INHIBITION OF HEPATITIS B VIRUS USING HELPER-DEPENDENT ADENOVIRAL VECTORS EXPRESSING PRI-MIRNAs FROM LIVER-SPECIFIC MTTR PROMOTER

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

of

Master of Science in Medicine

Johannesburg, 2017

DECLARATION

I, Anele Mdunyelwa 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

To my family:

My loving parents, Siyakubonga and Nompendulo Mdunyelwa

My supportive siblings Mthetheleli, Ntombozuko and Nomakhwezi Mdunyelwa

CONFERENCE PRESENTATIONS

1. Mdunyelwa Anele, Maepa Betty and Arbuthnot Patrick; Inhibition of Hepatitis B Virus using PEGylated Helper-dependent adenoviral vectors expressing pri-miRNAs from MTTR promoter; MBRT research day 3rd of December 2015.

2. Mdunyelwa Anele, Smit Duodane', Maepa Betty and Arbuthnot Patrick; The development and application of adenoviral nanoparticles for the treatment of hepatitis B virus; DST-NRF Nanotechnology symposium 27-28 June 2016.

3. Mdunyelwa Anele, Maepa Betty and Arbuthnot Patrick, MAL-PEGylated helper-dependent adenoviral vectors expressing pri-miRNAs for prolonged inhibition of hepatitis B virus replication, 25th SASBMB Congress 10-14 July 2016.

4. Mdunyelwa Anele, Maepa Betty and Arbuthnot Patrick; Suppression of hepatitis b virus replication using mal-PEGylated Helper-Dependent Adenoviral vectors expressing pri-miRNAs; Faculty of Health Sciences Research Day, 1 September 2016

ABSTRACT

Approximately 240 million people are estimated to be chronic carriers of hepatitis B virus (HBV), placing them at high risk for complications like hepatocellular carcinoma (HCC) and cirrhosis. Infection with the virus is predominantly high in sub Saharan Africa, East and Southeast Asia. Current treatments are limited by the emergence of viral resistance and adverse side effects. These challenges prompted development of new therapy for HBV. The application of RNA interference (RNAi) as a form of treatment has shown successful HBV silencing. However, safe and efficient delivery of anti-HBV sequences remains a challenge. Recombinant adenoviruses (Ads) have a natural tropism for the liver, making them suitable for anti-HBV sequence delivery. Their use in gene therapy is limited by their ability to induce the innate and adaptive immune response, thus leading to diminished transgene expression. To overcome immune stimulation, third generation helper-dependent adenoviral vectors (HDAds), which are devoid of all their viral coding sequences, were developed. This study investigated the use of HDAds expressing primary micro RNA (pri-miRNA) sequences from the liver-specific MTTR promoter with the aim of inhibiting HBV replication. The anti-HBV pri-miRNA expression cassettes were successfully cloned into the adenoviral bearing genome and used for HDAd production. Infection of liver derived cell line with these HDAds resulted in efficient pri-miRNA expression and processing into expected guide sequences. This was accompanied by a significant inhibition of HBV replication. Injection of the HDAds into mice resulted in efficient liver transduction and no significant induction of the inflammatory response or liver toxicity. However, as a result of high levels of HBV replication markers produced in the transgenic mice used, this did not translate into significant HBV replication inhibition. This study therefore highlights the safety and therapeutic potential of HDAds against HBV infection. To demonstrate HBV gene silencing *in vivo*, transgenic mice expressing HBV replication markers will be used.

ACKNOWLEDGEMENTS

1. I would like to thank my supervisors Dr Betty Maepa and Prof. Patrick Arbuthnot for their endless support, guidance, and patience that has led me to complete this degree.
2. I would like to thank Dr Abdullah Ely and Duadané Smit for their assistance and advice regarding various aspects of this study.
3. I would like to thank my colleagues at the Antiviral Gene Therapy Research Unit for their assistance.
4. I would like to thank Brendan Lee from Baylor College, USA for donating the Adenoviral plasmid for the study.
5. I would like to thank Monica Birkhead from the National Institute for Communicable Diseases, division of the National Health Laboratory Services for assisting with Transmission Electron microscopy (TEM).
6. I sincerely thank the following funding bodies for making it possible for me to pursue this degree, South African National Research Foundation (NRF) and Poliomyelitis Research Foundation (PRF).
7. I am grateful to Kotoanyane Sefali for his constant support, motivation and encouragement throughout.
8. A special thank you to my family and friends for your love, support and for believing in me.

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AAV - adeno-associated virus

Ad - adenovirus

ALT - alanine aminotransferase

ANAs - altritol nucleic acids

apo - apolipoprotein

bp - base pair

CAS - central animal service

CBA - cytometric bead array

cccDNA - covalently closed circular DNA

CMV - cytomegalovirus

CPE - cytopathic effect

CsCl - caesium chloride

CTL - cytotoxic T lymphocyte

DAB - 3,3'-Diaminobenzidine

ddH_2O – double deionised water

DGCR8 - DiGeorge syndrome chromosomal region

dH_2O - distilled water

DMEM – Dulbecco's Modified Eagles Medium

dsDNA – double-stranded DNA

DTT - dithiothreitol

eGFP - enhanced green fluorescent protein

ELISA - enzyme linked immunosorbent assay

EMEM - Eagle's minimum essential medium

FBS - fetal bovine serum

FDA- food and drug administration

HBcAg - hepatitis B core antigen

HBeAg - hepatitis B e protein

HBsAg - hepatitis B surface antigen

HBV - hepatitis B virus

HCC - hepatocellular carcinoma

HCV - hepatitis C virus

HDAd - helper-dependent adenovirus

HDAdcys - cysteine modified HDAd

HEK293 - human embryonic cell line

HIV - human immunodeficiency virus

Huh7 - human hepatoma cell line

HV - helper virus

HVR - hypervariable region

IFN - interferon

ifu - infectious units

IL - interleukin

ITR - inverted terminal repeat

JEMEM - Joklik Eagle's Minimum Essential Medium

kb – kilobase

LA - Luria Bertani agar media

LB - Luria Bertani media

LVs - lentiviral vectors

Mal-PEG - maleimide-activated PEG

MCP-1 - monocyte chemotactic protein-1

miRNA – microRNA

MLP - major late promoter

mPEG-SPA - Monomethoxy polyethylene glycol-succinimidyl propionate

mRNA - messenger RNA

MOI - multiplicity of infection

MTTR - murine transthyretin receptor

NHS - N-hydroxysuccinimide

nm - nanometre

nt – nucleotide

NTCP - Sodium Taurocholate Co-Transporting Polypeptide

NVV - non-viral vector

OD - optical density

OH – hydroxyl group

ORF - open reading frame

OTC - ornithine transcarbamylase

PAMAM - polyamidoamine

PBS – phosphate buffered saline

PEG - polyethylene glycol

PEI – polyethyleneimine

PEPCK - phosphoenolpyruvate carboxykinase

pgRNA - pregenomic RNA

PLL - poly-L-(lysine)

PNK - polynucleotide kinase

Pol - polymerease

pre-miRNA - precursor miRNA

pri-miRNA - primary miRNA

Q-PCR - quantitative polymerase chain reaction

rcDNA - relaxed circular DNA

RE - restriction enzyme

RISC - RNA induced silencing complex

RNAi - RNA interference

RT - reverse transcriptase

scAAV - self-complementary AAV

SEM - standard error of the mean

shRNA - short hairpin RNA

SIN - self-inactivating

siRNA - small interfering RNA

TEM - transmission electron microscopy

TCEP – tris (2-carboxyethyl)phosphine)

TNF- tumour necrosis factor

UV – ultraviolet

VPEs - particle equivalents

vps – viral particles

VV - viral vector

X-gal - 5-bromo-4-chloro-indolyl-β-D-galactopyranoside

CHAPTER 1

1. INTRODUCTION

1.1 Hepatitis B Virus (HBV)

1.1.1 Hepatitis B Virus infection

Hepatitis B virus (HBV) mainly infects the liver to cause Hepatitis B disease. Approximately 240 million people are chronically infected with HBV and about 686 000 people die every year from the infection globally (http://www.who.int/mediacentre/factsheets/fs204/en/). Infection with HBV can cause a variety of liver diseases, ranging from acute to chronic hepatitis, hepatocellular carcinoma (HCC) and cirrhosis. HBV infection is transmitted either perinatally from infected mother to child at birth, parenterally through sexual contact or through contact of infected blood and bodily fluids [Reviewed in (MacLachlan and Cowie, 2015, Arbuthnot et al., 2007)].

