 6228bp and 763 bp
	Pme I	30262 bp and 2949 bp

Fragments in blue migrate close together

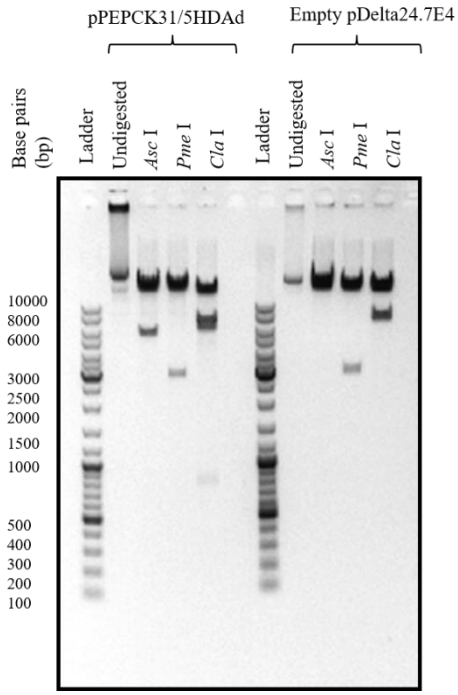


Figure 3.4: Validation of positive HDAd clone carrying the PEPCKpri-miR31/5 cassette.
1 % Agarose gel of the empty pΔ24.7E4 and positive clone was validated with *Asc I*, *Pme I* and *Cla I* digestion. Band sizes were determined with standard molecular weight marker; O' GeneRuler DNA Ladder (Thermo Fisher Scientific, MA, USA).

Figure 3.5 shows the confirmation of pPEPCK31/589HDAd construct with the PEPCKpri-miR31/589 insert using *Asc* I, *Cla* I and *Pme* I and compared to an empty pΔ24.7E4. The expected bands indicated in Table 3.5 were visible, however bands that migrate close together could not be differentiate (indicated in blue). The banding pattern thereby demonstrates the successful construction of pPEPCK31/589HDAd.

Table 3.5: Expected bands sizes for pPEPCK31/589HDAd after restriction digest

Plasmid type	Enzyme Type	Expected base pair sizes
pΔ24.7E4	*Asc* I	27459 bp
	Cla I	20065 bp and 7394 bp
	Pme I	30303 bp and 2949 bp
pPEPCK31/589HDAd	*Asc* I	27465 bp and 6097 bp
	Cla I	18847 bp, 7394 bp, 6228 bp and 1093 bp
	Pme I	30613 bp and 2949 bp

Fragments in blue migrate close together

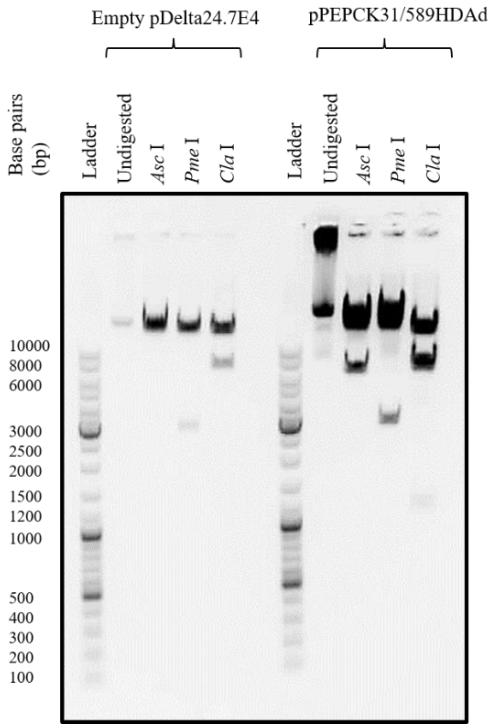


Figure 3.5: Validation of the HDAd clone carrying the PEPCKpri-miR31/589 cassette.
1 % Agarose gel of the standard molecular O' GeneRuler DNA Ladder (Thermo Fisher Scientific, MA, USA) and the positive clone and empty pΔ24.7E4 plasmid validated with *Asc I*, *Pme I* and *Cla I* restriction digestion.

3.2 Anti-HBV Helper Dependent Adenoviral vector production

3.2.1 Amplification of AdNG163Cys Helper virus

One of the limitations for efficient and safe delivery of transgenes, is the activation of the host innate immune response to the adenoviral viral capsid proteins (Crowther *et al*., 2008, Croyle *et al*., 2005, O'Riordan *et al*., 1999, Yang *et al*., 1995). To prevent activation of the host innate immune response to viral capsid proteins, PEGylation can be performed. The introduction of cysteine residues into the hypervariable region 5 of the hexon proteins have shown to aid in a more specific thiol reactive PEGylation (Prill *et al*., 2011). This type of PEGylation results in a more uniform viral particle size and had less interference with intercellular trafficking. To enable production and propagation of HDAd vectors with cysteine modification, a plasmid carrying a helper virus (AdNG163Cys) genome with cysteine modification within the HVR 5 was donated by Philip Ng (Baylor college of Education, USA). This plasmid was previously used to produce AdNG163Cys. In this

study, AdNG163Cys was amplified and used for HDAd production and amplification. Successful amplification was determined by immunostaining of the infected cells. The infected cells are represented by the dark brown stained cells in Figure 3.6. Despite the successful production and amplification of this helper virus, infection of HEK293T cells showed consistent uneven distribution of positively stained cells, with brown cells always together (Figure 3.6). This made it difficult to determine accurate viral infectious units. Therefore, qPCR was used to determine total viral particles and 4.88×10^{10} vps in 20 ml were obtained, which were enough to use for HDAd production and amplification.

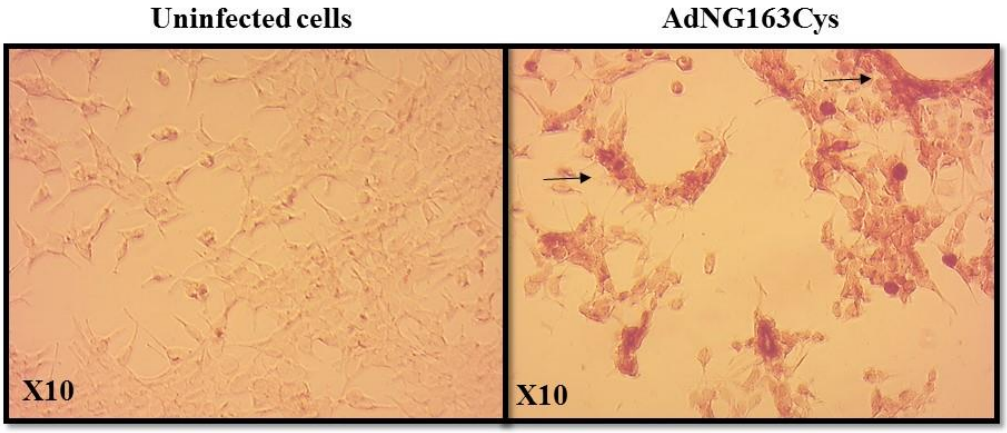


Figure 3.6: Immunostaining of HEK293T cells infected with produced AdNG163Cys. Immunostaining was performed on both uninfected and infected HEK293T cells. The HEK293T cells were infected with different dilutions of AdNG163Cys followed with a 48 hour incubation period and immunocytochemical staining to quantify the produced HV. All microscopic images were taken at 10× magnification. Image taken from cells infected with undiluted AdNG163Cys was used as a representative. AdNG163Cys infected cell clumping is indicated by the arrows.

3.2.2 AdNG163Cys does not serve as an effective HV for HDAd propagation

Because of the difficulty in determining AdNG163Cys infectious units, total viral particles were used to determine the amount of HV required for different MOIs tested for HDAd production. Interestingly, application of this helper virus in the production of *β*-galactosidase expressing HDAd, resulted in an HDAd vector that bears similar signs of possible viral aggregation before or during infection of cells. This was indicated by clumped blue cells after X-gal staining in Figure 3.7. In addition, HDAd could only be produced with AdNG163Cys when a higher MOI of 50 was used. Amplification of this HDAd with AdNG163Cys at different MOIs was also unsuccessful (data not shown).

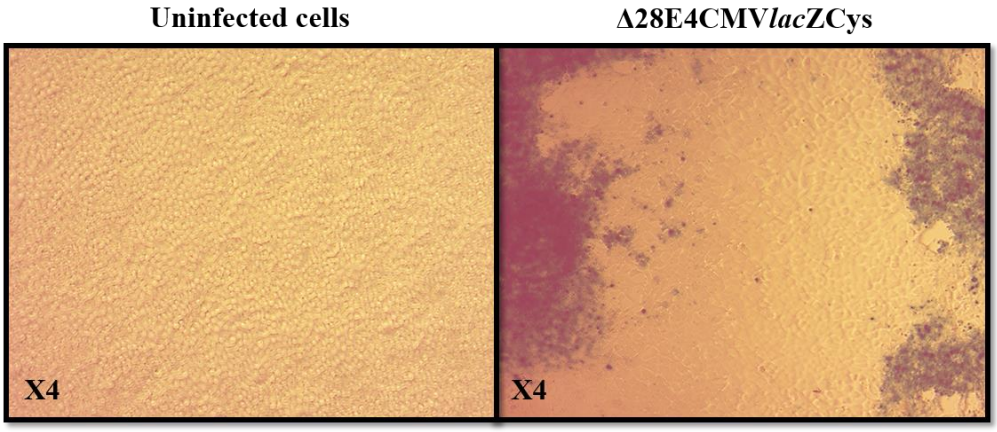


Figure 3.7: X-gal staining of 116 cells infected with Δ28E4CMV*lac*ZCys.
X-gal staining was performed on both uninfected and infected 116 cells. The 116 cells were infected with different dilutions of Δ28E4CMV*lac*ZCys and incubated for 48 hours before X-gal staining. Image taken from cells infected with undiluted HDAd sample was used as a representative.

To confirm if the aggregation of the virus is as a result of the cysteine modification, a previously produced AdNG163 helper virus with wild type HVR 5 was used to infect cells followed by the immunostaining. Unlike with AdNG163Cys, single brown stained cells were visible and could be counted to accurately determine the infectious units (Figure 3.8). When the AdNG163 virus was used, HDAd could be successfully produced at AdNG163 MOI 5 (determined using total viral particles). After X-gal staining of cells with Δ28E4CMV*lac*Z produced with AdNG163, single blue cells were observed and could be counted to determine infectious units (Figure 3.9). The observed clumping of infected cells and failure of AdNG163Cys to effectively serve as helper virus for HDAd amplification suggest that the cysteine capsid modification may alter the capsid structure and affect HV infection efficiency.

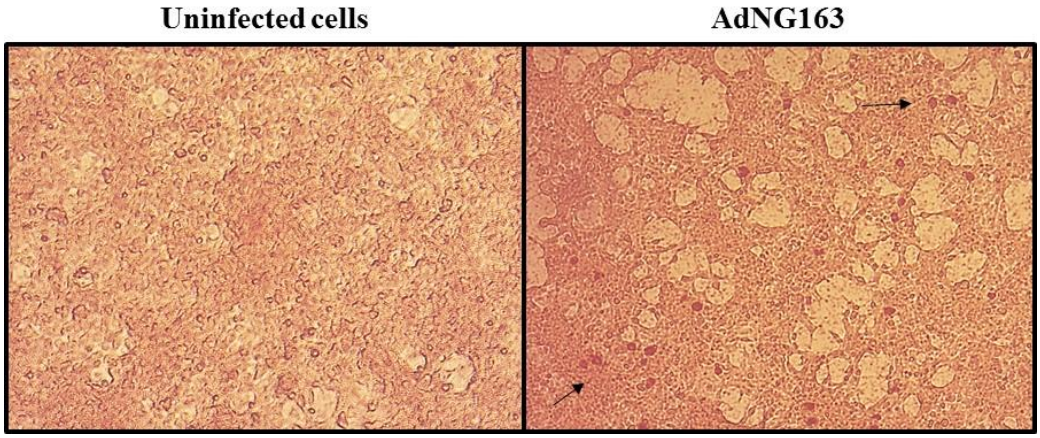


Figure 3.8: Immunostaining of HEK293T cells infected with AdNG163 Helper virus. Immunostaining was performed on both uninfected and AdNG163 infected HEK293T cells to determine if aggregation occurs. Briefly, HEK293T cells were infected with different dilutions of the helper virus and incubated for 48 hours at 37 ˚C before immunocytochemical staining. All imaging was performed at 10 × magnification. Positively stained cells are presented by the darker brown cells indicated by the arrows. Image taken from cells infected with 10^{-5} AdNG163 dilution was used as representative.

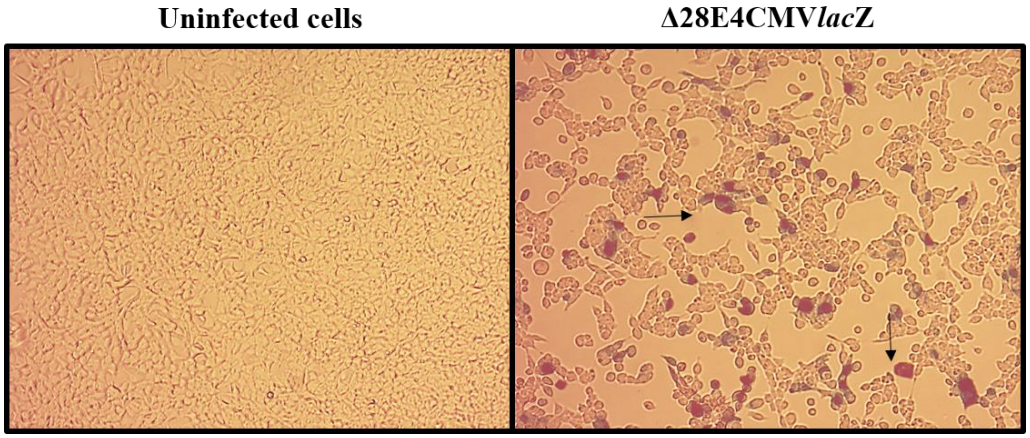


Figure 3.9: X-gal staining of Δ28E4CMV*lac*Z produced with AdNG163 helper virus. X-gal staining was performed on both uninfected and infected 116 cells to confirm whether aggregation occurs. Briefly, 116 cells were infected with different dilutions of Δ28E4CMV*lac*Z and incubated for 48 hours at 37 ˚C before X-gal staining. Ten times magnification was used for the imaging of the cells infected with undiluted Δ28E4CMV*lac*Z virus. Positively stained cells are presented by the blue cells indicated by the arrows.

3.2.3 AdNG163Cys has a distorted particle structure

To determine whether the aggregation and unsuccessful amplification of HDAd using AdNG163Cys was as a result of capsid structure modification, Transmission Electron

Microscopy (TEM) was performed. The TEM images presented in Figure 3.10d showed morphological deformation of the cysteine modified AdNG163Cys helper virus when compared to the unmodified AdNG163 helper virus (Figure 3.10b). The AdNG163 helper virus diameter was between 70-80 nm and showed the expected icosahedral shape. When comparing the AdNG163Cys helper virus the particle morphology was deformed, with the icosahedral shape lost. This suggests that the cysteine modification influenced the helper virus shape and structure. Confirming the X-gal staining data with Δ28E4CMV*lac*ZCys, this virus showed similar structural deformities to the AdNG163Cys (Figure 3.10c) as compared to Δ28E4CMV*lac*Z (Figure 3.10a). In addition all imaging of AdNG163 and HDAd was of large scale productions, which is not the case for AdNG163Cys and HDAdCys. Due to the low HV efficiency of AdNG163Cys large scale production of HDAdCys could not be performed.