Areas with high incidences of HBV infection are sub-Saharan Africa and Asia [Reviewed in (MacLachlan and Cowie, 2015)]. HBV has ten genotypes (A-J) that have been described; with some of these genotypes further divided into sub-genotypes. These genotypes vary in their geographical distribution. Genotype A is commonly found in sub-Saharan Africa, India, Europe and North America; genotypes B and C are endemic in East and Southern Asia; genotype D is endemic in the middle East, India and Mediterranean regions; genotype E is endemic in sub-Saharan Africa; genotype F is rarely found; genotype G is endemic in USA, Germany and Mexico; genotype H is endemic in Mexico; genotype I has been recently found in Vietnam and Laos, whereas genotype J has been reported in Ryukyu Islands in Japan [Reviewed in(Sunbul, 2014)].

1.1.2 HBV genome structure

HBV is a member of a family of small, DNA containing viruses known as *Hapadnaviridae*. It has an enveloped, partly double stranded, circular DNA genome also known as relaxed-circular DNA (rc-DNA) that is 3.2 kb in length. The viral genome is made up of four open reading frames

(ORFs): the nucleocapsid (core), virus replicase (polymerase), envelope (surface) and the X ORF. The core ORF encodes the structural protein [the hepatitis B core antigen, (HBcAg)] as well as the secreted non-structural hepatitis B e antigen (HBeAg) [Reviewed in (Pollicino et al., 2014, Arbuthnot et al., 2007)]. The polymerase ORF encodes the viral polymerase enzyme which has a reverse transcriptase (RT) and ribonuclease activities. The surface ORF encodes viral surface envelope protein (HBsAg), which is divided in to pre-S1, pre-S2 and the S proteins. The X ORF encodes the regulatory X (HBx) protein, which is highly conserved and expressed during infection in humans and in wood chucks (Leupin et al., 2005, Liang, 2009, Nordin et al., 2014). The viral genome also contains promoters, enhancers, negative regulatory elements and a single polyadenylation signal at which transcription of all four major mRNAs is terminated. [**Figure 1.1**, (Decorsiere et al., 2016, Moolla et al., 2002)].

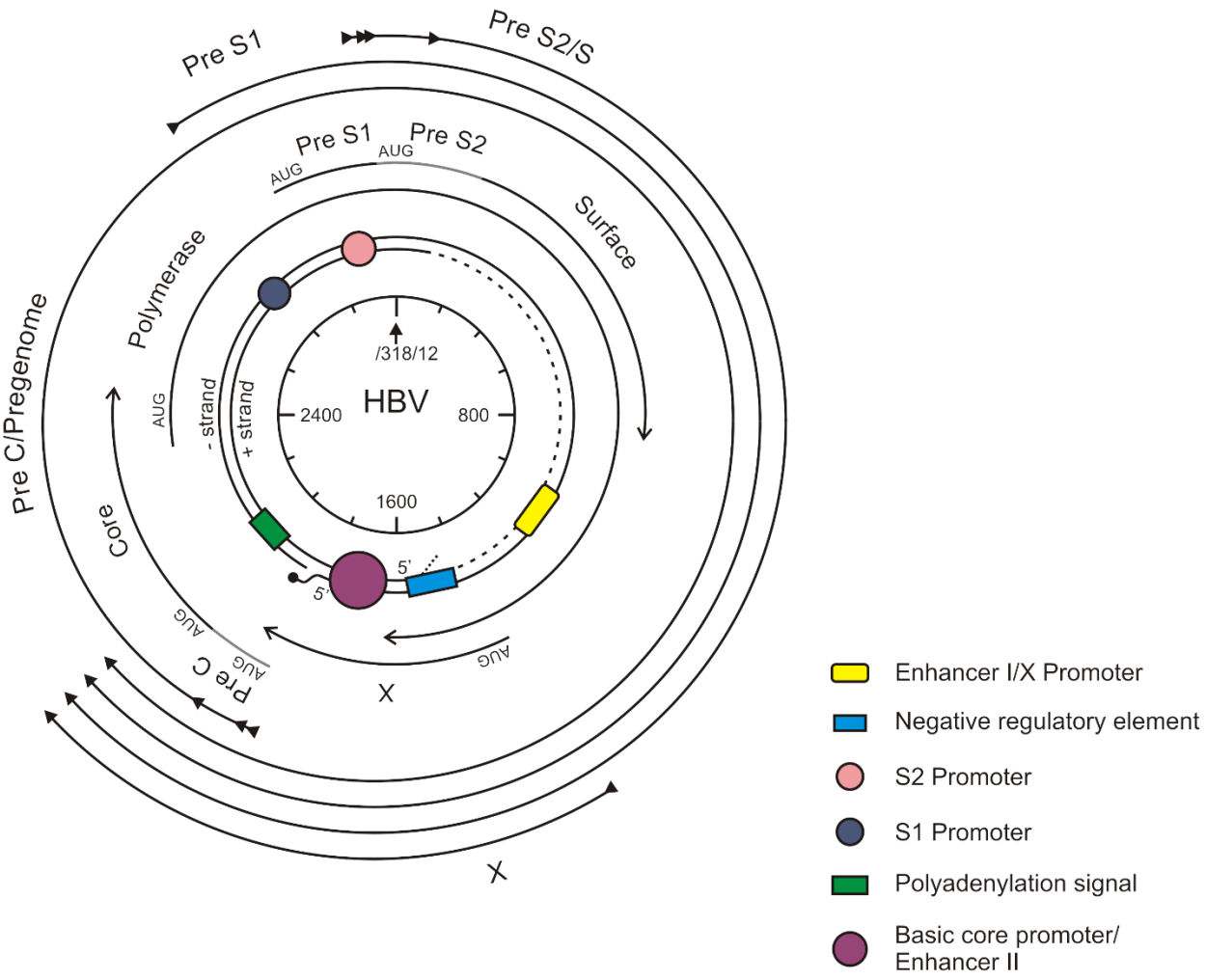

Figure 1.1: Genome organization of the hepatitis B virus (HBV). The HBV genome is made up of a circular, partly double stranded DNA. The circular and rectangular symbols show the elements that regulate HBV transcription. The arrows show the overlapping open reading frames (ORFs). Diagram adapted from (Arbuthnot et al., 2007).

1.1.3 HBV replication

Since HBV has a natural tropism for the liver, viral DNA replication occurs in the hepatocytes. The partially double stranded DNA (dsDNA) genome of HBV is enveloped into the nucleocapsid which is surrounded by the surface glycoproteins L (large), M (medium) and S (small). The binding and receptor recognition of the nucleocapsid is initiated by the L and M proteins. Sodium Taurocholate Co-Transporting Polypeptide (NTCP) was discovered to be the host entry receptor that interacts with HBV membrane proteins initiating entry into the host hepatocytes. During

infection, hepatitis virions bind to the NTCP receptor on the hepatocytes and viral entry occurs through endocytosis. Following entry, the viral capsid is transported to the nucleus. The relaxed-circular DNA (rcDNA) is released and converted to covalently closed circular DNA (cccDNA). RNA polymerase II transcribes the cccDNA into four subgenomic messenger RNAs (mRNAs) and pregenomic RNA (pgRNA). The mRNAs are transported from the nucleus to the cytoplasm where they are translated into viral proteins. Proximal to the 5'-end, pgRNA has a ε-stem loop, which when bound to polymerase triggers its encapsidation. The encapsidated pgRNA is then reverse transcribed in to rcDNA. The rcDNA containing nucleocapsid is either enveloped and secreted or transported back to the nucleus to produce more cccDNA [Reviewed in (Seeger and Mason, 2000, Lempp and Urban, 2014, Yan et al., 2012, Morikawa et al., 2016)].

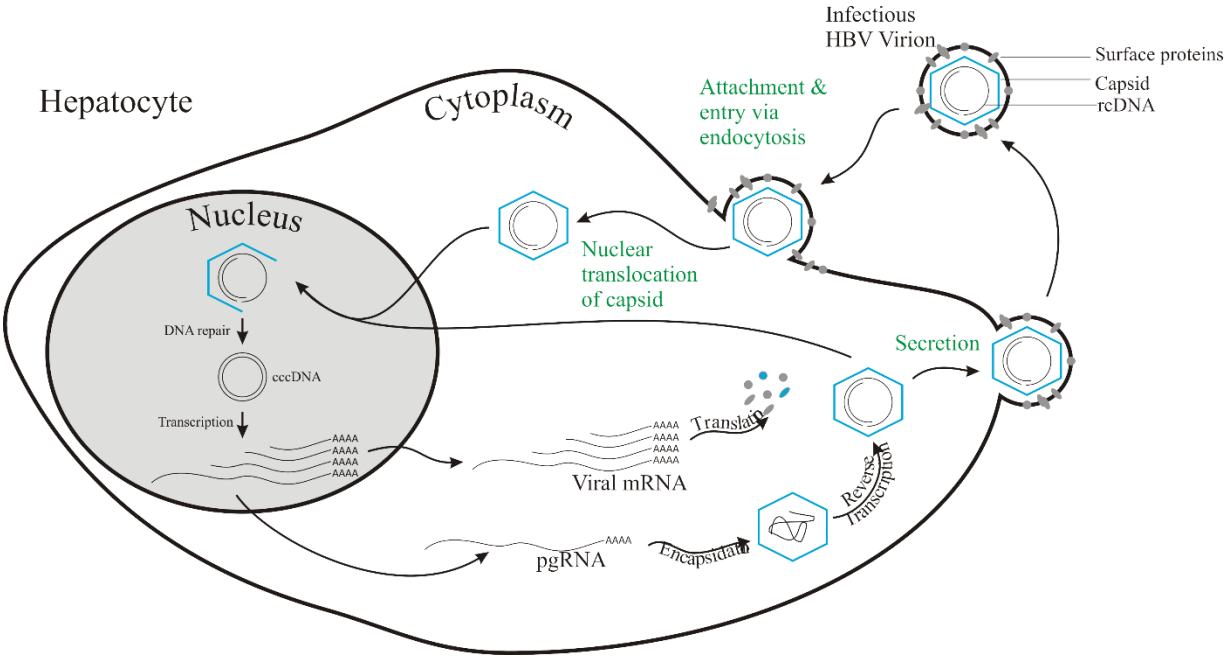

Figure 1.2: Hepatitis B virus (HBV) replication cycle. The hepatocytes are infected by the virus; the virions bind to the receptor on the hepatocytes and enter through endocytosis. The nucleocapsid is transported to the nucleaus where the rcDNA is converted to cccDNA. RNA Pol II transcribes cccDNA into pgRNA and four mRNAs which are translated into viral proteins in the cytoplasm. The pgRNA interacts with polymerase and it is packaged and reverse transcribed into rcDNA. This capsid may either be recycled back into the nucleus and converted into cccDNA or it gets secreted out of the cell. The figure was adapted from (Morikawa et al., 2016).

1.1.4 Current treatment and vaccination against Hepatitis B

The first HBV vaccine was approved in 1980 by the US Food and Drug Administration (FDA) and made available for use by 1982. Thus far there are three generations of HBV vaccine. First-generation vaccines also known as plasma-derived vaccines were made from HBsAg. HBsAg was extracted from the blood of people who were chronically infected with HBV through a process known as plasmapheresis and then it was inactivated before use. Second-generation vaccines also known as yeast-derived vaccines were developed in the mid-1980s. These vaccines were created in yeast (*Saccharomyces cerevisiae*) transfected with HBV-DNA sequences coding for small HBV surface protein. Third-generation vaccines were produced in mammalian cells, these vaccines induce immunity against HBV and either contain a single Pre-S2 envelope protein or a combination of Pre-S1 and Pre-S2 envelope proteins [Reviewed in (Edey et al., 2010, Shouval et al., 2015)].

Even though these vaccines are effective against HBV, they are unable to eradicate existing HBV infections. Hence, HBV therapeutics are needed to manage hepatitis B. Thus far there are seven drugs that have been approved by FDA for the treatment of chronic hepatitis B. These are: nucleoside analogues (lamivudine, telbivudine and entecavir), nucleoside analogues (adefovir and tenofovir) and immune modulators [interferon alfa (IFN-α) and PEGylated version (PEG IFN-α)].

Nucleoside and nucleotide analogues are phosphorylated, hence mimics naturally occurring nucleosides/nucleotides. However, once incorporated in to the growing DNA strands they act as chain terminators by inhibiting DNA polymerase [Reviewed in (Arbuthnot et al., 2007)]. Lamivudine was the first oral nucleoside analogue to be approved for treating chronic HBV infection and it has been shown to normalize alanine aminotransferase (ALT) levels (a marker of liver damage), induce seroconversion of HBeAg and reverses necroinflammatory activity(Lok, 2003, Xu et al., 2015). Adefovir was approved in 2002 for treating chronic hepatitis B after it showed great efficacy against lamivudine-resistant mutant, whereas entecavir was approved in 2005. Telbivudine was approved in 2006 and has been shown to be more effective in treating chronic hepatitis B as compared to lamivudine. Immune modulatory agents IFN- α and PEG IFN-α are antiviral cytokines which are secreted by the host during viral infections. Interferons function by augmenting the natural human immune system to fight infection caused by HBV [Reviewed in

(Pham et al., 2016, Piratvisuth, 2008, Karayiannis, 2003, Fung et al., 2011)]. PEGylated IFN- α replaced IFN- α as it had improved efficacy and pharmacokinetic properties [Reviewed in (Manzoor et al., 2015)].

The above mentioned therapeutics have several limitations e.g. poor tolerability, limited efficacy and higher rates of drug resistance is usually observed. Therefore, this calls for a need for novel therapeutics that will result in long term HBV replication inhibition with little or no resistance and toxicity. Several studies have explored and showed promising outcomes with RNA interference (RNAi-) based gene therapy approach to combat HBV infection. RNAi-based therapeutics have been developed in the past years as potential novel alternatives to current anti-HBV drugs and have shown great success (Ely et al., 2008, Grimm et al., 2006, Crowther et al., 2014).

1.2 RNA interference

RNAi is a nucleic acid-based mechanism used in eukaryotic systems to regulate gene expression. A well-studied RNAi pathway in multicellular organisms such as plants, humans, and yeast is a naturally occurring pathway in which small RNA molecules called microRNAs (miRNAs) serve as mediators of gene silencing (Uprichard, 2005, Aagaard and Rossi, 2007). These miRNAs are small (19-24 nucleotides), endogenous, single-stranded, non-coding RNA molecules that regulate gene expression transcriptionally through complementary base pairing with messenger RNA (mRNA) to direct site-specific cleavage of the target or repress translation (Gentile et al., 2013).