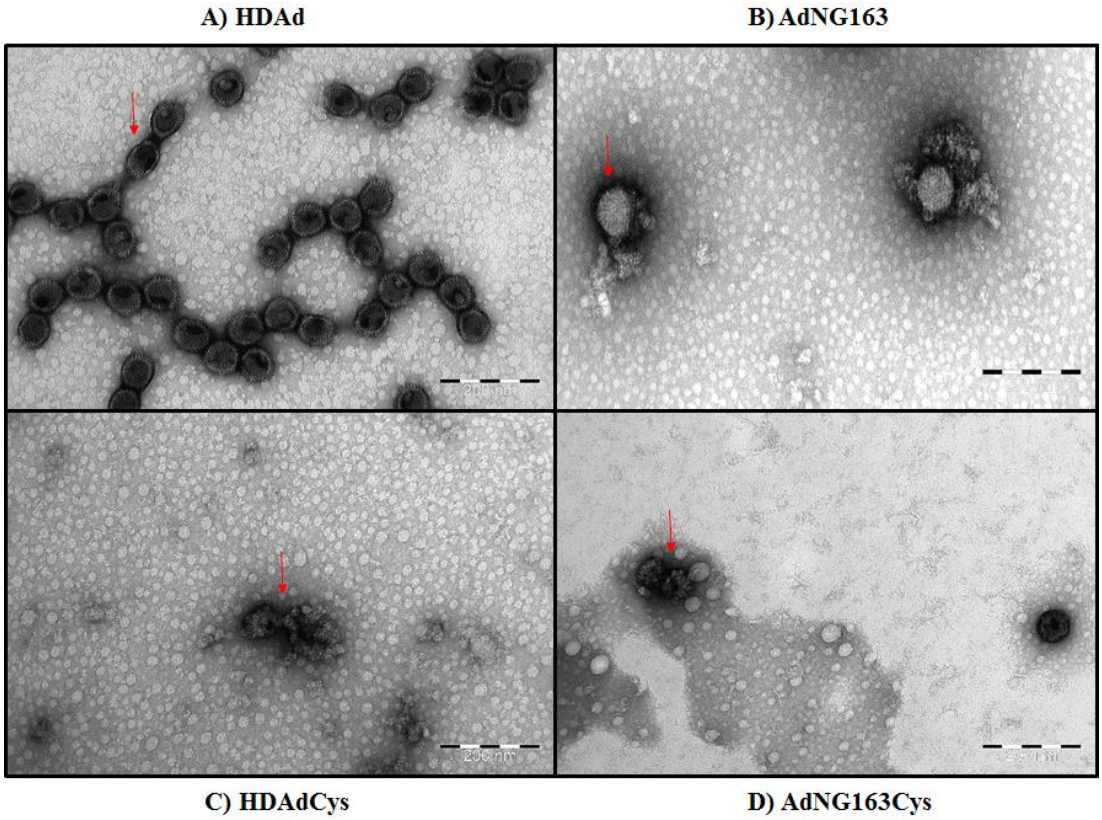


Figure 3.10: Transmission Electron microscopy of Cysteine modified AdNG163Cys/ HDAdCys and unmodified AdNG163/HDAd.
The samples were added to 0.25 % formvar-coated 300 mesh copper grids and incubated for 5 minutes. The grids coated with the samples were then stained with 4 % aqueous uranyl acetate for 5 minutes and dried overnight at room temperature. The samples were viewed at 80kV on a FEI BioTwin Spirit TEM. **A)** HDAd virus prepared with AdNG163 helper virus measured at 200 nm, **B)** AdNG163 virus measured at 200 nm **C)** HDAd virus prepared with AdNG163Cys helper virus measured at 200 nm, and **D)** AdNG163Cys helper virus measured at 200 nm. Red arrows indicating the viral particles.

3.2.4 Cysteine modification on the HVR 5 alters AdNG163Cys helper virus infection efficiency

Upon infection and viral gene expression, adenoviruses such as a helper virus (E1 deficient Ad vector) effect morphological change in the cultured cells mainly characterized by rounding of infected cells called cytopathic effect (CPE). This was taken advantage of to determine if the cysteine modification and deformities of the AdNG163Cys influenced the infection efficiency of the helper virus. The infection efficiency was determined by infecting 116 cells with AdNG163 and AdNG163Cys helper virus at an MOI of 2 (determined from total viral particles for both HVs). The cells were viewed for CPE after 48 hours. The comparison between the cysteine modified AdNG163Cys and unmodified AdNG163 helper viruses presented a clear difference between the CPE induced by the two helper viruses (Figure 3.11). Cytopathic effect of 90-100 % was observed for AdNG163 after 48 hours. However, AdNG163Cys presented little or no cytopathic effect after 48 hours. The findings indicated that the cysteine modification of the viral capsid and the deformities of the AdNG163Cys influenced the infection activity of the AdNG163Cys helper virus.

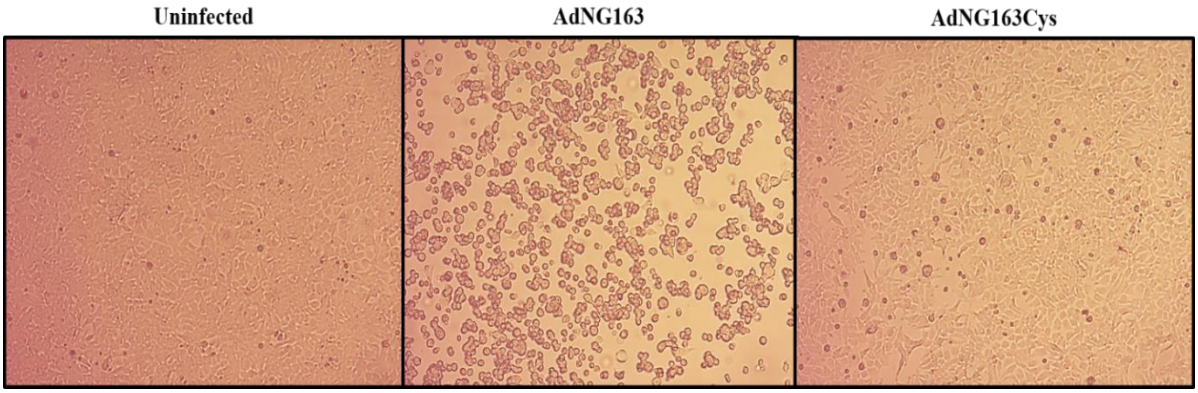


Figure 3.11: Cytopathic effect of AdNG163 and AdNG163Cys.
116 cells were infected with both AdNG163 and AdNG163Cys at a MOI of 2, respectively. The cytopathic effect of both helper viruses were viewed after 48 hours at 10× magnification.

3.2.5 Successful production and amplification of HDAds with unmodified AdNG163 helper virus

As a result of deformities and reduced infection efficiency associated with cysteine introduction into the AdNG163 capsid, an unmodified AdNG163 was therefore used for all viral productions and propagations in this study. As a result of the absence of a *lac* Z reporter gene in the designed HDAd constructs, a HDAd carrying the *lac* Z gene was produced and amplified in parallel and subjected to X-gal staining to determine the number of infectious units. HDAd viral production consisted of three steps; rescue, amplification and large scale production. The rescue step required the conversion of the HDAd plasmid into an HDAd virus. Successful rescue of the different HDAd plasmids were achieved with the production of about 3.160×10^4 infectious particles/ml. The produced viral particles were subjected to further passaging known as amplification to increase the amount of virus that was produced. Each passaging of the HDAd bearing a *lac* Z reporter gene was subjected to X-gal staining to confirm successful amplification. Figure 3.12, shows that an increase in the amount of positively stained cell (blue cells) occurred with each passage.

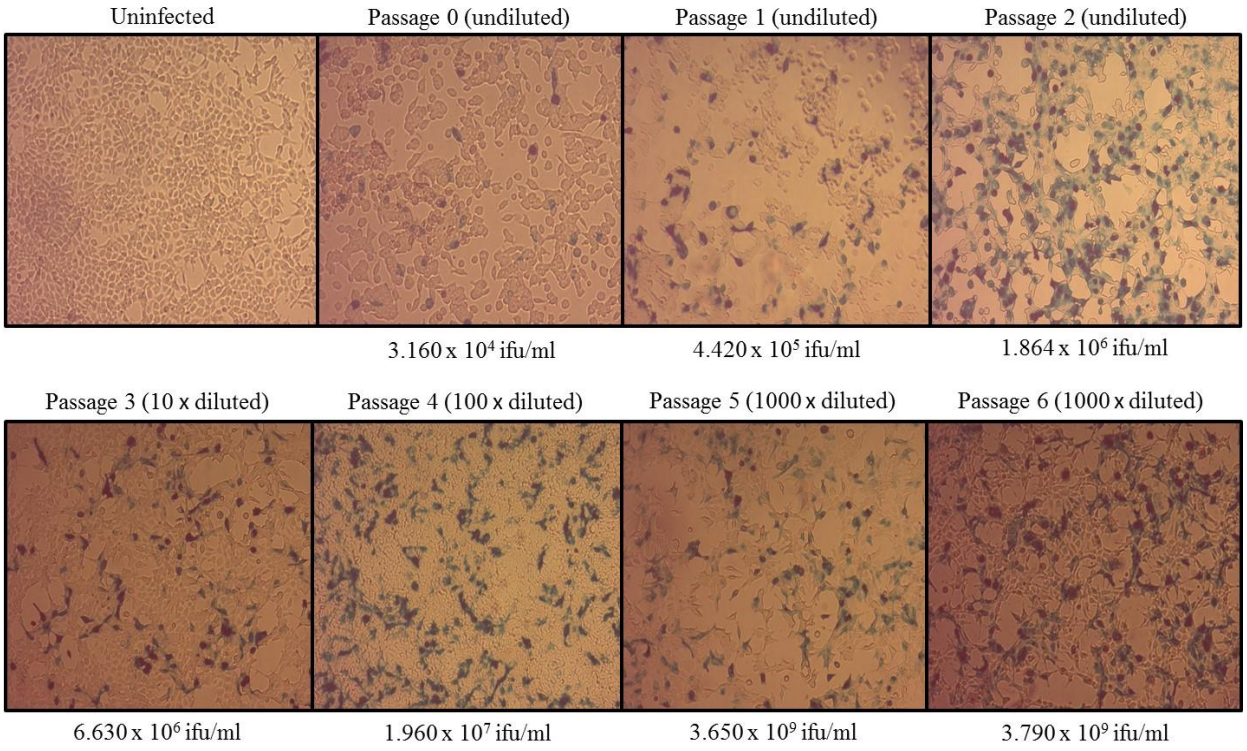


Figure 3.12: X-gal staining to monitor the amplification of the HDAd constructs. Amplification was performed in 116 cells with Δ28E4CMV*lac*Z in parallel to determine if the amplifications were successful. The 116 cells were infected with dilutions of the lysates and incubated for 48 hours, followed by X-gal staining. All microscopic images were captured at 10× magnification.

A fold increase of between 1 and 186 in the number of infectious units was observed during amplification until maximum viral titers were achieved (Table 3.6). Passage 5 and 6 indicated that optimal titers were achieved in the 60 mm plates, which could be seen by the slight difference in the viral titers obtained for both passages. Once optimum titers were achieved, the lowest passage with the highest amount of HDAd virus was used for amplification in 150 mm plates. Due to the slight difference in the infectious units obtained between passage 5 and 6, passage 5 was used for the amplification in 150 mm plates. The amplification resulted in a 1 fold increase in the amount of HDAd virus produced, yielding 4.260×10^9 ifu/ml, which was in the desired range for large scale production. The HDAd virus (passage 6) produced in the 150 mm plate was then used to co-infect cells with AdNG163 at MOI 2 and amplified in a 3 L suspension culture, followed by viral purification using a CsCl step gradient. A single band was observed after all CsCl gradient steps (Figure 3.13).

Table 3.6: Infectious units and fold changes obtained for each passage during HDAd amplification

Passages	Plate size	Infections units/ml (ifu/ml)	Fold change
HDAd Rescue (Passage 0)	60 mm	3.160×10^4	
Passage 1	60 mm	4.420×10^5	~ 14
Passage 2	60 mm	1.864×10^6	~ 4
Passage 3	60 mm	6.630×10^6	~ 3.5
Passage 4	60 mm	1.960×10^7	~ 3
Passage 5	60 mm	3.650×10^9	~ 186
Passage 6	60 mm	3.790×10^9	~ 1
Large Scale (Passage 6)	150 mm	4.260×10^9	~ 1

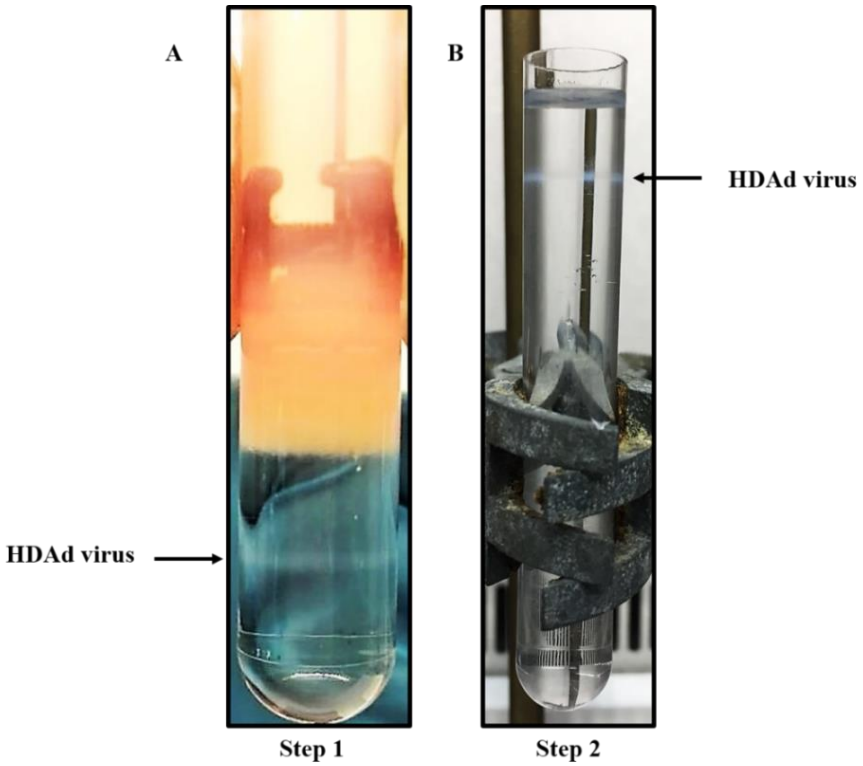


Figure 3.13: A representative of a CsCl step and continuous gradients of a purified HDAd virus.
A) CsCl step gradient with purified HDAd virus indicated by the arrow. B) CsCl continuous gradient with purified HDAd virus (indicated by arrow).

The HDAd24.7E4, PEPCK31/5HDAd, PEPCK31/589HDAd and PEPCK*Fluc*HDAd were successfully produced and purified in large scale. The viral titers of each CsCl gradient purified vector was quantified with qPCR and varied between ~ 10^{11} and 10^{13} total viral

particles as presented in Table 3.7 (~ 10^{11} and 10^{12} viral particles per ml). The helper virus contamination was quantified routinely and ranged between 0.07 % and 1 % after CsCl gradient purification, which was within the acceptable range.

Table 3.7: Viral quantification of CsCl purified HDAd viruses

HDAd	Total viral particles (VPs)	Helper virus contamination (%)
HDAd empty vector	1.078×10^{13}	0.07 %
PEPCK31/5HDAd	2.840×10^{12}	1 %
PEPCK31/589HDAd	3.870×10^{11}	1 %
PEPCK*Fluc*HDAd	5.385×10^{12}	0.5 %

3.3 Efficient expression of *Firefly luciferase* from PEPCK promoter by HDAds

A problem that is often faced in gene therapy is the expression of therapeutic transgenes in the untargeted tissue. Owing to HBV infecting the liver, optimal expression of the therapeutic transgene (anti-HBV sequences) within the liver is required for sufficient HBV silencing. The use of liver specific Pol II promoters have shown to improve the expression of anti-HBV sequences and lead to the silencing of HBV gene expression (Ivacik *et al*., 2015, Mowa *et al*., 2014). Although liver-specific promoters have been tested in our laboratory for expression of anti-HBV sequences, the use of the highly efficient liver specific PEPCK has not been investigated.

To determine the activity of PEPCK promoter in liver derived cells in our hands, PEPCK*Fluc*HDAd vector expressing *Firefly luciferase* from a PEPCK promoter was constructed and tested in Huh 7 cells. Briefly, Huh 7 cells were infected with PEPCK*Fluc*HDAd or HDAd24.7E4 at an MOI of 2000. After 72 hours, cell lysates were used to measure luciferase activity using luciferase assay. A significant expression of *Firefly luciferase* was observed ($p = 0.0045$) relative to the empty HDAd vector (Figure 3.14). The findings suggest that PEPCK promoter is efficiently active in liver derived cells.

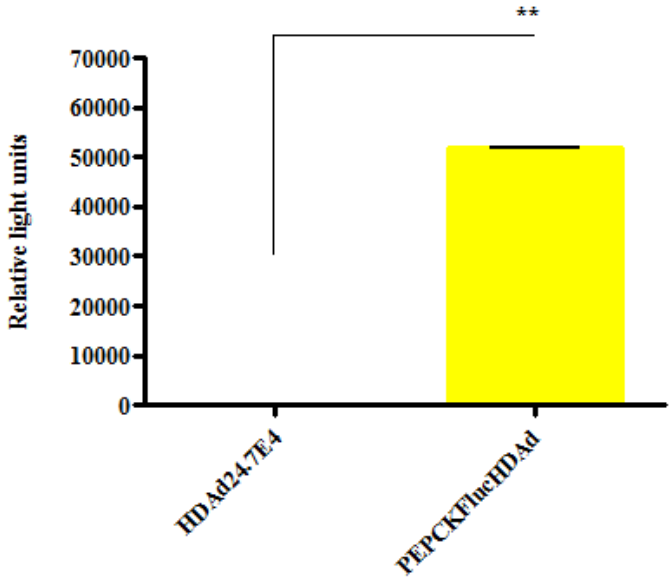


Figure 3.14: *In vitro Firefly luciferase* expression under the control of a PEPCK promoter.
Huh 7 cells were infected with PEPCK*Fluc*HDAd or HDAd24.7E4 (negative control) at a MOI of 2000. Luciferase reporter assay was performed 72 hours after infection. ** indicate significant *Fluc* gene expression, p = 0.0045, T-Test, two tailed, paired. Error bar indicates the normalized Standard error of the mean (SEM) (n=4).

To validate the liver specific gene expression of *Firefly luciferase* from PEPCK promoter, HBV transgenic mice were injected with 1×10^{10} vps of PEPCK*Fluc*HDAd or HDAd24.7E4. All mice were administered with luciferase substrate D-luciferin intraperitoneally at 3 days, 14 days (2 weeks) and 28 days (4 weeks) post infection and bioluminescence was measured. Bioluminescence from 60 seconds exposure was quantified using the Living Image® software (Figure 3.15b). After 3 days, luminescence at 2.75×10^5 relative light units was measured. After two weeks post infection, luminescence within the livers had significantly increased ($p= 0.0495$) to 1.31×10^6 relative light units. This was maintained at 28 days post infection (period of experiment). As expected, bioluminescence was not detected with saline and HDAd24.7E4 infected mice (Figure 3.15a). The results therefore indicated that the PEPCK promoter was capable of driving a sustained *Firefly luciferase* expression within the mice livers.

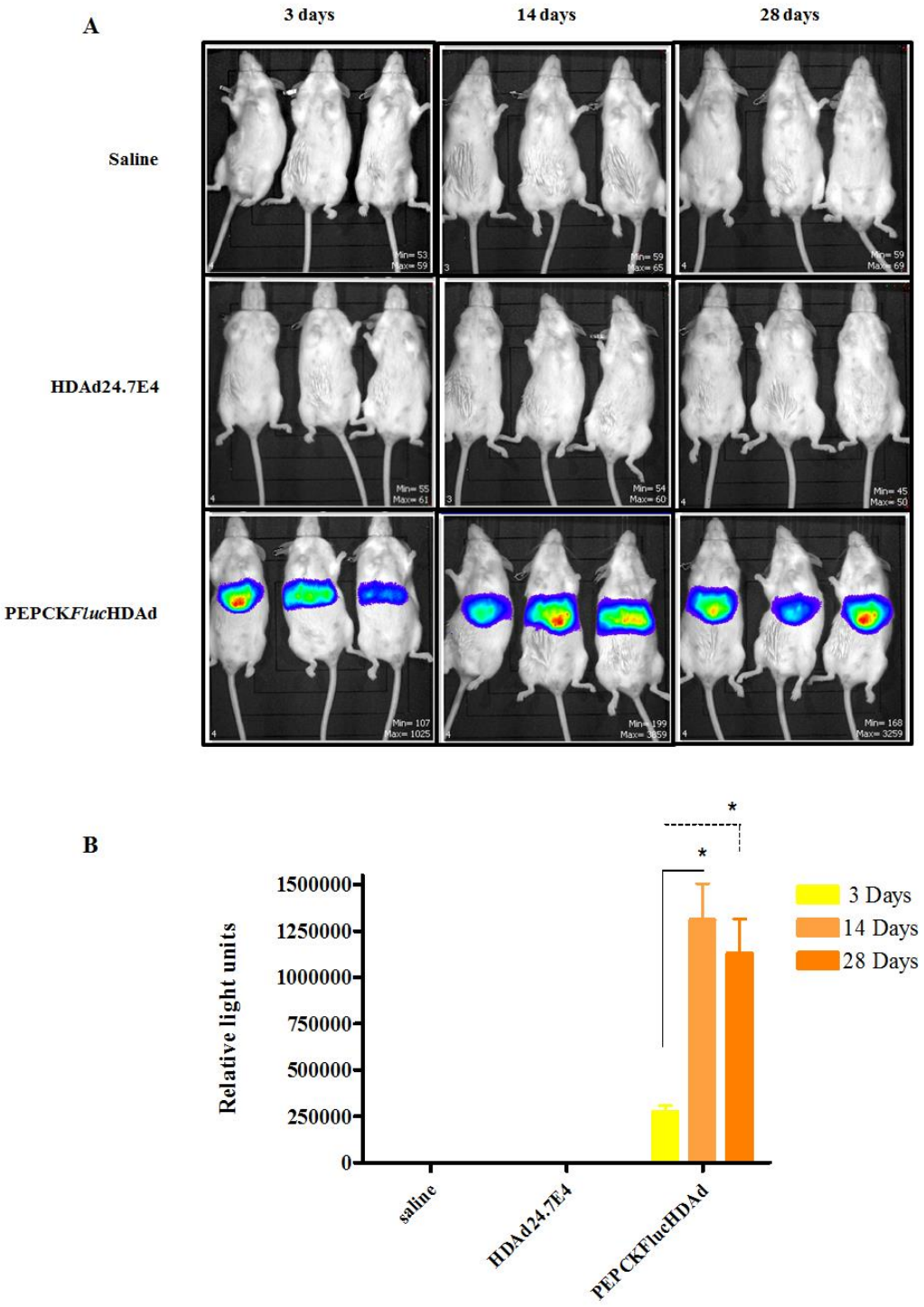


Figure 3.15: Bioluminescence imaging of HBV transgenic mice injected with PEPCK*Fluc*HDAd.

Mice injected with saline or HDAd24.7E4 or PEPCK*Fluc*HDAd was injected with D-luciferin. Bioluminescence imaging at 60 seconds was performed on 3, 14 and 28 days post infection. **A)** Bioluminescence of mice at 60 seconds exposure. **B)** Bioluminescence was quantified using the Living Image® software. * Significant increase ($p<0.05$), T-Test, two tailed, paired. Errors bar indicate the normalized Standard error of the mean (SEM) (n=4).

3.4 Assessment of HBV gene silencing by anti-HBV pri-miR's *in vitro*

The high *in vivo* and *in vitro* expression levels observed with *Firefly luciferase* expressed from the PEPCK promoter, prompted the investigation into applying the PEPCK promoter for HBV silencing through the expression of anti-HBV pri-miR sequences. The HBsAg is a surface glycoprotein, which is produced in excess and secreted by HBV infected liver cells. The HBsAg therefore offers as a good marker for active HBV infection and in turn for HBV gene expression. Hence, HBV knockdown was determined by measuring the reduction of HBsAg secretion using ELISA in Huh 7 cells transfected with pCH-9/3091 target plasmid and infected with HDAd24.7E4 or PEPCK31/5HDAd or PEPCK31/589HDAd. After 48 hours, HBV knockdown by PEPCK31/5HDAd and PEPCK31/589HDAd was determined relative to HDAd24.7E4 as a negative control.

Previous studies have shown that MOI of 500 to 1000 are enough to cause significant HBV replication inhibition (Mowa *et al*. 2014). Hence, HBV gene expression knockdown was first performed at a MOI 1000. At this MOI a significant HBsAg knockdown ($p<0.01$) was observed. However, it was only about 9 % for both PEPCK31/5HDAd and PEPCK31/589HDAd, which was less than observed in previous studies conducted with anti-HBV pri-miR sequences (Figure 3.16a). To determine whether the low levels of HBV expression knockdown was as a result of the inefficient processing and expression of the anti-HBV pri-miR sequences. Northern blotting was performed with RNA isolated from Huh 7 cells infected with PEPCK31/5HDAD or PEPCK31/589HDAD at MOI of a 1000 (Figure 3.16b). RNA isolated from Huh 7 cells infected with HDAd expressing pri-miR 31/589 from MTTR promoter at an MOI of 1000 was used as a positive control. After hybridisation with probes specific for guide 5, 8 and 9, a 21 nt band was detected with the positive control. Guide 5 probe also showed a higher molecular weight band representing unprocessed or partially processed intermediates. However, the guide sequences were not detectable in the RNA samples for the anti-HBV pri-miR sequences expressed from a PEPCK promoter, indicating inefficient expression of the anti-HBV pri-miRs. This analysis revealed that the PEPCK promoter could not efficiently express the anti-HBV pri-miR sequences.

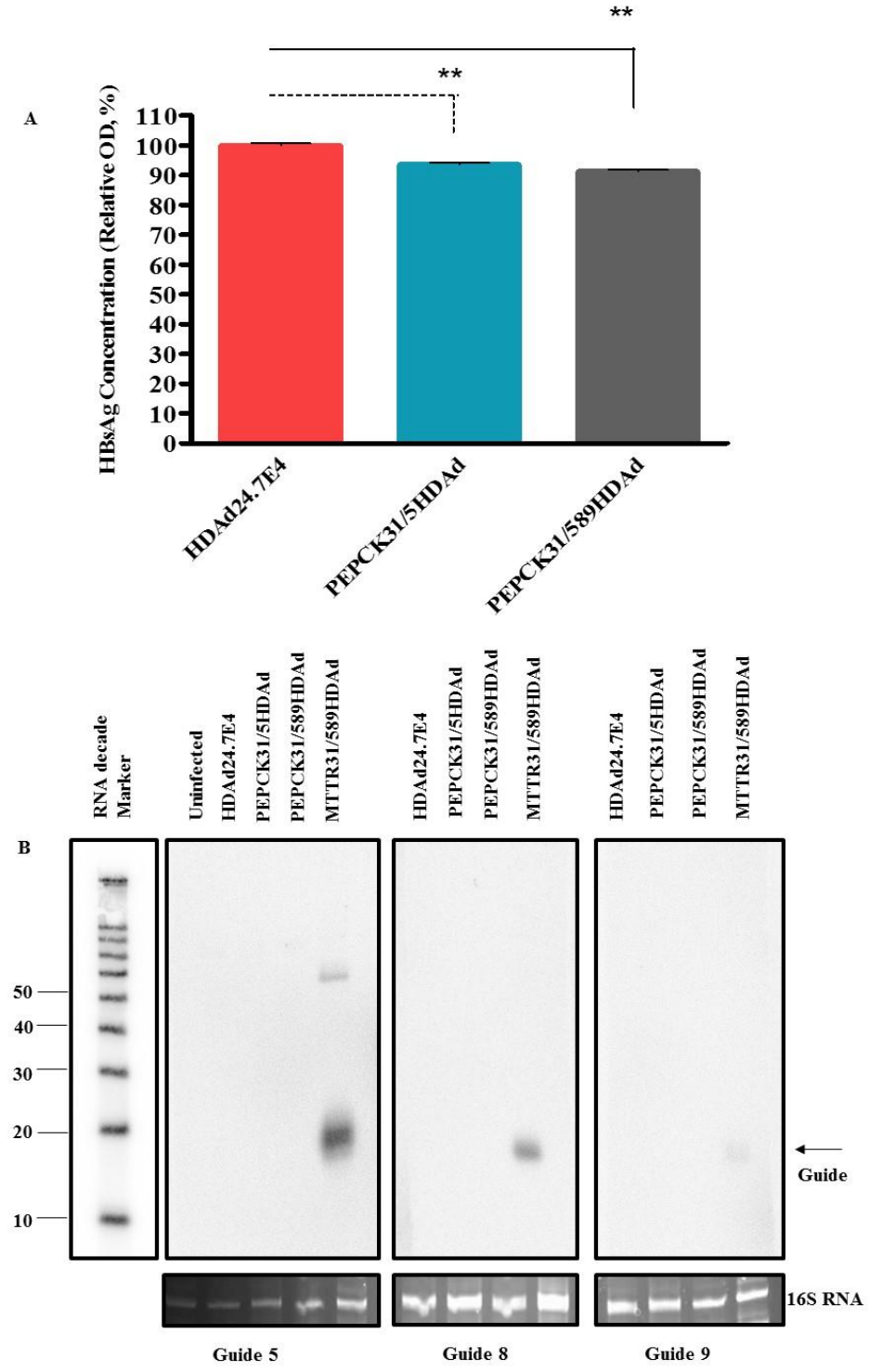


Figure 3.16. *In vitro* analysis of HBV knockdown and expression of anti-HBV pri-miR sequences.

A) Huh 7 cells were transfected with pCH-9/3091 and infected 5 hours later with HDAd24.7E4 or PEPCK31/5HDAd or PEPCK31/589HDAd at MOI 1000. Supernatant was collected after 48 hours and ELISA was performed to determine HBV knockdown by reduction in HBsAg concentration. PEPCK31/5HDAd and PEPCK31/589HDAd was normalized to HDAd24.7E4. ** Significant ($p<0.01$), T-Test, two tailed, unpaired. Error bars represented as Standard error of the mean (SEM) (n=4). **B)** *In vitro* Northern blot

analysis of 30µg of RNA extracted from Huh 7 cells infected with HDAd24.7E4 or PEPCK31/5HDAd or PEPCK31/589HDAd at a MOI of 1000. Probing was performed with probes complementary to guide 5, -8 and -9. Equal loading of wells was determined by the visualization of the RNA after Ethidium bromide staining.

To determine which MOI will result in enough pri-miR expression and HBV gene silencing HBsAg ELISA and Dual Luciferase assay were performed at different MOIs. A dose-dependent reduction between 10 % and 40 % in the HBsAg secretion levels were observed (Figure 3.17). The higher and significant reduction ($p<0.01$) of HBsAg expression HBsAg at an MOI of 20000 was observed with PEPCK31/589HDAd. This indicated that PEPCK31/589HDAd was more effective in HBV silencing compared to PEPCK31/5HDAd. However, the HBV knockdown was non-significant ($p>0.05$) for remainder of the MOI's 2000 and 10000 for PEPCK31/5HDAd and PEPCK31/589HDAd. Based on these observations and compared to previous studies (Mowa *et al*., 2012, Mowa *et al*., 2014), a high amount of virus is required to obtain a higher and significant level of HBV knockdown by the anti-HBV pri-miR's expressed from PEPCK promoter. This suggests that anti-HBV pri-miR expression was too low to be detected on a northern blot analysis when MOI 1000 was used.