1.2.1 Micro RNA biogenesis

The biogenesis of miRNAs begins in the nucleus where genes encoding miRNAs are transcribed by RNA polymerase II to form capped and polyadenylated primary miRNA (pri-miRNA). An RNAse III enzyme called Drosha together with DiGeorge critical region 8 (DGCR8) (microprocessor complex) which is a double-stranded RNA-binding partner, catalyses the processing of the pri-miRNA to form stem-loop hairpin structures known as precursor miRNAs (pre-miRNAs). These are transported by exportin-5 from the nucleus into the cytoplasm through a nuclear pore in an ATP dependent manner where they are further processed by Dicer to produce mature miRNA duplexes of 21-23 nt long. One miRNA strand (guide strand) of the miRNA duplex

is incorporated into the RNA induced silencing complex (RISC), whereas the other miRNA strand (passenger strand) is degraded by unknown mechanism. This RISC complex directs the bound guide miRNA strand to the target messenger RNA (mRNA) where it either directs site specific cleavage or represses translation [**Figure 1.3,** (Gonzalez-Alegre and Paulson, 2007, Khraiwesh et al., 2012, Sun et al., 2013)].

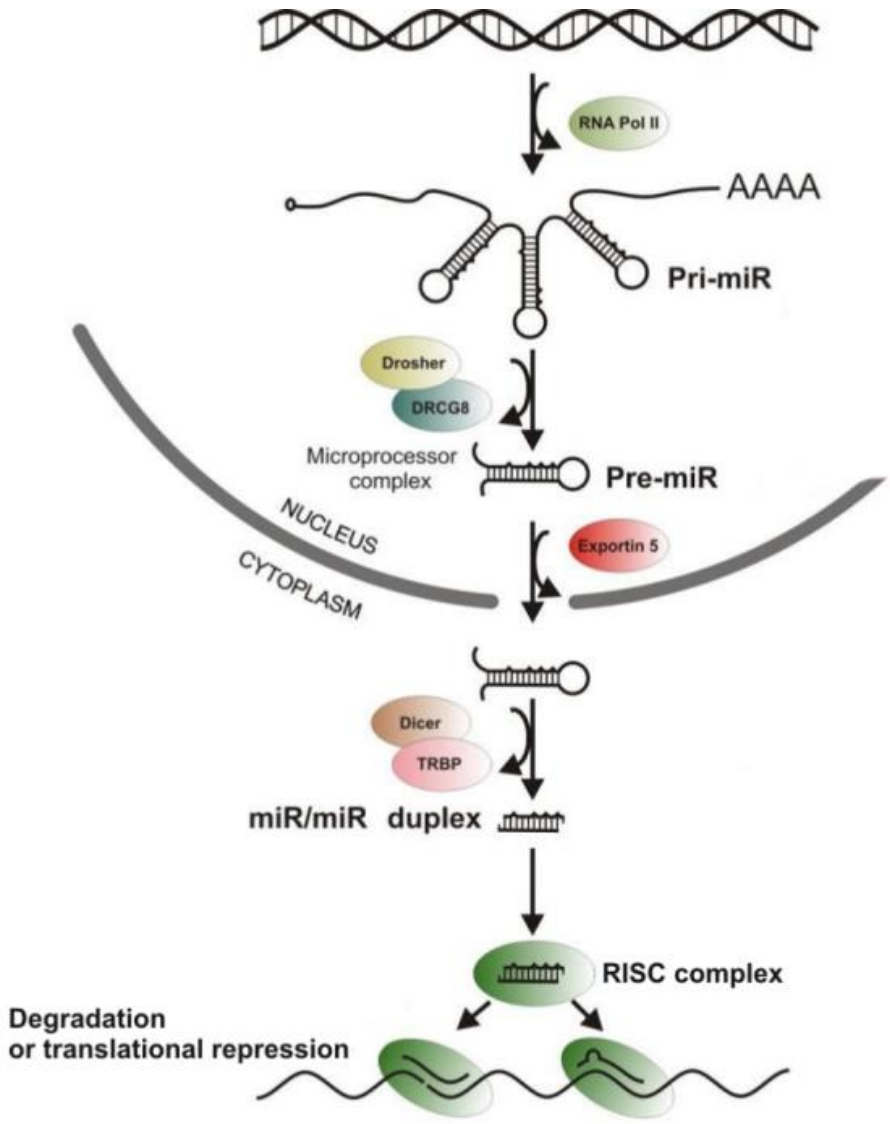

Figure 1.3: The microRNA RNA interference pathway. The biogenesis of miRNA begins in the nucleus where RNA Pol II transcribes miRNA genes into pri-miRNAs. Drosha and DGCR8 catalyse pri-miRNAs into pre-miRNAs which are transported to the cytoplasm by exportin 5. Dicer processes pre-miRNAs into mature miRNA duplexes, the guide strand is incorporated into the RISC complex which directs the guide miRNA sequence to the target messenger RNA (mRNA) where it either directs site specific cleavage or represses translation. Figure adapted from (Macfarlane and Murphy, 2010).

1.2.2 Manipulation of RNAi for Hepatitis B treatment

The ability to tap into RNAi pathway has led to the development of RNAi-based therapeutics that target disease causing genes. The RNAi pathway may be activated by exogenously supplied synthetic or expressed RNAi activators. Synthetic small interfering RNAs (siRNAs) mimic mature endogenous miRNAs whereas the expressed RNAi intermediates are derived from exogenous DNA templates and they mimic pri-/pre-miRNA of the RNAi pathway [Reviewed in (Mowa et al., 2010)].

An advantage of using siRNAs includes easier dose regulation and easy chemical modifications to reduce immune stimulation, off targeting effects and improve stability (Robbins et al., 2009). Chemicals such as 2'-fluoro, 2'-O-methyl and 2'-de-oxy sugars, phosphorothioate linkages have been used to modify siRNAs. siRNAs modified with these chemicals improved the stability of siRNAs and showed effective HBV silencing in cultured cells (Morrissey et al., 2005). However, siRNA application in gene therapy against chronic diseases like hepatitis B is limited by their short term effects and the requirement of a repeated administration for sustained therapeutic effects.

Expressed RNAi activators are best suited for treating chronic infection with HBV as they are easy to propagate and are more stable with sustained efficacy as they are continuously produced from a stable DNA template. Most antiviral expression cassettes that have been used in RNAi therapeutics to inhibit HBV replication contain Pol III promoters such as U6 and H1 promoters with pre-miRNA mimics. Pol III promoter derived pre-miRNA mimics have been shown to effectively silence HBV replication. Previous studies used adeno-associated vector serotype 8 (AAV8) to express pre-miRNA mimics (short hairpin RNAs, shRNAs) from human U6 or H1 promoters. Complete liver transduction was observed and shRNAs were effectively expressed in

the liver. These shRNAs were also able to inhibit HBV replication in Huh 7 liver-derived cells as well as in HBV transgenic mice. U6-promoter driven shRNA however had a less sustained suppression effect as serum HBV DNA levels increased after two weeks. Severe liver damage was also observed following high dose injection of the shRNAs expressing AAVs. The shRNAs toxic effects resulted in mice weight loss and hepatic necrosis was induced along with elevated serum ALT activity. These studies also showed that these toxic effects were as a result of over expression of RNAi effectors from Pol III U6 or H1 promoters which saturate the endogenous RNAi pathway (Sun et al., 2013, Grimm et al., 2006). As a result of these limitations, expression of shRNA from a Pol II liver-specific ApoE/hAAT promoter was assessed and compared to that from U6 promoter. As expected, expression of shRNA from U6 promoter was detected in spleen, kidney and the heart, whereas expression of shRNA from ApoE/hAAT was only detected in the liver. Toxicity was also assessed by measuring ALT levels post injection. In mice injected with shRNA expressed from the U6 promoter, elevated levels of ALT were observed and the mice died after 3 weeks of injection. On the other hand, in mice injected with shRNA expressed from the ApoE/hAAT promoter, stable and low ALT levels were observed throughout the 96 day period of the measurement. Results obtained in this study therefore show that Pol II promoters offer tissue specificity with minimal or diminished toxicity (Giering et al., 2008) .

To further exploit the use of tissue specific Pol II promoters, pri-miRNA mimics which are more compatible with Pol II promoters have been widely used. A study by (Yang et al., 2010) used miRNA-17-92 cluster to generate a polycistronic pri-miRNA that is processed into 5 mature miRNA strands that target various regions of the hepatitis C virus (HCV) genome. A liver specific Pol II promoter was used and to deliver this miRNA cluster, a self-complementary AAV (scAAV) vector was used. All anti-HCV pri-miRNAs expression cassettes were efficiently delivered to the liver and resulted in gene silencing *in vitro* and *in vivo* without inducing toxicity. Previous studies in our lab have also generated cytomegalovirus (CMV) promoter cassettes transcribing mimics of naturally occurring pri-miRNA-122 (pri-miRNA 122/5) and pri-miRNA-31 (pri-miRNA31/5 or polycistronic pri-miRNA31/5-8/9) against HBV (Ely et al., 2009). These pri-miRNA mimics were able to be expressed and processed to form mature miRNAs of 21 nucleotides and suppress HBV replication significantly (Ely et al., 2008, Ely et al., 2009). In a study by (Mowa et al., 2014), murine transthyretin receptor (MTTR) liver-specific promoter driven pri-miRNAs were used to inhibit hepatitis B virus replication. Results obtained from this study showed that the MTTR

promoter results in a prolonged pri-miRNA expression in mice relative to the CMV promoter (Mowa et al., 2014). Hence this study further investigated the use of MTTR promoter for anti-HBV RNAi activator expression.

Despite the availability of highly effective anti-HBV RNAi activators, finding the effective and the safest delivery system remains a daunting obstacle to progression of these sequences in to the clinic. For successful gene silencing, RNAi activators need to be safely and efficiently delivered to the target cell. To accomplish this, various methods such as viral vectors (VVs) and non-viral vectors (NVVs) have been explored.

1.3 Vectors for HBV gene silencers

1.3.1 Non-viral vectors (NVVs)

NVVs are easy and not costly to produce in large amounts therefore making them preferred alternatives for gene delivery [Reviewed in (Mali, 2013)]. NVVs systems include either chemical methods such as liposomes and polymers, or physical methods such as naked DNA electroporation and hydrodynamic injection. When liposome or polymers interact with nucleic acids, they results in the formation of lipoplexes or polyplexes. These mask the negative charge of DNA and condense DNA molecules into smaller structures to facilitate interaction with the cell membrane for nucleic acid delivery. Liposomes can be made up of cationic lipids and neutral lipids also known as helper lipids. Cationic lipids are composed of both hydrophobic and hydrophilic regions and, positive polar heads containing hydrophobic groups [Reviewed in (Sunbul, 2014)]. Advantages of liposomes include low toxicity, targeted delivery and ability to incorporate hydrophilic and hydrophobic drugs (Ziady et al., 1999, Hofland et al., 2002, Son et al., 2000, Mastrobattista et al., 2002). A study by (Hean et al., 2010) used lipoplexes containing altritol nucleic acids- (ANAs) modified siRNAs. These ANA siRNAs resulted in inhibition of HBV replication of about 50 % without an induction of toxicity or immunostimulation. Even though lipoplexes have shown promising results, their downfall is their rapid degradation and inability to achieve drug delivery over a prolonged period of time (Son et al., 2000).

Cationic polymers differ from liposomes as they do not have any hydrophobic groups, thus making them completely soluble in water (Elouahabi and Ruysschaert, 2005, De Smedt et al., 2000). The

most commonly used cationic polymers are polyethyleneimine (PEI), poly-L-(lysine) (PLL) and polyamidoamine (PAMAM). PEI is frequently used for *in vitro* gene delivery, however it is not biodegradable and due to it being highly positively charged, it results in dose dependent toxicity (Fischer et al., 1999, Gosselin et al., 2001).

Physical methods for gene delivery have an advantage of achieving direct nucleic acid delivery into cells [Reviewed in (Mehier-Humbert and Guy, 2005)]. Most commonly used methods include electroporation and hydrodynamic injection. Electroporation entails firstly injecting DNA to target tissue then applying electric pulses and voltage. This technique is efficient; however it results in a lot of cell death and has limitations in *in vivo* safety. Hydrodynamic tail vein injection is a commonly used technique for delivering genetic material into the liver of small animals. It does so by using rapid, large volume intravascular injection of liquid in to the vein. This induce high intravascular pressure, resulting in liver size increase and concomitant nucleic acid delivery in to the permeabilised cells [Reviewed in (Al-Dosari and Gao, 2009)]. RNAi activators delivered via hydrodynamic tail injection into BV transgenic mice efficiently transduced the liver and resulted in significant knockdown of HBsAg expression at 3 and 5 days post injection (Ely et al., 2009). Even though electroporation and hydrodynamic injection have been widely used as delivery techniques, they are not suited to deliver HBV-targeting RNAi therapeutics.

Even though non-viral vectors have their own advantages, the delivered nucleic acids are exposed to mononuclear phagocyte system clearance and prone to degradation by nucleases (Mehier-Humbert and Guy, 2005). They are also not suitable for large expressed RNAi activator cassettes.

1.3.2 Viral vectors (VVs)

Viral vectors are highly efficient and are more compatible with antiviral RNAi expression cassettes. The commonly used viral vectors to deliver anti-HBV RNAi effector sequences are adeno-associated viruses, lentiviral vectors and adenoviruses (Park et al., 2000).

1.3.2.1Adeno-associated viral vectors (AAVs)

AAVs are small, non-enveloped viruses that belong to the *Parvaviridae* family and the *Dependoviridae* genus. These viruses have a linear single stranded genome of 4.7 kb [Reviewed

in (Goncalves, 2005)]. AAVs have been used with great success in gene therapy mainly because they have a sustained transgene expression and are not pathogenic. In addition, following infection, AAVs require a helper-virus (herpesvirus or adenovirus) to replicate [Reviewed in (Daya and Berns, 2008)]. Thus far 12 AAV serotypes (AAV1-AAV12) and more than 100 non-human and human primate AAV variants have been discovered (Lisowski et al., 2014). Various studies have developed RNAi and AAV based gene therapeutics to treat chronic HBV infection. Three hepatotropic double stranded (ds) AAV vectors, dsAAV7, dsAAV8 and sdAAV9 were used and their transduction efficiency *in vivo* and their ability to inhibit HBV gene expression and replication were compared. These AAVs were able to deliver shRNAs to the liver efficiently and resulted in suppression of serum and liver HBV gene expression (Chen et al., 2009). Various studies have showed that by using AAVs as delivery vectors, inhibition of HBV replication is possible, however as high doses are usually required, a study by (Grimm et al., 2006) showed that this is associated with the over saturation of the endogenous RNAi pathway. Other limitations of AAVs include decreased delivery efficiency due to the high prevalence anti-AAV neutralizing antibodies in the majority of the human population and their limited transgene capacity of ~ 4.5 kb [Reviewed in (Santiago-Ortiz and Schaffer, 2016)].

1.3.2.2Lentiviral vectors (LVs)

LVs are derived from human immunodeficiency virus-1 (HIV-1) (Delenda, 2004) and they are single-stranded RNA viruses that belong to the *Retroviridae* family. These viruses replicate by reverse transcribing viral RNA genome into DNA before integration into the host genome [Reviewed in (Parr-Brownlie et al., 2015)]. LVs have an advantage of being able to transduce both diving and non-diving cells and they can be generated in high amounts (Naldini et al., 1996, Montini et al., 2009). In a study by Ivacik, self-inactivating (SIN) LVs expressing artificial HBV-silencing primary microRNAs (pri-miRNAs) from a liver specific MTTR promoter were generated. Anti-HBV sequences were efficiently processed into mature miRNAs in liver-derived Huh 7 cells and resulted in significant knockdown of HBV gene expression. Expression from the MTTR promoter lasted for over a period of 12 months in livers of HBV transgenic mice without evidence of toxicity. However, the major concern with using LVs is their ability to integrate in to the host genome. While this result in a prolonged transgene expression, it may also result in an undesired essential genes inactivation or oncogene activation (Ivacik et al., 2015).