To validate the results obtained with ELISA, Dual luciferase reporter assay was performed. The psiCHECK-*HBx* plasmid, which contains the *HBx* sequence downstream of the *Renilla luciferase* ORF was used. This renders the *Renilla luciferase* susceptible to silencing by anti-*HBx* sequences such as pri-miR, while the *Firefly luciferase* expression remains unaffected. Therefore, HBV knockdown is determined by the ratio of *Renilla luciferase* to *Firefly luciferase.* This entailed the transfection of Huh 7 cells with psiCHECK-*HBx*, followed by infection with HDAd24.7E4 (negative control) or PEPCK31/5HDAd or PEPCK31/589HDAd at the same MOI's of 2000, 10000 and 20000. Cell lysates were then used for luciferase assay. To determine HBV knockdown, the ratio of *Renilla luciferase* expression to *Firefly luciferase* expression for PEPCK31/5HDAd and PEPCK31/589HDAd was normalized to HDAd24.7E4 ratios.

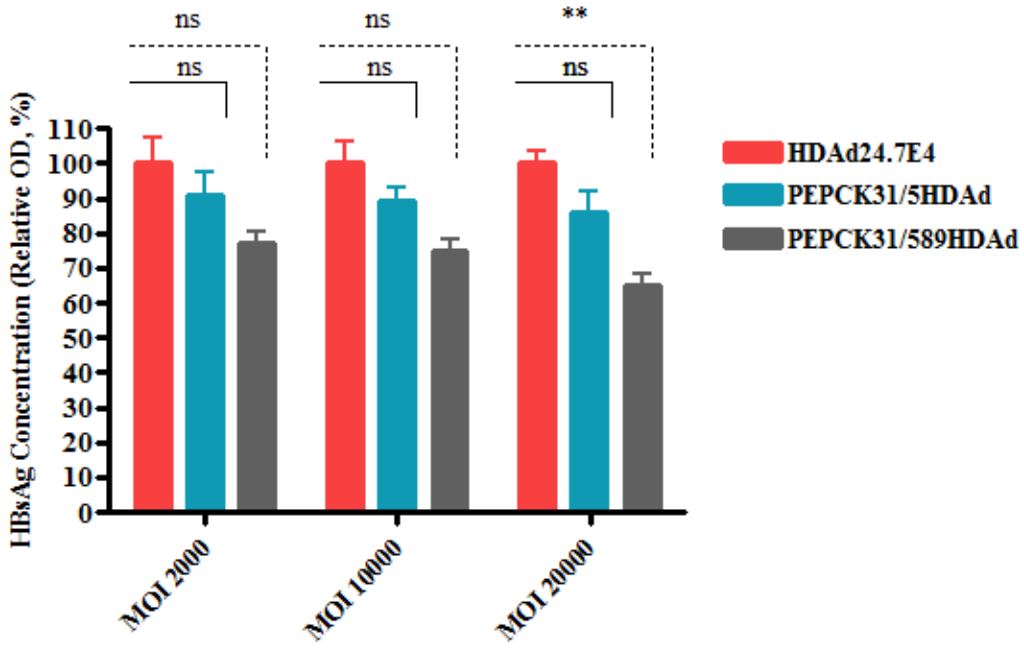


Figure 3.17: Assessment of HBV knockdown by anti-HBV pri-miR by ELISA.
Huh 7 cells were transfected with pCH-9/3091. After 5 hours the cells were infected with HDAd24.7E4 or PEPCK31/5HDAd or PEPCK31/589HDAd at MOI's of 2000, 10000 and 20000. Supernatant was collected after 48 hours and ELISA was performed to determine HBV knockdown by reduction in HBsAg concentration. The PEPCK31/5HDAd and PEPCK31/589HDAd was normalized to HDAd24.7E4. ** Significant ($p<0.01$), ns – Non-significant ($p>0.05$), T-Test, two tailed, unpaired. Error bars represented as Standard error of the mean (SEM) (n=4).

Similar to ELISA, a reduction in HBV expression between 10 % and 50 % was obtained for both PEPCK31/5HDAd and PEPCK31/589HDAd at the different MOI's (Figure 3.18). A significant ($p<0.05$) decrease was observed at MOI 2000 and 20000 for PEPCK31/589HDAd, which had a greater percentage of HBV silencing compared to PEPCK31/5HDAd. Non-significant ($p>0.05$) reduction at the remainder of the MOI's for both PEPCK31/589HDAd and PEPCK31/5HDAd was observed. The highest MOI of 20000 caused the greatest decrease in HBV expression, again confirming the requirement of a higher MOI for effective HBV replication inhibition by these HDAds.

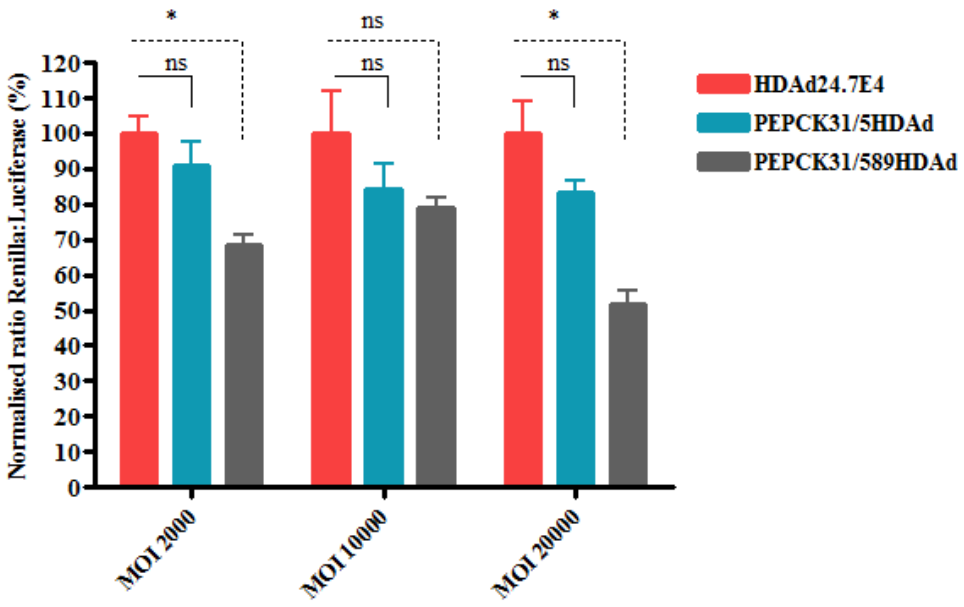


Figure 3.18: Dual luciferase reporter assay to determine HBV knockdown by anti-HBV pri-miR.
Huh 7 cells were transfected with psiCHECK-*HB*x. Five hours after transfection, the cells were infected with HDAd24.7E4 or PEPCK31/5HDAd or PEPCK31/589HDAd at MOI of 2000, 10000 and 20000. Cells were harvested in lysis buffer after 48 hours and Dual Luciferase reporter assay was performed to determine HBV knockdown as a ratio of *Renilla* luciferase:*Firefly luciferase*. PEPCK31/5HDAd and PEPCK31/589HDAd were normalized to HDAd24.7E4. * Significant ($p<0.05$), ns – Non-significant ($p>0.05$), T-Test, two tailed, unpaired. Error bars represented as Standard error of the mean (SEM) (n=4).

3.5 Assessment of pri-miR expression and HBV knockdown *in vivo*

Previous studies have shown a high PEPCK promoter driven transgene expression from HDAds *in vivo* (Brunetti-Pierri *et al*., 2006, Mian *et al*., 2004). To determine if PEPCK promoter is stronger *in vivo* than *in vitro*, mice studies were performed. As PEPCK31/589HDAd showed better effect against HBV *in vitro*, it was used to assess the inhibition of HBV replication in mice. HBsAg levels were measured in mice injected with saline or 1×10^{10} vps of HDAd24.7E4 or PEPCK31/589HDAd. Blood serum was collected one week and two weeks after injection and ELISA was performed to determine HBV knockdown relative to the control HDAd24.7E4. However, there was no HBsAg levels reduction observed after both one week and two weeks. This observation showed that there was no significant reduction in the *in vivo* HBV replication inhibition by the anti-HBV pri-miR HDAd (Figure 3.19a). This was supported by lack of anti-HBV pri-miR

expression after one week injection of PEPCK31/589HDAd and Northern blot analysis (Figure 3.19b).

These observations could be as a result of ineffective liver transduction by the HDAd viral particles or induction of a strong immune response that lead to early vector clearance.

3.6 Liver transduction by HDAd vectors expressing anti-HBV pri-miR

To determine if hepatocyte transduction was achieved, viral particle equivalents were measured in HDAd infected mice livers by qPCR (Figure 3.20). Mice livers were harvested one week post-injection with saline or 1×10^{10} vps of HDAd24.7E4 or PEPCK31/589HDAd. DNA extracted from the livers was used for qPCR with primers specific for HDAd. As expected, the results showed that mice administered with saline had no HDAd viral particles present. On the other hand, the mice subjected to HDAd24.7E4 or PEPCK31/589HDAd infection showed the presence of HDAd viral particles between 2 to 3×10^5 VPEs/µg DNA. Thus, efficient delivery of HDAd24.7E4 and PEPCK31/589HDAd viral particles to hepatocytes was achieved. Meaning that the therapeutic HDAd viral particles transduced the liver effectively and compared to previous study (Mowa *et al*., 2014) HDAd present within the livers was enough to result in a significant HBV knockdown.

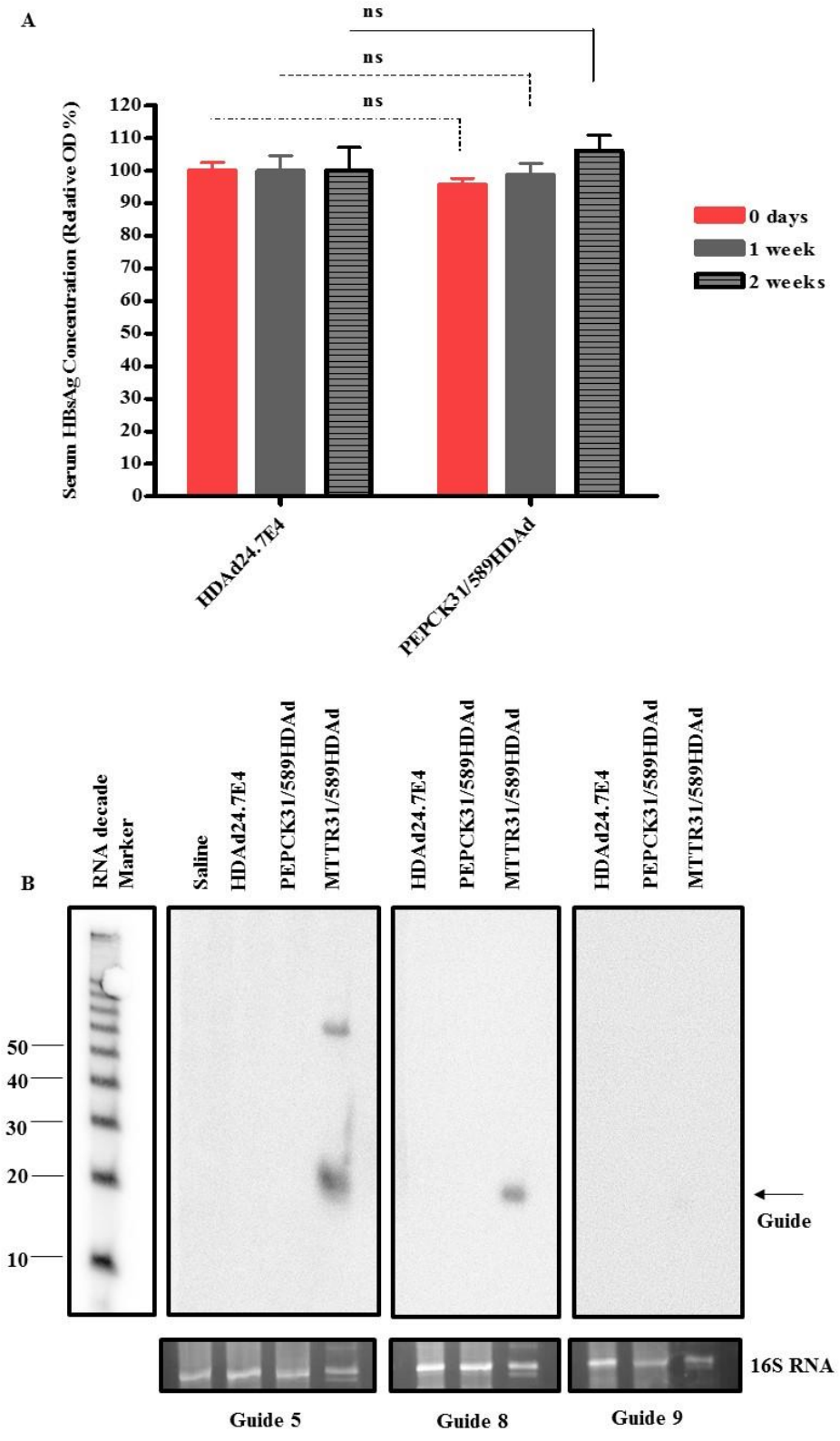


Figure 3.19: Assessment of *in vivo* knockdown of HBV replication and anti-HBV pri-miR expression from a PEPCK promoter.

The mice were administered separately with Saline or 1×10^{10} vps of HDAd24.7E4 or PEPCK31/589HDAd through tail vein injection. **A)** Blood serum was collected through retro-orbital puncturing before injection one week and two weeks after injection and ELISA was performed. Knockdown of HBV gene expression by PEPCK31/589HDAd was

determined relative to the control virus HDAd24.7E4. T-test, unpaired, two-tailed, ns-non-significant Error bar presents as standard error of the mean (n=6). **B)** Liver infected with HDAd24.7E4 or PEPCK31/589HDAd were harvested and 30 µg of RNA was analysed and probed with oligonucleotides complementary to guide 5, 8 and 9. RNA from Huh 7 cells infected with HDAd expressing pri-miR 31/589 from MTTR promoter at MOI of 100 was used as a Northern blot control. Equal loading of wells were determined by the visualization of RNA after Ethidium bromide staining.