1.3.2.3Adenoviral vectors (Ads)

As a result of their natural liver tropism, non-integrative genomes, larger transgene capacity and prolonged transgene expression, Ads are more relevant for development of nucleic acid therapeutics against chronic HBV infection. Adenoviruses belong to the *Adenoviridae* family; they are non-enveloped and have icosahedral capsids which contain a linear double-stranded DNA genome of ~ 30-40 kb. Within the capsid there are 240 homotrimeric proteins as well as 12 pentameric located at each of the apices of the capsid, whereas the homotrimeric fibre monomers extend from the penton base [Reviewed in (Mowa et al., 2010)]. There are ~50 human adenovirus (Ad) serotypes and six subgroups (A-F). Serotypes 2 (Ad2) and 5 (Ad5) of sub-group C are the most extensively studied and have been used to develop vectors for gene transfer. Ad2 and Ad5 genome is a 36-kb linear dsDNA with inverted terminal repeats (ITRs) at each end. ITRs are sequences required in cis for viral DNA replication and located near the left ITR is a cis-acting packaging signal, which is required for genome encapsidation. The Ad genome is separated into three transcriptional units based on the stage at which they are transcribed during replication. These are the early, delayed early and the late transcriptional units. The early transcriptional unit encode the E1A, E1B, E2, E3 and E4 proteins. The delayed early transcriptional unit encode the proteins IX and IVa, as well as VA RNA I and II. The late transcriptional unit, encode L1 to L5 proteins. [**Figure 1.5,** Reviewed in (Ng and Graham, 2002, Ng et al., 2002)].

E1A and E1B early genes are essential and important for transcriptional regulation. They interact with tumour suppressor proteins Rb (retinoblastoma protein) and p53 to prevent or inhibit cell cycle progression and programmed cell death while permitting establishment of viral replication (Miller et al., 2007). Proteins required for viral DNA replication are encoded for by E2 region. The E3 region encodes at least seven proteins, which play a role in host immune evasion, and this region is unnecessary for viral growth in cell culture. On the other hand, the E4 region encodes at least six proteins, which aid in DNA replication, enhancement of late gene expression and decrease host protein synthesis. The IX protein is encoded by a gene that is collinear with E1B region, however their promoters differ. The VA RNA genes are produced by RNA polymerase III during infection, and protect cells from the antiviral response as a result of interferon-α. Late region transcripts are expressed from a major late promoter (MLP) as one transcript that is later processed

by alternative splicing to produce multiple mRNAs that mainly encode virion structural proteins [L1-L5, Reviewed in (Brunetti-Pierri and Ng, 2013, Ng et al., 2002, Punga et al., 2013)].

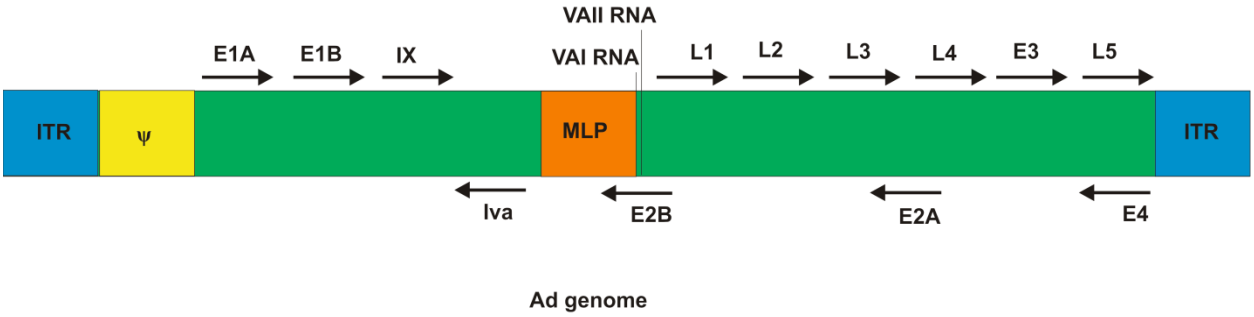

Figure 1.4: Map of wild-type adenovirus serotype 5 genome. Blue boxes at the end represent inverted terminal repeats (ITRs), ψ indicates the packaging signal and the orange box represents the major late promoter (MLP). E1-E4 represents the early genes, whereas L1-L5 shows the late transcripts. The arrows indicate direction of the transcription. Image adapted from (Palmer and Ng, 2008a).

Recombinant adenoviral vectors are the most commonly used delivery vehicles in gene therapy because they are able to transduce both dividing and non-dividing cells, have the ability to accommodate large DNA inserts, and they are easy to produce in high titres. In addition, a well-established biology of Adenoviruses makes it easy for the viruses to be manipulated for therapeutic purposes (Kreppel and Kochanek, 2008, Wonganan and Croyle, 2010).

Development of adenoviruses as gene therapy vectors is well advanced and there are mainly three types of adenoviral vectors generated: first-generation, second-generation and third-generation (**Figure 1.5**). First-generation adenoviruses have been widely applied in gene therapy for many years; these vectors do not have the viral E1 gene making them replication defective. In addition to E1 deletion some first generation vectors have E3 deleted (Ndi et al., 2013). U6 promoter shRNA cassettes against HBV have been previously delivered using 1st generation adenoviral vectors. These shRNAs were efficiently processed; shRNA5 and shRNA6 resulted in~80-100 % knockdown of HBV replication *in vitro* and *in vivo*. Even though these expression cassettes showed effective HBV inhibition, adenoviral gene expression from these vectors triggered a strong, vector dose dependant immune response (Crowther et al., 2008). Other studies have also showed that expression of adenoviral genes in these vectors triggers cytotoxic T lymphocyte (CTL)

immune response towards infected cells, resulting in the elimination of transduced cells and loss of therapeutic gene expression (Alba et al., 2005, Parks et al., 1996).

To overcome these limitations, second-generation adenoviral vectors were developed. In addition to E1 and E3 deletion, these vectors have the E2/ E4 genes removed, which reduces immunostimulatory effects; prolongs transgene expression and increases vector cloning capacity [Reviewed in (Kreppel and Kochanek, 2008)]. Various studies have used second-generation adenoviral vectors in gene therapy. Adenoviral vectors with E1 and E4 deletions and expressing β-galactosidase were constructed and these resulted in~80 % liver transduction and expression of β-galactosidase for up to 3 months in mice. Compared to first-generation, second-generation adenoviral vectors showed reduced cytopathic effect, resulted in prolonged transgene expression *in vivo* with minimal virus-specific immune response (Wang et al., 1997). A study described results of a phase I clinical trial of an E1 and E4-deleted adenovirus vector administered into the right hepatic artery of people with partial ornithine transcarbamylase deficiency (OTCD). Eighteen people were chosen to be subjects of the study and they were receiving alternate pathway therapy (sodium phenylbutyrate and citrulline/arginine) for OTCD during this study. Each subject received a single dose of E1 and E4-deleted adenoviral vector containing human OTC cDNA. The vector was infused into the right hepatic artery by using a catheter placed under fluoroscopic guidance. Following vector delivery, increase in temperature, hepatic transaminases, thrombocytopenia and hypophosphatemia were observed in all the subjects. Very toxic effects were also observed which resulted in the death of subject 18. Findings obtained in this study therefore showed that residual viral gene expression in second-generation adenoviral vectors results *in vivo*-associated immunogenicity and toxicity (Raper et al., 2002). As a result, third-generation vectors also called gutless vectors have been generated. These vectors are also called helper-dependent adenoviral vectors (HDAds) as a result of the fact that they require a helper adenovirus (HV) to replicate. They are also referred to as high-capacity adenoviruses since they can accommodate up to ~37 kb of DNA. HDAds have all their viral coding sequences removed and only retain the ITRs required for DNA replication as well as the packaging signal (ψ) required for DNA encapsidation (Alba et al., 2005).

Figure 1.5: First-, second-, and third-generation Ad vectors. First-generation vectors are devoid of E1 only, which is usually replaced by a transgene, or E1 and E3 deleted, second-generation vectors are devoid of E1 and E3 and additionally have E2/E4 deletion, whereas third-generation or HD adenoviral vectors have all the viral sequences deleted. This vector contains the 5' and 3' inverted terminal repeats (ITRs), the packaging signal (ψ), transgene, and a stuffer DNA which is there to adjust the genome size so that it is more or less the same as the wild type genome. Δ: shows deletion mutation and the purple colour shows the remaining adenoviral sequence. Image adapted from (Palmer and Ng, 2008a).

Because of their significantly reduced immune stimulation and *in vivo* long-term transgene expression with insignificant toxicity, HDAds are appropriate for use in gene therapy (Alba et al., 2005, Suzuki et al., 2010). To produce HDAds, a helper virus (HV) is required to complement for adenoviral genes required for viral production and amplification. Graham and co-workers developed the first competent and most reliable HV system for the generation of HDAds commonly used today in gene therapy. This uses the Cre/loxP system, where E1 and Cre-expressing 293 cells (293Cre) are transfected with HDAd genome and infected with the first generation adenoviral vector which carries a packaging signal flanked by loxP sites. In this system, HV genome is unpackageable as a result of Cre-mediated packaging signal excision **(Figure 1.6).** Sequential co-infection of 293Cre cells with HDAds and HV result in selective packaging of HDAd genome and increases HDAd titer which is further purified by caesium chloride (CsCl) ultracentrifugation (Palmer and Ng, 2003b).

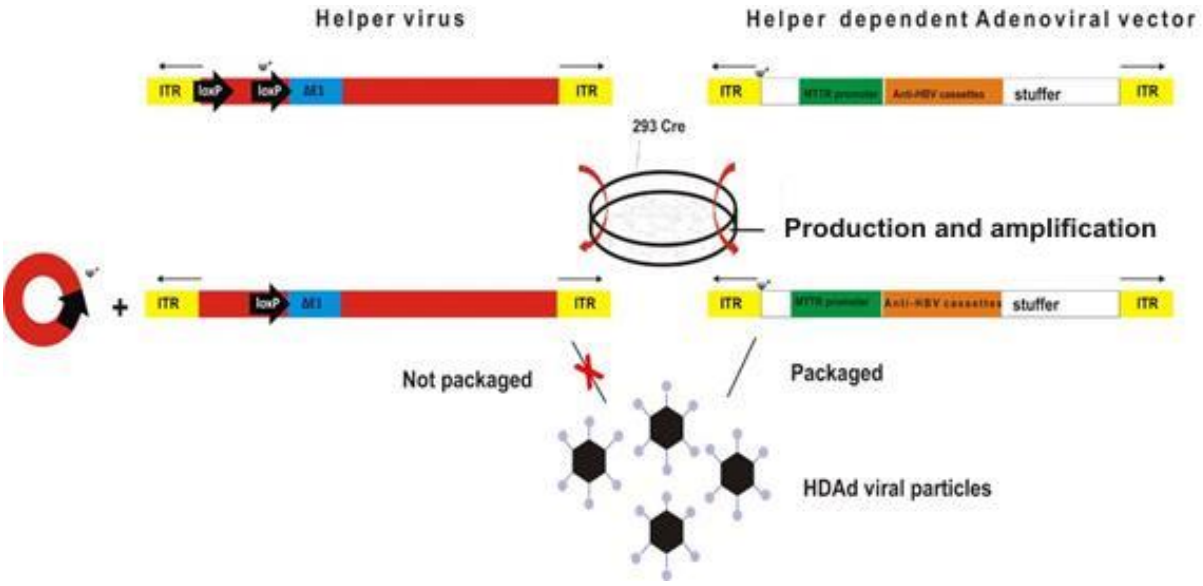

Figure 1.6: Propagation of HDAds. For production of HDAd, Cre recombinase and E1 expressing cell line (293cre) is transfected with HDAd genome and infected with HV. This results in homologous recombination of the HV loxP sites and excision of the packaging signal. The excision of this packaging signal makes the HV genome unpackagable and results in selective packaging of the HDAd genome. Image adapted from (Palmer and Ng, 2008b).

1.4 Application of HDAds for development of antiviral RNAi based therapy

For a delivery vector to be considered for therapeutic use, it needs to be safe, offer prolonged transgene expression with no induction of immune response. HDAds have been the vector of choice for various studies in gene therapy and have shown tremendous therapeutic potential. Vectors containing apolipoprotein (apo) E gene were constructed, these vectors were: i) FG-Ad5-cE which is a serotype-5 first-generation adenoviral vector containing apoE complementary DNA (cDNA) driven by a cytomegalovirus (CMV) promoter; ii) HD-Ad-cE, a helper-dependent adenoviral vector expressing apoE cDNA from a phosphoenolpyruvate carboxykinase (PEPCK) promoter; iii) HD-Ad-gE, a helper-dependent adenoviral vector expressing native apoE gene with

a liver-specific enhancer at the 3' end; and iv) HD-Ad-0, an empty helper-dependent adenoviral vector without any transgene. A single dose of the different vectors was injected via the tail vein of the 12-14 week-old apoE deficient hypercholesterolemic mice. All the apoE-Ads besides the empty vector resulted in a decrease in plasma cholesterol levels; however the effect of FG-Ad5-cE was short-lived as the plasma cholesterol levels returned to control levels after 28 days and were undetectable at day 140. Contrary to this, HD-Ad5-gE treated mice had sustained levels of plasma apoE similar to wild type for > 4 months, whereas HD-Ad5-cE had normal plasma cholesterol levels for about a year. A drastic decrease in plasma cholesterol without any signs of toxicity was also observed for HD-Ad5-cE and HD-Ad5-gE vectors, whereas FG-Ad5-cE induced an elevation in liver enzymes (Kim et al., 2001). Application of HDAds in RNAi-based therapeutics has shown great successes. First studies to demonstrate the use HDAds against HBV expressed IFN-α or IL-12 in a woodchuck or mouse model of acute or chronic hepatitis B. This resulted in specific intrahepatic expression of transgenes and sustained anti-HBV effects (Crettaz et al., 2009, Aurisicchio et al., 2000). Rauschhuber and co-workers used HDAds to deliver shRNA coding sequences expressed from the U6 promoter into HBV transgenic mice. An HDAd vector expressing β-galactosidase was used as a control. The transduction efficiency was 100 % three days post injection in hepatocytes. The shRNAs were also efficiently processed into the mature intended guide strands and resulted in about 68 % decrease in HBsAg levels *in vitro* and *in vivo* respectively. Surprisingly, the control vector resulted in 86 % decrease in HBsAg levels. Mice treated with the control vector (HD28E4*lacZ*) had elevated transaminase levels; this was assumed to be a result of immune response against β-galactosidase (Rauschhuber et al., 2008).

In a study by (Crowther et al., 2014) HDAds were used to deliver shRNAs under the control of a Pol III promoter. On the other hand, (Mowa et al., 2012) produced HDAds expressing pri-miRNA mimics also under the control of the Pol II CMV promoter. HDAds used in these studies were able to efficiently deliver anti-HBV RNAi activators to the hepatocytes and resulted in pri-miRNA expression and processing into mature guide strands. These HDAds also resulted in significant knockdown of HBV replication in liver-derived Huh 7 cells as well as in HBV transgenic mice. The only downfall about these HDAds was that they resulted in a short term transgene expression and anti-HBV effects. And this was attributed to the use of non-liver specific promoters that may be silenced in the liver. To allow for tissue-specific expression and prolong anti-HBV effects HDAds expressing pri-miRNA mimics from the liver-specific MTTR promoter were produced.

The anti-HBV pri-miRNAs were efficiently delivered *in vitro* and *in vivo* thereby may result in significant knockdown of HBV replication and prolonged transgene expression compared to CMV promoter expressed pri-miRNAs (Mowa et al., 2014).