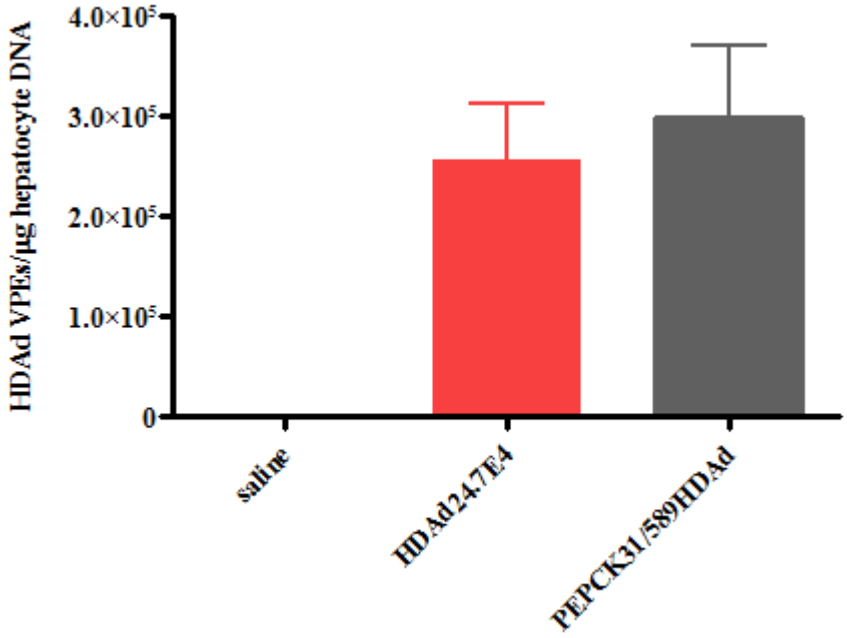


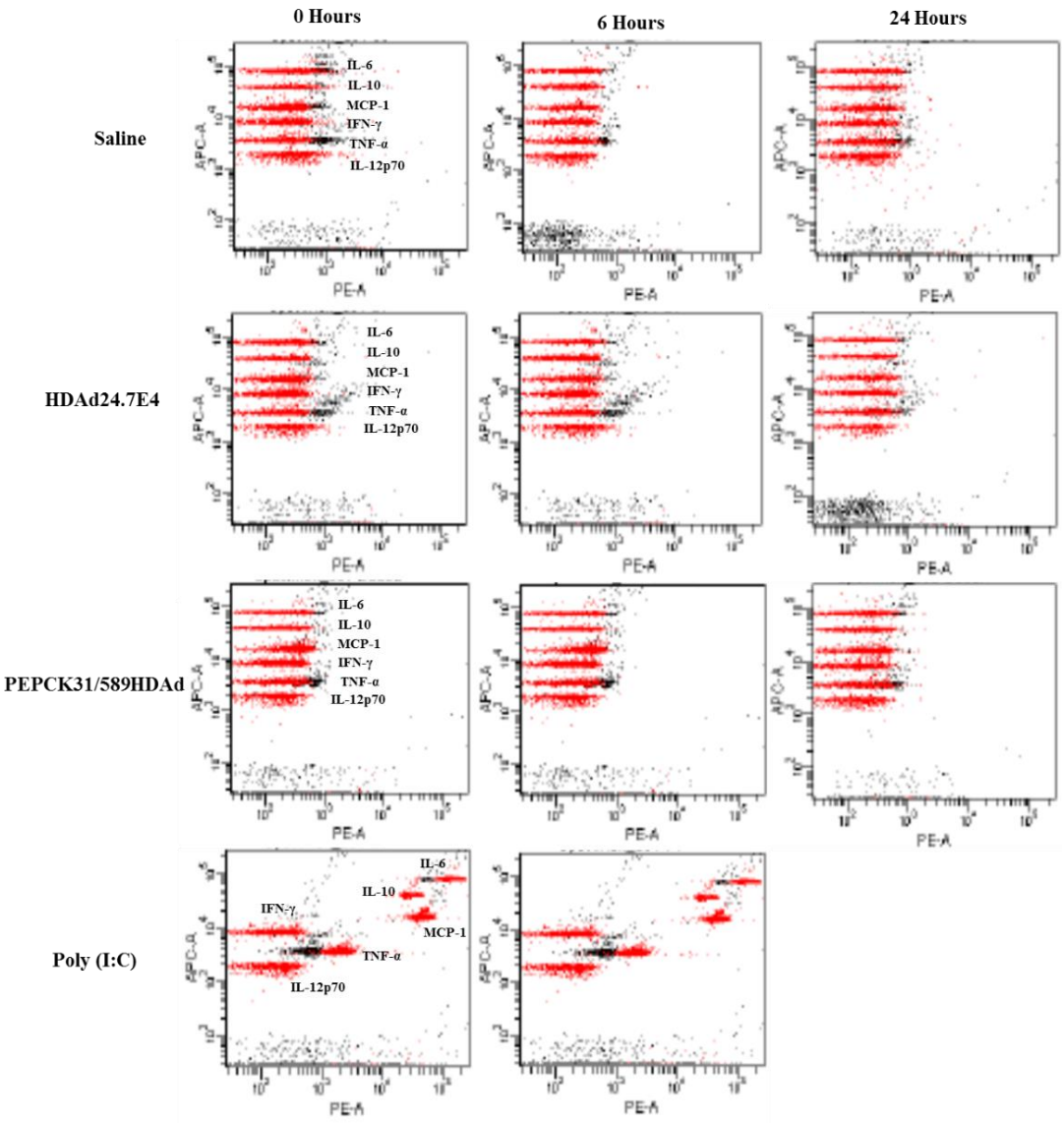
Figure 3.20: Intrahepatic HDAd viral particle levels.
HBV transgenic mice were injected with saline or 1×10^{10} vps of HDAd24.7E4 or PEPCK31/589HDAd. One week post-infection, DNA was extracted from the infected livers and qPCR was performed with HDAd specific primers. Error bars represented as standard error of the mean (SEM) (n=4).

3.7 HDAd viral particles did not induce a pronounced inflammatory response or liver toxicity

To determine if HDAds administered induced a strong acute inflammatory response that may result in toxicity and vector clearance, levels of secreted pro-inflammatory cytokines and liver toxicity marker Alanine Aminotransferase (ALT) were measured. IL-12p70, IFN-γ, TNF, MCP-1, IL-10 and IL-6 levels were measured at 0, 6 and 24 hours after the administration of saline or 1×10^{10} HDAd particles in mice using CBA assay. Poly (I:C) was used as a positive control and mice were euthanized after 6 hours due to high toxicity induced by this RNA. Mice injected with Poly (I:C) showed a significant (*P<0.05 and

**P<0.01) elevation of TNF, MCP-1, IL-10 and IL-6 but not for IL-12p70 and IFN-γ after 6 hours. In the contrary, out of all the cytokines measured only MCP-1 and IL-12p70 levels were elevated when compared to saline control for PEPCK31/589HDAd and HDAd24.7E4 after 6 hours respectively. However, MCP-1 and IL-12p70 increase was not statistically significant (p>0.05) and both cytokines were back to normal levels at 24 hours post infection (Figure 3.21a,b). Therefore, no concerning inflammation was induced by the HDAds.

A)



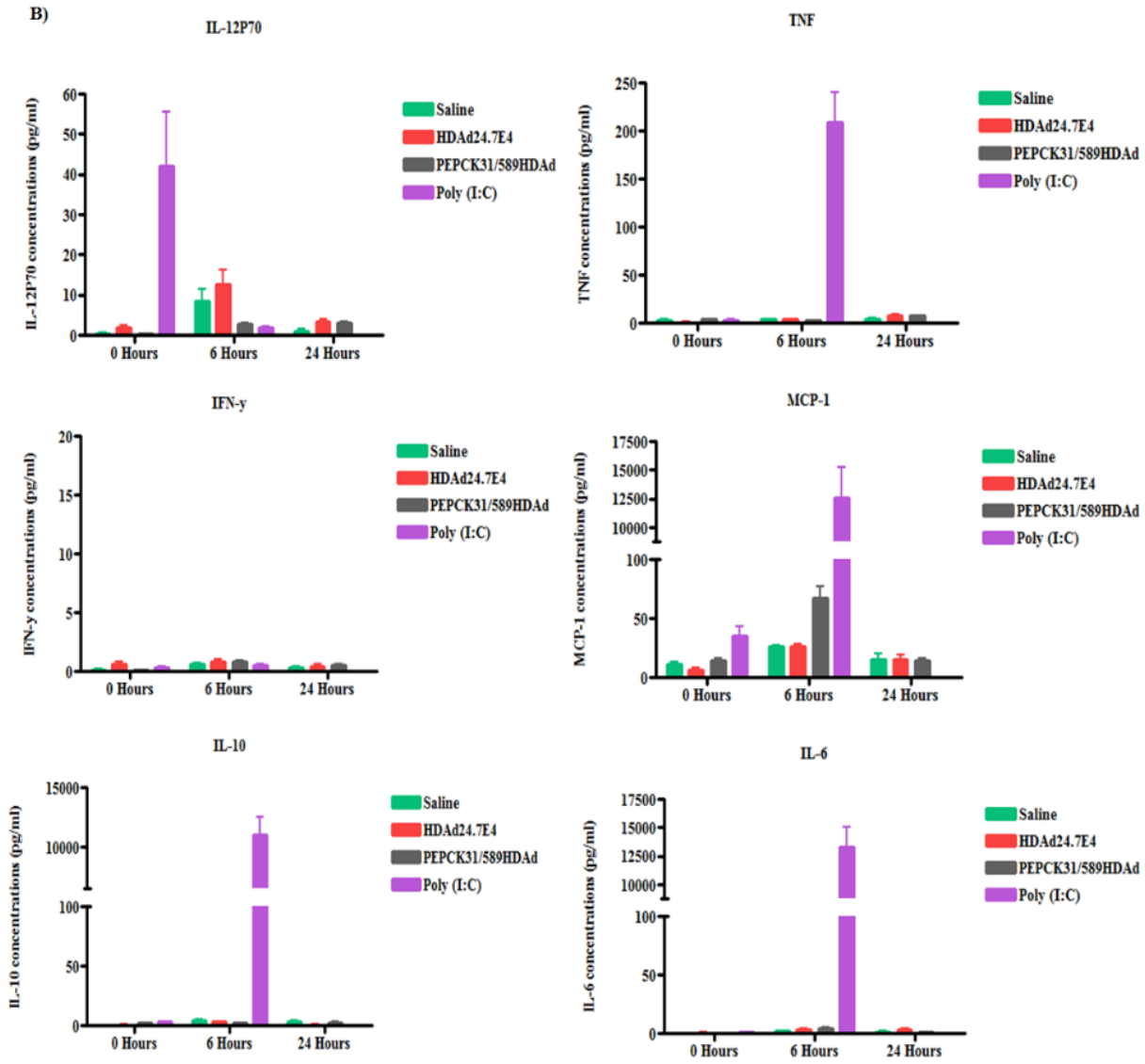


Figure 3.21: CBA analysis after HDAd viral particle administration.
The single dose of saline, Poly (I:C) or 1 × 10^{10} vp of HDAd24.7E4 and PEPCK31/589HDAd was administered to HBV transgenic mice. Blood was collected through retro-orbital puncturing at 0, 6 and 24 hours after injection. The pro-inflammatory cytokines; IL-12p70, IFN-γ, TNF, MCP-1, IL-10 and IL-6 were measured with the CBA assay. **A)** Representation dot plot from CBA, PE-A - Phycoerythrin conjugate and APC-A – Allophycocyanin conjugate. **B)** Serum cytokine concentrations. Serum cytokine concentrations were determined relative to saline for that time point. T-test, two-tailed, unpaired, * significant ($p<0.05$), ** significant ($p<0.01$). Error bars represented as standard error of the mean (SEM) (n=5).

The hepatotoxicity of HDAd vectors were determined by measuring ALT levels present in the liver as a marker of toxicity (Figure 3.22). Serum from blood collected from the mice at 3 days, 7 days and 14 days after HDAd administration was used. The ALT levels varied between 25 - 44 U/L for all the groups over 14 days. These ALT levels were within the

acceptable level of 100 U/L. Lack of hepatotoxicity was confirmed by normal weight maintained by all the mice over the period of the experiment (Figure 3.23).

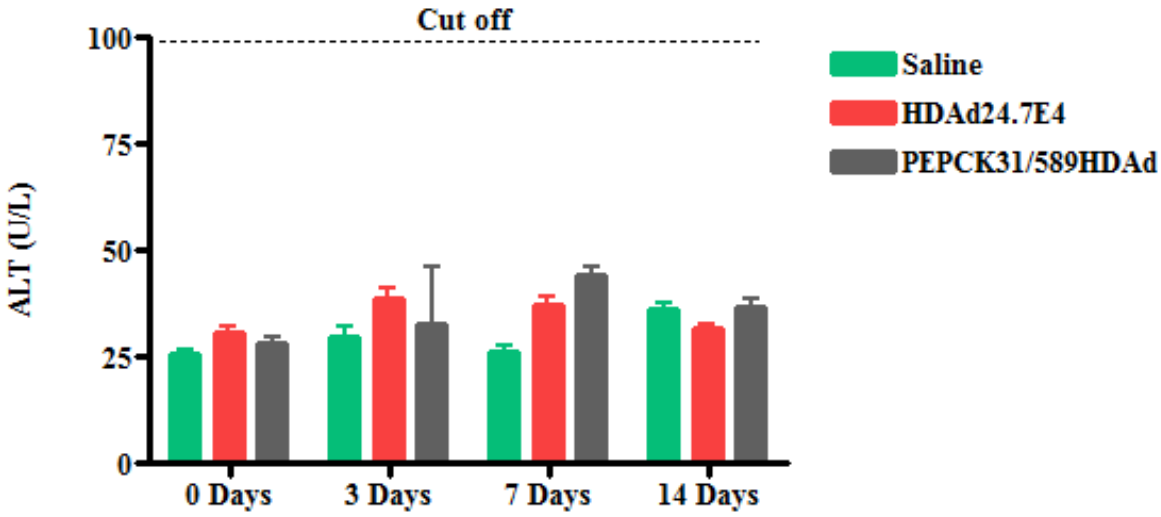


Figure 3.22: Alanine Transaminase activity after HDAd vector administration.
Mice were injected with Saline or 1×10^{10} vps of HDAd24.7E4 or PEPCK31/589HDAd. Blood serum was collected and ALT levels were measured at 0 days, 3 days, 7 days and 14 days. Error bars represented as standard error of the mean (SEM) (n=6). Dotted line indicates acceptable range for ALT levels of up to 100 (U/L).

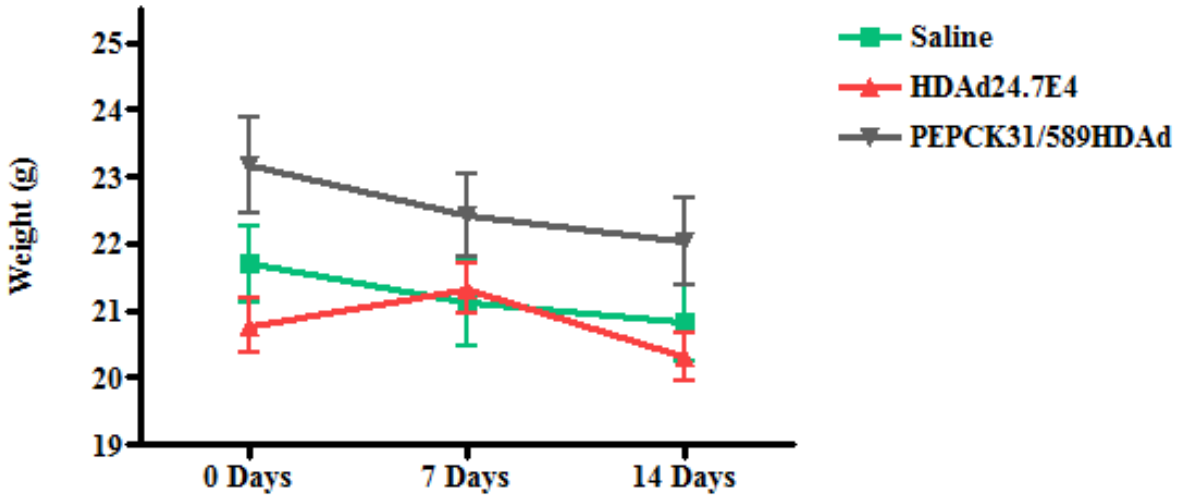


Figure 3.23: Line graph representing the weights of the mice.
Mice were weighed before and weekly post-infection with Saline or HDAd24.7E4 or PEPCK31/589HDAd.

CHAPTER 4

4 DISCUSSION

Hepatitis B is a global health problem that infects approximately 2 billion people worldwide of which 240 million are chronic cases. The discovery of effective therapies against HBV infection is key to reducing the spread of this condition. The exploitation of the RNAi pathway for the sequence specific silencing of genes is well established as a research tool in gene therapy (Elbashir *et al*., 2001, McBride *et al*., 2008, McCaffrey *et al*., 2003, Peng *et al*., 2005). Studies have shown the successful therapeutic application of the RNAi technology (RNAi activators) for the treatment of serious viral diseases. Harnessing of RNAi to inhibit HBV replication has shown a lot of promise. (Peng *et al*., 2005, Gitlin *et al*., 2002, Hamasaki *et al*., 2003, Jacque *et al*., 2002, Lee *et al*., 2002, Wilson *et al*., 2003). RNAi activators such as monocistronic (pri-miR 31/5 and pri-miR 122/5) and trimeric (pri-miR31/589) anti-viral expression cassettes have successfully been designed within AGTRU. These anti-viral expression cassettes, expressing anti-HBV pri-miR from Pol II promoters have shown to be successful against HBV (Ely *et al*., 2009, Ivacik *et al*., 2015, Mowa *et al*., 2012, Mowa *et al*., 2014).

The lack of safe, efficient delivery and expression system for these anti-HBV pri-miR's are one of the obstacles that needs to be overcome before these RNAi effectors can reach clinical application. This study therefore focused on obtaining efficient, tissue specific expression and a safer delivery system for anti-HBV RNAi activators.

4.1.1 Successful Construction of *lac Z* deficient HDAd genome plasmids carrying PEPCK-pri-miR cassettes.

Mowa and colleagues performed one of the first experimental studies using HDAds expressing anti-HBV pri-miR from a CMV promoter. These vectors transduced 90 % of hepatocytes and inhibited HBV replication efficiently *in vivo* without toxic side effects for 1 to 5 weeks (Mowa *et al*., 2012). To prolong the transcription and HBV inhibition, the CMV promoter in the anti-HBV cassettes were replaced with a liver specific modified mouse transthyretin (MTTR) promoter and delivered using lentiviral vectors (LVs) or

HDAds (Ivacik *et al*., 2015, Mowa *et al*., 2014). Data collected indicated that LVs expressed the transgenes and silenced the HBV genes over a course of 1 year within the liver of the mice (Ivacik *et al*., 2015). However, *in vitro* and *ex vivo* gene delivery with LVs is still limited by the low titers produced during viral production (Kubo and Mitani, 2003, Nguyen *et al*., 2002). Furthermore, another concern is the potential oncogene activation and/or tumor suppressor genes caused by LV integration into the host genome. As well as the pathogenicity that may be caused by the recombination and regeneration of the wild type virus, HIV-1 (Matrai *et al*., 2010, Manjunath *et al*., 2009).

HDAds carrying anti-HBV pri-miR expressed from a liver-specific MTTR promoter resulted in expression and processing of designed anti-HBV pri-miRs that is more sustained when compared to that of the CMV promoter (Mowa *et al*., 2014). However, HBV knockdown was over a short time period and an increase in HBsAg levels were observed after 3 weeks for the monomeric pri-miR cassettes and after 5 weeks for the trimeric pri-miR cassettes (Mowa *et al*., 2012, Mowa *et al*., 2014). Improvement of these MTTR-pri-miR carrying HDAds is currently being investigated in our lab. The use of vectors bearing a PEPCK promoter has shown long-term liver specific gene expression in various studies (Brunetti-Pierri *et al*., 2007, Mian *et al*., 2004). Mian and colleagues observed the rectification of orotic aciduria with considerable OTC activity for 12 months (length of experiment) in the absence of chronic liver toxicity. By expressing human ornithine transcarbamylase (hOTC) in the presence or absence of a Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) from a PEPCK promoter (Mian *et al*., 2004). Brunetti-Pierri and co-workers observed long-term (> 1 year) and stable hepatic expression of the α-fetoprotein (bAFP) from a PEPCK promoter in non-human primates in the absence of chronic liver toxicity (Brunetti-Pierri *et al*., 2007, Brunetti-Pierri *et al*., 2006). However, the application of this promoter to express RNAi activators has never been explored. For that reason, this study investigated the possibility of using the PEPCK promoter for a sustained liver specific expression of anti-HBV pri-miR mimics.

To detect successful delivery and tissue transduction, reporter genes such as *lac* Z have been incorporated into delivery vectors such as FGAd and HDAd. However, an induction by β-galactosidase of the adaptive immune response and/or cellular immune response of the host results in loss of viral gene expression and destruction of transduced cells (Mian *et al*., 2005, Morral *et al*., 1997, Rauschhuber *et al*., 2008). Studies performed showed

similar results which presented the activation of the immune response and production of neutralizing antibodies against Adenoviral vectors bearing the *lac* Z report gene (Sullivan *et al*., 1997, Crowther *et al*., 2014, Mowa *et al*., 2014). As a result, expression of the transgenes were short-lived due to clearance of the Ad vectors by the activation of the immune response. Hence, the removal of the *lac* Z reporter gene from HDAd vectors could lengthen liver transduction and antiviral sequence expression by reducing immune stimulation, which in turn could prolong HBV knockdown.

Because of their larger size (~ 27- 32 kb), cloning into HDAd genome carrying plasmids is difficult. This is as a result of difficulties to ligate into the HDAd backbone and the lower transformation efficiencies that are usually obtained. Hence, some studies have used homologous recombination between a shuttle vector carrying the transgene and an Adenoviral backbone in place of the conventional ligation. In this system, the shuttle vector is transformed into the *E. coli* cells containing the Ad plasmid and recombinants selected. This method eliminates the need for large Ad backbone manipulation by restriction digest and ligations (He *et al*., 1998, Luo *et al*., 2007). In addition, transformation efficiency can be increased by the use of highly effective transformation techniques like electroporation. Although the generation of *lac* Z deficient Helper Dependent Adenoviral plasmids expressing anti-HBV pri-miR's from a liver-specific PEPCK promoter was challenging, the construction was successful. The constructed anti-HBV HDAd plasmids were confirmed by multiple restriction digests and used to produce anti-HBV HDAds vectors.

4.1.2 Cysteine modification in the Adenoviral capsid alters the particle structure and impairs helper virus role in HDAds production

In addition to the removal of reporter genes in HDAds, immune stimulation by the viral capsid proteins through site specific PEGylation can be performed. To facilitate residue specific and thiol reactive PEGylation, cysteine residues were previously incorporated into the HVR5 of Ad helper virus [AdNG163Cys, (Kreppel *et al*., 2005, Prill *et al*., 2011)]. Hence, packaging and propagation of the HDAd genomes in capsid encoded by this helper virus will bear the modification in their capsid. PEGylation of Ad capsid proteins have shown to reduce the cytotoxicity and innate and adaptive immune response against the

viral proteins, thereby extending the duration of transgene expression (Croyle *et al*., 2001, Mok *et al*., 2005, Crowther *et al*., 2008). Furthermore, unlike amine reactive PEGylation, site specific thiol reactive PEGylation showed an increase in hepatocyte transduction, higher particle uniformity by allowing precise shielding at the desired sites, and a reduction in the amount of polymers required to shield the capsid epitopes (Kreppel *et al*., 2005, Prill *et al*., 2011).

An attempt to use AdNG163Cys HV in this study revealed interesting features of the HV as a result of the HVR5 cysteine modification. During the propagation of the AdNG163Cys helper virus, immunostaining showed the aggregation of the helper virus. This was confirmed by the fact that HDAd produced using the AdNG163Cys HV also seemed to be aggregating. Failure to amplify HDAd using this HV also suggest impairment in transduction. The comparison of infection efficiency between the modified and unmodified (AdNG163) virus confirmed that the infection efficiency of the cysteine modified virus was diminished, indicated by minimal cytopathic effect induced by AdNG163Cys (Figure 3.11). The introduction of specific genetic mutations could possibly bring about the misfolding of the modified viral proteins, influencing the structure of the viral capsid, as well as the function of resultant virus itself. TEM images (Figure 3.10) obtained for both cysteine modified (AdNG163Cys) and unmodified helper virus (AdNG1563), showed that the AdNG163 was 70-80 nm in size, icosahedral in shape and the intact capsids were visible. Interestingly, a distinct change in the morphology of AdNG163Cys was observed. The icosahedral shape could not be seen and the capsid was deformed. These changes suggest that the cysteine modification influenced the helper virus protein folding, assembly, and biological function. However, these findings were not in agreement with studies performed by other authors, which indicated that the structure and biological function of the virus with the cysteine modification at the HVR5 was not influenced by the capsid modification with the cysteine residues (Kreppel *et al*., 2005, Prill *et al*., 2011). Prill and colleagues applied this site specific PEGylation method to improve the delivery of Adenoviral vectors to the liver by using thiol reactive PEG, which react with thiol groups in cysteine residues that was introduced into the hypervariable region 5 (HVR 5) of the hexon protein successfully. However, they showed that certain single point mutation could either decrease or increase hepatocyte transduction, and particle uniformity (Prill *et al*., 2011).

On the other hand, Kreppel and colleagues performed the study with E1 deleted Ad5 adenoviral vectors that have been genetically modified with cysteine residues into the H1 loop of the Ad fiber protein. During the process of viral purification, aggregates were observed. Suggesting that the thiol groups formed interparticle disulphide bonds, which could be reduced by the addition of a reducing agent. However, aggregates could not be sufficiently resolved for Ads carrying one (Ad1Cys) or three (Ad3Cys) genetically introduced cysteine residues (Kreppel *et al*., 2005). With the challenge to reduce the aggregation that have been observed with the AdNG163Cys, HDAd production in this study can be attempted under reducing condition with either Dithiothreitol (DTT) or Tris(2-carboxyl)phosphine) [TCEP] reducing agents that could control the reactivity of the thiol groups. However, to overcome deformities observed in our hands by TEM, the HV virus used will need to be sequenced to determine the possibility of other mutation within the hexon gene.

4.1.3 Production, propagation and activity of unmodified HDAds *in vitro* and *in vivo*

Unlike with AdNG163cys, HDAds were produced effectively using AdNG163. Based on the β-galactosidase expressing vector produced in parallel, about 3.160×10^4 infectious viral particles were produced. This could easily be amplified to about 4.260×10^9 ifu/ml after five passages. These results obtained are in correlation with the findings and protocol by Palmer and Ng, (2008b), which indicated that optimal 10^8 - 10^9 ifu/ml could be obtained after production and amplification in plates. All the HDAds were successfully produced in a large scale production and purified with a two-step CsCl gradient. A single band was observed after each CsCl gradient step (Figure 3.13). Quantification by qPCR indicated that the total viral particles of the purified viruses varied between 10^{11} and 10^{13} while the helper virus contaminations were routinely between 0.07 % and 1 %. The HDAd quantification and percentage of HV contamination was within the expected range of 10^{11} to 10^{13} purified vector particles with HV contamination of $\leq$ 1 % that is normally obtained during large scale production that uses the Cre/loxP system (Ng *et al*., 2002, Palmer and Ng, 2008a).

To test for activity of PEPCK promoter in our hands a *lac Z* deficient HDAd expressing *Firefly luciferase* was produced and characterised *in vitro* and *in vivo*. Experiments in both

Huh 7 cells and mice showed a significant expression of *Firefly luciferase* (Figure 3.14, Figure 3.15). Bioluminescence in mice illustrated sustained liver specific expression of *Firefly luciferase* over a time period of 4 weeks post infection (period of the experiment), with the maximum expression observed at 2 weeks post infection (Figure 3.15). Similar results were obtained previously where a significant *Firefly luciferase* gene expression was observed with a plasmid expressing *Firefly luciferase* under the control of PEPCK (pPEPCK-Fluc) in cancerous liver cells and non-tumorigenic liver cells (Song and Lee, 2006). The results confirm that the PEPCK promoter is highly active in the liver. However, to confirm even longer sustainable expression in liver, *Firefly luciferase* expression is currently being monitored over a period longer than four weeks. Nevertheless, this motivated the aim to design PEPCK promoter driven pri-miR constructs to achieve liver specific expression and HBV replication silencing.

The proven liver specificity and long-term transgene expression observed in the liver with the PEPCK promoter from this study and others (Brunetti-Pierri *et al*., 2007, Brunetti-Pierri *et al*., 2006, Mian *et al*., 2004, Song and Lee, 2006), made it a suitable promoter for anti-HBV pri-miR expression and sustained HBV knockdown analysis. However, the HBV knockdown analysis only showed marginal HBV gene expression silencing, with higher quantity of HDAd (MOI 20000) required to obtain ~50 % knockdown *in vitro* (Figure 3.17 and Figure 3.18). Regardless of the low percentage of HBV inhibition, the trimeric pri-miR expressing HDAd demonstrated more efficient HBV silencing. This agrees with previous HBV knockdown studies performed by Mowa and colleagues whereby the trimeric pri-miR 31/589 showed a significant decrease in HBV gene expression over a longer period (5 weeks) compared to the monomeric pri-miR 31/5 and pri-miR 122/5 (Mowa *et al*., 2014, Mowa *et al*., 2012). The requirement of high dosages for PEPCK31/5HDAd and PEPCK31/589HDAd in culture contradicts with previous studies performed with anti-HBV pri-miR's HDAd expressed from a MTTR promoter. The study performed showed efficient HBV replication knockdown of ~91 % at MOI's of 500 and 1000 in Huh 7 cells (Mowa *et al*., 2014). There are several possibilities that may result in marginal pri-miR expression and HBV gene silencing observed in this study. While reporter gene removal is desirable for *in vivo* studies, it also comes with its limitations. Meaning that the qPCR quantification method used does not discriminate between infectious and uninfectious viral particles. Similar conclusions were made by Crettaz *et al.,* (2008) using the qPCR based method to determine the total viral particles in

the viral stock, whereby the total viral particles were ~10^{11} and the infectious units of the same viral stock was ~10^{10} (Crettaz *et al*., 2008). To improve the quantification and obtain a more accurate measurement of the produced infectious units of HDAds, a DNA-based technique known as the slot-blot assay was describe by (Kreppel *et al*., 2002), briefly cells are infected with diluted HDAd virus and incubated for 15 hours. The cells are then harvested and lysed. The lysates are then slot blotted onto a nylon membrane submerged in wash buffer. The membrane is then cross-linked and hybridized with [^{32}P] labelled probes specific for the left ITR of Ad5 and probes specific for the HV. Signal from exposure is then measured using a phosphoimager.

The other possibility could be that PEPCK promoter might not be a strong enough promoter to drive RNAi activator expression such that the desired HBV knockdown is achieved. This was supported by no expression of pri-miRs observed when RNA was isolated from cells infected with HDAds at MOI of 1000 (Figure 3.16b). Studies performed within primary hepatocytes to determine the strength of expression of different promoter revealed that PEPCK had a lower activity *in vitro* (primary hepatocytes) compared to viral promoters such as CMV. However, when tested in transgenic mice the promoter activity could be stimulated for high mRNA expression (Ponder *et al*., 1991). In addition, most of the studies that showed long-term transgene expression by the PEPCK promoter, were conducted *in vivo* (Brunetti-Pierri *et al*., 2006, Brunetti-Pierri *et al*., 2009, Brunetti-Pierri *et al*., 2007, Mian *et al*., 2004). We therefore speculated that PEPCK promoter could be highly active *in vivo* than *in vitro*. Because previous studies have shown that total viral particles determined by qPCR are usually about half a log to a log higher than infectious units (Crettaz *et al*., 2008), *in vivo* studies were performed by injecting mice with 1×10^{10} viral particles per mouse, which was double the standard dose of 5×10^{9} viral particles per mouse. However, the *in vivo* analysis of HBV knockdown showed no significant change in the HBV replication at 1 or 2 weeks post HDAd infection (Figure 3.19a). Supporting the *in vitro* data, there was no pri-miR expression detected in mice at one week post infection with PEPCK31/589HDAd (Figure 3.19b).

Even though expression of pri-miR at HDAd MOI of 1000 could not be detected by Northern as seen in previous studies (Mowa et al., 2012, Mowa et al., 2014) , a lower but significant HBV knockdown was observed (Figure 3.16a,b). Indicating that the PEPCK promoter was not efficient for the *in vitro* expression of pri-miR. Furthermore, the

complete lack of HBV gene silencing *in vivo* could also be explained by the nature of the mouse model used in this study. In our hands mice with medium HBsAg (OD 0.4 - 0.6) are the relevant ones and demonstrate HBV gene silencing by RNAi activators. However, as the majority of the transgenic pups have very high HBsAg levels (OD 0.8 - 1), which may not mimic the exact situation in human, these high titer mice were used. Hence, these experiments need to be repeated with medium HBsAg level mice. Alternatively, we speculated that HDAds used may not transduce the liver efficiently or induce an immune response that results in earlier vector clearance and toxicity.

4.1.4 HDAds transduce the liver efficiently and do not induce a significant inflammatory response and toxicity

HBVs ability to infect almost 100 % of liver hepatocytes, prompts the use of Ad vectors as a viable treatment method. The efficient hepatotropism of HDAd vectors have been proven beyond reasonable doubt (Brunetti-Pierri *et al*., 2012, Brunetti-Pierri and Ng, 2011, Brunetti-Pierri *et al*., 2006, Mian *et al*., 2004, Crowther *et al*., 2014, Mowa *et al*., 2012, Mowa *et al*., 2014). However, the lack of expected HBV replication inhibition by HDAds used in this study raised the necessity to confirm liver transduction. Transduction was assessed by qPCR using DNA isolated from mice liver at 1 week post infection. About 2×10^5 HDAd particles/µg DNA were detected in the livers (Figure 3.20). The VPEs obtained in this study were comparable to those obtained in the study by Mowa and co-workers where about 3×10^5 VPEs/µg were present in the hepatocytes at 1 week post infection and these gave about 90 % HBV knockdown. To determine if the removal of the *lac* Z reporter gene resulted in sustainable liver transduction (prevented or reduced the clearance of the therapeutic HDAd), the VPE's will have to be measured over a longer time period. However, this shows that the amount of vector administered results in enough liver transduction to effect HBV replication inhibition.

The activation of the immune response by Adenoviral vectors forms great reason for concern with the use of Ad vectors as a delivery system. This entails the activation of an acute innate inflammatory response and toxicity that is set in progress and Ad viral particles are cleared from the blood circulation (Brunetti-Pierri *et al*., 2009, Morral *et al*., 1997, Schnell *et al*., 2001, Tomasec *et al*., 2007). However, HDAd vectors were generated

to minimize the immune response and toxicity that have been associated with FGAd vectors by the removal of all the viral genes. This aids in diminishing the long-term humoral and cell-mediated adaptive immunity. Nevertheless, an immune response is activated by the viral capsid proteins of both infectious and non-infectious viral particles (Crowther *et al*., 2008, Crowther *et al*., 2014, Muruve *et al*., 2004, Schnell *et al*., 2001, Croyle *et al*., 2005). Therefore, the induction of inflammation by the administered PEPCK31/589HDAd and empty HDAd24.7E4 was determined using the CBA assay. Even though not comparable to Poly (I:C) control, there was an elevation in the MCP-1 and IL-12p70 (non-significant) in HDAd injected mice, however this returned to normal levels after a 24 hour period (Figure 3.21b). This finding correlates with previous studies performed within AGTRU, whereby HDAd vectors expressing shRNA (Crowther *et al*., 2008, Crowther *et al*., 2014) or pri-miR (unpublished) resulted in an elevation of MCP-1 6 hours after receiving the HDAd viral particles and reverted back to normal levels after 24 - 48 hours post-infection.

The expression of anti-HBV shRNAs have been reported to saturate the endogenous RNAi pathway, which results in liver toxicity (Grimm *et al*., 2006). To determine if anti-HBV pri-miR HDAd vectors would result in liver toxicity, the ALT levels were determined and found to be within the acceptable range of 40 - 100 U/L (Figure 3.22). This is in agreement with results obtained for the administration of anti-HBV pri-miR HDAd bearing a CMV promoter (Mowa *et al*., 2014). Supported by the healthy average weights of the mice, this data indicated that the anti-HBV pri-miR HDAds used in this study were not toxic to the mice (Figure 3.23).

CHAPTER 5

5 CONCLUSION

This dissertation reports the first study of the incorporation of RNAi activators into a *lac* Z deficient HDAd vector under the control of a liver-specific PEPCK promoter. Liver-specific and sustainable gene expression from a PEPCK promoter was validated by the *Firefly luciferase* reporter gene expression *in vitro* and *in vivo*. While a PEPCK promoter is suitable for the expression of other genes such as *Fluc*, it is not adequate for RNAi activator expression. This work confirms that the removal of the viral genes from HDAd vectors markedly attenuates the immune response, which limits hepatotoxicity. Therefore, presenting the therapeutic potential and utility of HDAd vectors for hepatotropic delivery of antiviral sequences.

5.1.1 Future studies

To improve the expression of anti-HBV pri-miR, a different liver-specific promoter like MTTR could be used. Furthermore, the method of quantification will have to be optimized to quantify the infectious unit of the *lac Z* deficient HDAd viral stock, whereby a slot-blot method could provide a possible solution. Whether the elimination of *lac Z* from HDAds results in the reduced immune stimulation that translates to prolonged HBV gene silencing needs to be investigated further by performing long-term studies. This would provide some insight into the design of more desirable HDAd vectors. In order to reduce or completely eradicate the immune stimulation caused by the capsid proteins, PEGylation of the viral particles needs to be performed, followed by a comparison study between PEGylated and unPEGylated HDAd vectors.

CHAPTER 6

6 APPENDIX

6.1 Bacterial methods solutions and recipes

6.1.1 Luria Bertani (LB) agar medium

Ten grams of tryptone, 5 g of Yeast extract, 5 g NaCl and 10 g of agar was added to 1 L of distilled water. The medium was autoclaved for 20 minutes at 121 ℃ and 1 kg/cm^2. The solution was cooled to approximately 55 ℃ and the desire antibiotic (100 µg/ml Ampicillin or 50 µg/ml Kanamycin) was added. The agar was poured into bacterial petri dishes and allowed to set over night.

6.1.2 Luria Bertani (LB) Medium

Ten grams of tryptone, 5 g of Yeast extract and 5 g NaCl was added to 1L of distilled water. The medium was autoclaved for 20 minutes at 121 ℃ and 1 kg/cm^2.

6.1.3 X-gal (5-bromo-4-chloro-3-indoyl-b-D-galactopyranoside) reagent

Twenty milligrams of X-gal was dissolved in 1 ml of dimethyl formamide. Due to the light sensitivity, the solution was cover in foil and stored at -20 ℃ in a dark area.

6.1.4 Ampicillin stock solution (100mg/ml) (PanReac Applichem inc., MO, USA)

One gram of ampicillin was weighed out and dissolved in 5 ml of sterilized dH_2O and 5 ml of absolute ethanol (100 %). The solution was filter sterilized and stored at -20℃.

6.1.5 Kanamycin stock solution (50mg/ml) (Sigma-Aldrich, MO, USA)

Kanamycin of 0.25 g was weighed out and dissolved in 5 ml of sterilized dH_2O. The solution was filter sterilized and aliquoted into 500 µl working stocks. Kanamycin is light sensitive and aliquots were covered with foil and stored at -20℃.

6.1.6 Transformation buffer

A 100 ml of transformation buffer was prepared with 100 mM $CaCl_2$ (1.47 g), 10 mM PIPES-HCl (0.3 g) and 15 % Glycerol (15 ml) added to 70 ml of dH_2O. The pH was adjusted to 7 with NaOH, the solution was made up to 100 ml with dH_2O. The solution was autoclaved for 15 minutes at 121℃ and 1 kg/cm^2 and stored at 4℃.

6.2 Small scale plasmid isolation buffers

6.2.1 Buffer P1- resuspension buffer

One liter of buffer P1 was prepared as follow: 6.06 g Tris base and 3.72 g $Na_2EDTA.{}_2H2O$ was added to 800 ml of dH_2O. The pH was adjusted to 8 with HCl and volume was made up to 1 L with dH_2O. The buffer was autoclaved and allowed to cool down before adding 100 mg of RNase A. Solution was mixed thoroughly and stored at 4℃.

6.2.2 Buffer P2- lysis buffer

One liter of buffer P2 was prepared as follow: 8 g NaOH pellets and 10 g SDS was dissolved in 500 ml dH_2O, volume was adjusted to 1 L with dH_2O and stored at room temperature.

6.2.3 Buffer P3- neutralization buffer

One liter of buffer P3 was prepared as follow: 294.5 g potassium acetate was dissolved in 500 ml dH_2O. The pH was adjusted to 5.5 with approximately 300 ml of acetic acid and adjusted volume to 1 L with dH_2O and stored at 4℃.

6.2.4 QC buffer- wash buffer

One liter of QC buffer was prepared as follow: 58.44 g NaCl and 10.46 g MOPS was dissolved in 800 ml dH_2O. The pH was adjusted to 7 with NaOH, 150 ml of isopropanol was added and volume was adjusted to 1 L with dH_2O and stored at room temperature.

6.2.5 QF buffer- elution buffer

One liter of QC buffer was prepared as follow: 73.05 g NaCl and 6.06 g Tris base was dissolved in 800 ml dH_2O. The pH was adjusted to 8.5 with HCl, 150 ml of isopropanol was added and volume was adjusted to 1 L with dH_2O and stored at room temperature.

6.2.6 0.5 M EDTA

To make 500 ml, 73.06 g EDTA was added to 300 ml of dH_2O. The pH was adjusted to 7.5 with NaOH pellets and then to pH 8 with 10 M NaOH. The final volume was adjusted to 500 ml with dH_2O.

6.2.7 1× TAE gel electrophoresis running buffer

A 50 × TAE stock was prepared as follow: 242 g Tris base [tris(hydroxymethyl)-aminomethane], 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA (pH 8) was dissolved in 800 ml, the volume was adjusted to 1 L with dH_2O. To make an 11.25 L of 1× TAE buffer, 225 ml of 50× TAE was added to 10 L of dH_2O, volume was adjusted to 11.25 L.

6.3 Tissue culture reagents

6.3.1 Dulbecco's modified Eagles medium (DMEM)

DMEM powder (Life Technologies, CA, USA)

One liter of DMEM was prepared as follow: 13.38 g DMEM and 3.7 g $NaHCO_2$ was dissolved in 500 ml of Sabex dH_2O. The pH was adjusted to 6.7 with HCl and volume was adjusted to 950 ml. The medium was filter sterilised and stored at 4℃.

6.3.2 Eagle's minimum essential medium (EMEM) (Life Technologies, CA, USA)

Three liters of EMEM was prepared as follow: 28.59 g EMEM and 6.6 g $NaHCO_2$ was dissolved in 500 ml of Sabex dH_2O. The pH was adjusted to 6.7 with HCl and volume was topped up to final volume of 1 L. The medium was supplemented with 2mM L-glutamine (Lonza, Verviers, Belgium), filter sterilised and stored at 4℃.

6.3.3 Joklik modified Eagle's minimum essential medium (EMEM) Eagle's minimum essential medium (JEMEM)

One liter of JEMEM was prepared as follow: 11.2 g JEMEM and 2 g $NaHCO_2$ was dissolved in 500 ml of Sabex dH_2O. The pH was adjusted to 6.7 with HCl and volume was topped up to 1000 ml. The medium was supplemented with 2 mM L-glutamine (Lonza, Verviers, Belgium), 5 % FCS, 100 units/ml Penicillin/Streptomycin and 0.1 mg/ml hygromycin filter sterilised and stored at 4℃.

6.3.4 1000× Penicillin and Streptomycin

Stock solutions of a 1000× were prepared and filter sterilised. This equated to 100,000 units/ml for penicillin and 100,000 µg/ml for streptomycin. For the supplementation of the medium, thus 0.5 ml 1000× stock into 500 ml culture medium, the working concentrations were penicillin 100 units/ml and streptomycin 100 µg/ml.

6.3.5 0.5× TrypLE™ Express for trypsinisation (Life Technologies, CA, USA)

TrypLE™ Express (1×) was diluted 2-fold with Saline-EDTA solution (saline + 0.01 % EDTA). The mixture was filter sterilised and stored at 4℃.

6.3.6 Fetal Bovine serum (FBS) (Life Technologies, CA, USA)

Gamma irradiated Fetal Bovine serum (FBS) was filter sterilized, aliquot into 50 ml working stocks and stored at -20℃. Fifty milliliters of FBS was added to supplement the medium with 10 % FBS and 25 ml for 5 % FBS.

6.3.7 4 mM Ferricyanide (Potassium Ferricyanide)

Potassium Ferricyanide (1.317 g) was added to 20 ml of PBS and filter sterlised. To prevent precipitation, add PBS first.

6.3.8 4 mM Ferrocyanide (Potassium Ferrocyanide)

Potassium Ferrocyanide (1.689 g) was added to 20 ml of PBS and filter sterlised. To prevent precipitation, add PBS first.

6.3.9 40 mg/ml X-Gal (5-bromo-4-chloro-3-indoyl-b-D-galactopyranoside) reagent (Sigma-Aldrich, MO, USA)

X-gal (0.8 g) was added to 20 ml of DMSO.

6.4 *In vitro* and *In vivo* reagents

6.4.1 15 % 1:19 Bis-acrylamide:acrylamide gel (Merck Chemicals (Pty) Ltd, Darmstadt, Germany)

To 10 ml of dH_2O 0.45 g of bis-acrylamide, 8.55 g acrylamide, 28.8 g Urea (~8 M) and 6 ml 10× TBE pH 8 was added. The solution was made up to 60 ml with dH_2O and dissolved in hot water. Before pouring of the gel 250 µl 1 % APS (0.1 g in 1 ml dH_2O) and 25 µl TEMED was added to the solution.

6.4.2 TE buffer

Added 10 mM Tris-HCl pH8 (1.58 g Tris-HCl in 600 ml dH_2O, adjust pH to 8 and make up to 1L) and 1 mM EDTA to 1 L dH_2O.

6.4.3 Sephadex

Five grams of Sephadex G-225 was added to 50 ml of TE buffer, the solution was stirred at room temperature overnight. Followed by centrifugation at 4000 rpm for 2 min. The TE buffer was poured off and 50 ml fresh TE buffer was added, repeated 2-3 times. The Sephadex was then resuspended in 50 ml TE buffer.

6.4.4 10× TBE (500 ml) (Autoclave)

Boric acid powder (27.5 g), 50 g Tris and 20 ml 0.5 M EDTA (pH 8) was added to 400 ml dH_2O. The pH was adjusted to pH 8 and made up to a final volume of 500 ml with dH_2O.

6.4.5 20× SSC

Added 3 M NaCl (175 g/L) and 0.3 M $Na_3C_6H_5O_7.2H_2O$ to dH_2O, adjusted the pH to 7 and made up to final desired volume.

6.4.6 10 % SDS

Ten grams of SDS was added to 100 ml dH_2O.

6.5 Alignments

6.5.1 Alignment of positive pTZ-*Fluc* clone sequence with parent sequence

```
            51
Reference seq                                   CAG GTGTCCACTC CCAGTTCAAT
 pTZ-Fluc seq                   GAT CGATATCCAG GTGTCCACTC CCAGTTCAAT
           101
Reference seq  TACAGCTCTT AAGGCTAGAG TACTTAATAC GACTCACTAT AGGCTAGCCT
 pTZ-Fluc seq  TACAGCTCTT AAGGCTAGAG TACTTAATAC GACTCACTAT AGGCTAGCCT
           151
Reference seq  CGAGGATTGG GGACCCTGCG CTGAACATGG AAGACGCCAA AAACATAAAG
 pTZ-Fluc seq  CGAGGATTGG GGACCCTGCG CTGAACATGG AAGACGCCAA AAACATAAAG
           201
Reference seq  AAAGGCCCGG CGCCATTCTA TCCGCTGGAA GATGGAACCG CTGGAGAGCA
 pTZ-Fluc seq  AAAGGCCCGG CGCCATTCTA TCCGCTGGAA GATGGAACCG CTGGAGAGCA
           251
Reference seq  ACTGCATAAG GCTATGAAGA GATACGCCCT GGTTCCTGGA ACAATTGCTT
 pTZ-Fluc seq  ACTGCATAAG GCTATGAAGA GATACGCCCT GGTTCCTGGA ACAATTGCTT
           301
Reference seq  TTACAGATGC ACATATCGAG GTGGACATCA CTTACGCTGA GTACTTCGAA
 pTZ-Fluc seq  TTACAGATGC ACATATCGAG GTGGACATCA CTTACGCTGA GTACTTCGAA
           351
Reference seq  ATGTCCGTTC GGTTGGCAGA AGCTATGAAA CGATATGGGC TGAATACAAA
 pTZ-Fluc seq  ATGTCCGTTC GGTTGGCAGA AGCTATGAAA CGATATGGGC TGAATACAAA
           401
Reference seq  TCACAGAATC GTCGTATGCA GTGAAAACTC TCTTCAATTC TTTATGCCGG
 pTZ-Fluc seq  TCACAGAATC GTCGTATGCA GTGAAAACTC TCTTCAATTC TTTATGCCGG
           451
Reference seq  TGTTGGGCGC GTTATTTATC GGAGTTGCAG TTGCGCCCGC GAACGACATT
 pTZ-Fluc seq  TGTTGGGCGC GTTATTTATC GGAGTTGCAG TTGCGCCCGC GAACGACATT
           501
Reference seq  TATAATGAAC GTGAATTGCT CAACAGTATG GGCATTTCGC AGCCTACCGT
 pTZ-Fluc seq  TATAATGAAC GTGAATTGCT CAACAGTATG GGCATTTCGC AGCCTACCGT
           551
Reference seq  GGTGTTCGTT TCCAAAAAGG GGTTGCAAAA AATTTTGAAC GTGCAAAAAA
 pTZ-Fluc seq  GGTGTTCGTT TCCAAAAAGG GGTTGCAAAA AATTTTGAAC GTGCAAAAAA
           601
Reference seq  AGCTCCCAAT CATCCAAAAA ATTATTATCA TGGATTCTAA AACGGATTAC
 pTZ-Fluc seq  AGCTCCCAAT CATCCAAAAA ATTATTATCA TGGATTCTAA AACGGATTAC
           651
Reference seq  CAGGGATTTC AGTCGATGTA CACGTTCGTC ACATCTCATC TACCTCCCGG
 pTZ-Fluc seq  CAGGGATTTC AGTCGATGTA CACGTTCGTC ACATCTCATC TACCTCCCGG
           701
Reference seq  TTTTAATGAA TACGATTTTG TGCCAGAGTC CTTCGATAGG GACAAGACAA
 pTZ-Fluc seq  TTTTAATGAA TACGATTTTG TGCCAGAGTC CTTCGATAGG GACAAGACAA
           751
Reference seq  TTGCACTGAT CATGAACTC  CTCTGGATCT ACTGGTCTGC CTAAAGGTGT
 pTZ-Fluc seq  TTGCACTGAT CATGAACTC  CTCTGGATCT ACTGGTCTGC CTAAAGGTGT
           801
Reference seq  CGCTCTGCCT CATAGAACTG CCTGCGTGAG ATTCTCGCAT GCCAGAGATC
 pTZ-Fluc seq  CGCTCTGCCT CATAGAACTG CCTGCGTGAG ATTCTCGCAT GCCAGAGATC
```

851
Reference seq CTATTTTTGG CAATCAAATC ATTCCGGATA CTGCGATTTT AAGTGTTGTT
pTZ-Fluc seq CTATTTTTGG CAATCAAATC ATTCCGGATA CTGCGATTTT AAGTGTTGTT
901
Reference seq CCATTCCATC ACGGTTTTGG AATGTTTACT ACACTCGGAT ATTTGATATG
pTZ-Fluc seq CCATTCCATC ACGGTTTTGG AATGTTTACT ACACTCGGAT ATTTGATATG
951
Reference seq TGGATTTCGA GTCGTCTTAA TGTATAGATT TGAAGAAGAG CTGTTTCTGA
pTZ-Fluc seq TGGATTTCGA GTCGTCTTAA TGTATAGATT TGAAGAAGAG CTGTTTCTGA
1001
Reference seq GGAGCCTTCA GGATTACAAG ATTCAAAGTG CGCTGCTGGT GCCAACCCTA
pTZ-Fluc seq GGAGCCTTCA GGATTACAAG ATTCAAAGTG CGCTGCTGGT GCCAACCCTA
1051
Reference seq TTCTCCTTCT TCGCCAAAAG CACTCTGATT GACAAATACG ATTTATCTAA
pTZ-Fluc seq TTCTCCTTCT TCGCCAAAAG CACTCTGATT GACAAATACG ATTTATCTAA
1101
Reference seq TTTACACGAA ATTGCTTCTG GTGGCGCTCC CCTCTCTA AGGAAGTCGG
pTZ-Fluc seq TTTACACGAA ATTGCTTCTG GTGGCGCTCC CCTCTCTA AGGAAGTCGG
1151
Reference seq GGAAGCGGT TGCCAAGAG GTTCCATCTG CCAGGTATCA GGCAAGGATA
pTZ-Fluc seq GGAAGCGGT TGCCAAGAG GTTCCATCTG CCAGGTATCA GGCAAGGATA
1201
Reference seq TGGGCTCACT GAGACTACAT CAGCTATTCT GATTACACCC GAGGGGGATG
pTZ-Fluc seq TGGGCTCACT GAGACTACAT CAGCTATTCT GATTACACCC GAGGGGGATG
1251
Reference seq ATAAACCGGG CGCGGTCGGT AAAGTTGTTC CATTTTTTGA AGCGAAGGTT
pTZ-Fluc seq ATAAACCGGG CGCGGTCGGT AAAGTTGTTC CATTTTTTGA AGCGAAGGTT
1301
Reference seq GTGGATCTGG ATACCGGGAA AACGCTGGGC GTTAATCAAA GAGGCGAACT
pTZ-Fluc seq GTGGATCTGG ATACCGGGAA AACGCTGGGC GTTAATCAAA GAGGCGAACT
1351
Reference seq GTGTGTGAGA GGTCCTATGA TTATGTCCGG TTATGTAAAC AATCCGGAAG
pTZ-Fluc seq GTGTGTGAGA GGTCCTATGA TTATGTCCGG TTATGTAAAC AATCCGGAAG
1401
Reference seq CGACCAACGC CTTGATTGAC AAGGATGGAT GGCTACATTC TGGAGACATA
pTZ-Fluc seq CGACCAACGC CTTGATTGAC AAGGATGGAT GGCTACATTC TGGAGACATA
1451
Reference seq GCTTACTGGG ACGAAGACGA ACACTTCTTC ATCGTTGACC GCCTGAAGTC
pTZ-Fluc seq GCTTACTGGG ACGAAGACGA ACACTTCTTC ATCGTTGACC GCCTGAAGTC
1501
Reference seq TCTGATTAAG TACAAAGGCT ATCAGGTGGC TCCCGCTGAA TTGGAATCCA
pTZ-Fluc seq TCTGATTAAG TACAAAGGCT ATCAGGTGGC TCCCGCTGAA TTGGAATCCA
1551
Reference seq TCTTGCTCCA ACACCCCAAC ATCTTCGACG CAGGTGTCGC AGGTCTTCCC
pTZ-Fluc seq TCTTGCTCCA ACACCCCAAC ATCTTCGACG CAGGTGTCGC AGGTCTTCCC
1601
Reference seq GACGATGACG CCGGTGAACT TCCCGCCGCC GTTGTTGTTT TGGAGCACGG
pTZ-Fluc seq GACGATGACG CCGGTGAACT TCCCGCCGCC GTTGTTGTTT TGGAGCACGG
1651
Reference seq AAAGACGATG ACGGAAAAAG AGATCGTGGA TTACGTCGCC AGTCAAGTAA
pTZ-Fluc seq AAAGACGATG ACGGAAAAAG AGATCGTGGA TTACGTCGCC AGTCAAGTAA
1701

```
Reference seq  CAACCGCGAA AAAGTTGCGC GGAGGAGTTG TGTTTGTGGA CGAAGTACCG
pTZ-Fluc seq  CAACCGCGAA AAAGTTGCGC GGAGGAGTTG TGTTTGTGGA CGAAGTACCG
         1751
Reference seq  AAAGGTCTTA CCGGAAAACT CGACGCAAGA AAAATCAGAG AGATCCTCAT
pTZ-Fluc seq  AAAGGTCTTA CCGGAAAACT CGACGCAAGA AAAATCAGAG AGATCCTCAT
         1801
Reference seq  AAAGGCCAAG AAGGGCGGAA AGATCGCCGT GTAAA
pTZ-Fluc seq  AAAGGCCAAG AAGGGCGGAA AGATCGCCGT GTAAACTAGA GTCGACCCGG
         1851
Reference seq  GCGGCCGCTT CCCTTTAGTG AGG
pTZ-Fluc seq  GCGGCCGCTT CCCTTTAGTG AGGGATATCG ATC
```

Figure 6.1: Alignment of Firefly luciferase reference sequence with the pTZFluc clone sequence.
Sequences in red indicates the primers with engineered *Eco* RV site (blue sequence) used for the amplification of the *Fluc* sequence from pCI-neo-CMV*Fluc*

6.6 Plasmid maps

6.6.1 Empty pTZ57RInsTA cloning vector

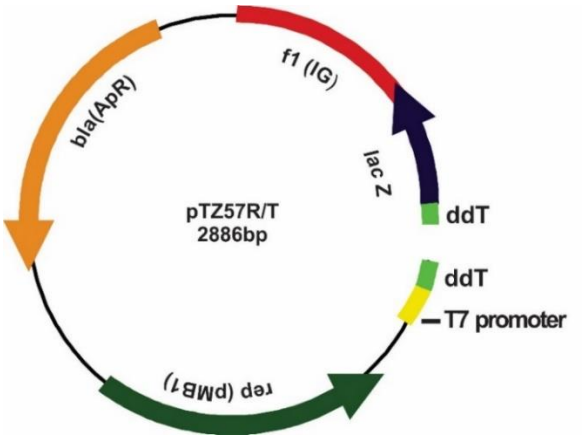


Figure 6.2: Plasmid map of pTZ57RInsTA plasmid used for the cloning of RNAi activators and *Firefly luciferase.*
The linear plasmid is 2886 bp and has ampicillin resistance that act as a selection marker.

6.6.2 Empty plasmid carrying a PEPCK promoter

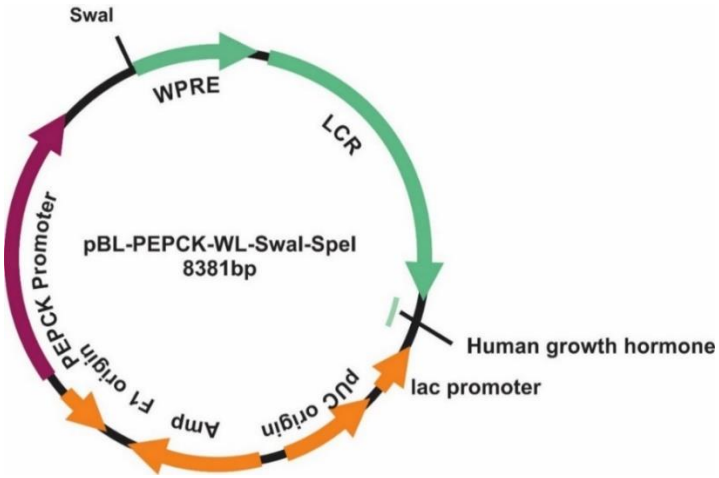
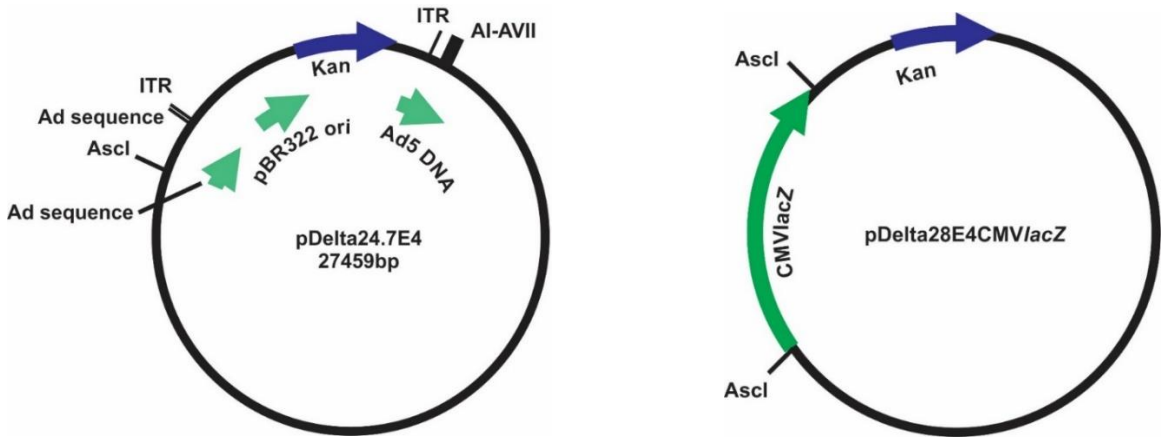


Figure 6.3: Plasmid map of pBL-PEPCK-WL-*Swa*I-*Spe*I plasmid used for engineering of PEPCK RNAi activator and *Fluc* cassettes.
Plasmid of 8381 bp with Ampicillin resistance as selection marker and a PEPCK promoter used for expression.

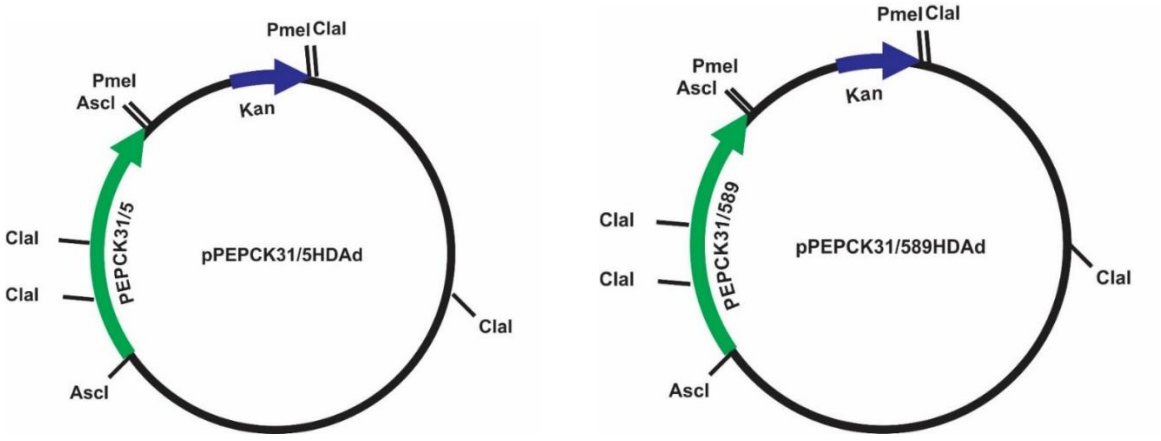
6.6.3 Empty recombinant Helper Dependent Adenoviral vector



A **B**

Figure 6.4: Plasmid map of pΔ24.7E4 and pΔ28E4CMV*lac*Z.
A) HDAd Plasmid of 27459 bp with Kanamycin resistance as selection marker. **B)** The *in vitro* control plasmid of 32133 bp with Kanamycin resistance and a *lac*Z reporter gene expressed by a CMV promoter.

6.6.4 Helper Dependent Adenoviral vector construction during cloning



A **B**

C

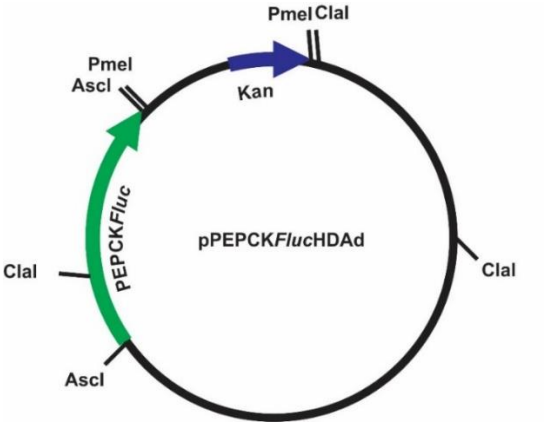


Figure 6.5: Constructed HDAd vectors used for *in vitro* and *in vivo* analysis.
A) HDAd plasmid with the anti-HBV pri-miR 31/5 expressed from a PEPCK promoter. B) HDAd plasmid with the anti-HBV pri-miR 31/589 expressed from a PEPCK promoter. C) HDAd plasmid with the reporter gene *Firefly luciferase* expressed from a PEPCK promoter.

6.7 Ethics clearance

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2016/01/01/C

APPLICANT: Ms B Maepa

SCHOOL: School of Pathology, Molecular Medicine and Haematology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Delivery of anti-HBV sequences in mice using adenoviral vectors

Number and Species

247 FVB/STC HBV transgenic mice

Approval was given for the use of animals for the project described above at an AESC meeting held on 2016/01/26. This approval remains valid until 2018/02/04.

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

None

Signed: _____ (Chairperson, AESC) Date: 10th February 2016

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

Signed: _____ (Registered Veterinarian) Date: 10/02/2016

cc: Supervisor: N/A
Director: CAS

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CHAPTER 7

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