Even though HDAds have all the adenoviral genes deleted, showed improved safety profile and are more effective than their counterparts, immunostimulatory effect from capsid proteins and sometimes from transgene encoded proteins is still a challenge.

1.4.1 Reducing immune stimulation by the capsid proteins

Shielding epitopes on the capsids by chemical modifications using polymers such as polyethylene glycol (PEG) have been shown by several studies to reduce non-specific interactions with non-target sites which may results in the induction of the immune response and elevated toxicity (Kreppel et al., 2005). PEG is a chemically inert compound consisting of either linear or branched repeat units of ethylene oxide. The covalent attachment of PEG to molecules is known as PEGylation and this technique has been employed to improve the stability, solubility and immunological properties of many biological compounds such as lipids, cells, viruses etc. (Wonganan and Croyle, 2010).

As a result of hydroxyl (-OH) groups, which are unable to react with the proteins, PEG molecules require activation before they can be coupled to proteins. This is achieved by substituting the single –OH group at the end of the PEG molecule with an electrophilic reactive group that can be covalently linked to the reactive site of the molecule of interest (Wonganan and Croyle, 2010). Target sites normally used for protein PEGylation are the epsilon-amino groups of lysine residues, N-terminus of the protein or thiol groups of cysteine residues. The surface of Ad vector capsid is dominated by the amine functional groups resulting in approximately 18 000 amine groups on human Ad5. N-hydroxysuccinimide (NHS) esters are activation groups required for amine PEGylation. Maleimide or dithiopyridyl are activation groups required for thiol PEGylation (Kreppel and Kochanek, 2008).

Monomethoxy polyethylene glycol-succinimidyl propionate (mPEG-SPA) was used to alter anti-HBV RNAi effector expressing first-generation vectors. From this study, PEG-modified vectors

resulted in a decrease in intrahepatic HBV mRNA and HBV viral particle equivalents (VPEs) following the first Ad injection. Following injection of PEGylated and unPEGylated Ad, serum levels of monocyte chemoattractant protein-1 (MCP-1, marker of inflammation) were elevated in mice which received unPEGylated vector (Crowther et al., 2008). Even though the amine PEGylation used in this study was able to reduce immunostimulation, studies have shown that this PEGylation method reduce Ad transduction efficiency (Kreppel and Kochanek, 2008).

To allow efficient thiol dependent PEGylation of Ad vectors, a helper-virus (HV) carrying a gene encoding the hypervariable hexon region of the capsid with a cysteine modification was developed. The HV (AdNG163cys) was generated by introducing a single point mutation into the hypervariable region 5 (HVR5) hexon of AdNG163 (with wild type HVR 5), where alanine was substituted by a cysteine residue. Ads produced using AdNG163cys were reacted with maleimide-activated PEG (mal-PEG) molecules of various sizes. After performing Western transfer and immunochemical detections with an anti-hexon antibody, 50-70 % of the hexon monomers were efficiently modified. It was also found out that cysteine modification does not alter vector tropism but rather increases the liver transduction efficiency (Prill et al., 2011). Hence, the application of HDAds with cysteine modification for anti-HBV RNAi effector delivery was investigated in this study.

1.4.2 Reducing the immune stimulation by transgene encoded proteins.

For a delivery vector to be regarded effective, it must be able to deliver efficiently the therapeutic to the target cells. The common way of checking for the efficiency of gene transfer or delivery is through the use of reporter genes. *Lac Z* which encodes for β-galactosidase is the commonly used reporter gene in gene delivery, mainly because of its ease of detection *in vitro* and *in vivo* (Naderian et al., 2011). In the various studies that have investigated the association of β-galactosidase and induction of immune response, common observations were that in the presence of β-galctosidase transgene expression is shortened and results in vector clearance (Chen et al., 1997, Morral et al., 1997, Sullivan et al., 1997). Elevation of β-galactosidase specific antibodies and short-term inhibition of HBV replication was also observed in a study where β-galactosidase was co-delivered with anti-HBV RNAi activator sequences (Crowther et al., 2014). To avoid such limiting results,

lacZ deficient HDAds expressing pri-miRNA mimics from a liver-specific promoter were produced in this study.

1.5 Aim and objectives

The aim of this study was to produce and characterize *lac* Z deficient recombinant HDAds expressing anti-HBV pri-miRNA sequences from MTTR liver-specific promoter. To achieve this aim the following objectives were set.

Objectives

1. Construction of adenoviral plasmids expressing anti-HBV pri-miRNAs from MTTR promoter.
2. Production, amplification and purification of anti-HBV helper-dependant adenoviral vectors using unmodified and cysteine modified helper virus
3. Measurement of *in vitro* pri-miRNA expression in HDAds infected cell line and in mice using Northern blot hybridization
4. Assessment of HBV knockdown *in vitro* and *in vivo* using ELISA.
5. Measurement of inflammatory cytokines in mice by using cytometric bead array (CBA).
6. Assessment of HDAd-induced liver toxicity by measuring serum alanine aminotransferase (ALT) levels.

CHAPTER 2

2. MATERIALS AND METHODS

2.1 Bacterial culturing, storage and manipulations

2.1.1 Culturing of *E.coli* cells

Xl-Blue and DH5α *Escherichia coli* (*E. coli*, Table 2.1) cells were grown in Luria Bertani media (LB, Appendix 7.1.1) or Luria Bertani agar media (LA, Appendix 7.1.2) at 37 °C. To grow strains carrying antibiotic resistance marker, media was supplemented with 100 µg/mL or 50 µg/mL of ampicillin (Appendix 7.1.3) or kanamycin (Appendix 7.1.4) respectively. The liquid cultures were incubated shaking at 100-150 g. All freezer stocks were frozen in 25 % glycerol at -80 ºC. Table 2.1 shows the *E.coli* strains used in this study along with their genotypes.

Table 2.1 Table of *E.coli* strains used in this study

Bacterial strain	**Genotype**	**Company/ Reference**
Xl-Blue cells	F- φ80*lac*ZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rk - , mk +) phoA supE44 thi-1 gyrA96 relA1 λ-	Thermo Fischer Scientific, MA,USA
DH5α cells	fhuA2 Δ(argF-*lacZ*)U169 phoA glnV44 ϕ80Δ (*lacZ*) M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	New England Biolabs®Inc, MA, USA

2.1.2 Preparation of chemically competent Xl-Blue cells

Xl-Blue cells from a freezer stock (2 µl) or from a single colony were inoculated in 10 mL of LB media (Appendix 7.1.1). The bacterial culture was incubated overnight at 37 °C with shaking at 100 – 150 rpm. The pre-culture was diluted 100× in LB media (Appendix 7.1.1) and incubated at 37 °C with shaking at 200 – 250 rpm. The optical density (OD) was measured until the absorbance reading at 600 nm was between 0.4 – 0.6. The cultures were poured into 50 mL Falcon tubes and

centrifuged at 6000 g for 20 minutes at 4 °C. The supernatant was discarded; the pellet was re-suspended in 10 mL of the transformation buffer (Appendix 7.1.6) and incubated on ice for 20 minutes. The cells were centrifuged at 6000 g for 15 minutes, the supernatant was discarded and the pellet was re-suspended in 1 mL of the transformation buffer (Appendix 7.1.6). Aliquots of 100 µl were transferred into sterile micro-centrifuge tubes and stored at -80 °C.

2.1.3 Transformation of chemically competent Xl-Blue cells

Chemically competent Xl-blue cells (100 µl) were added to a pre-chilled DNA. The mixture was incubated on ice for 10 minutes then heat shocked on a heating block at 42 °C for 90 seconds. Immediately after heat shock, 500 µl of pre- warmed LB (Appendix 7.1.1) was added to the mixture and incubated for an hour at 37 °C with shaking for phenotypic expression. The entire volume was plated on pre-warmed LB agar plates (Appendix 7.1.5) supplemented with the relevant antibiotic. The plates were allowed to dry at 37 °C and placed upside down in the incubator at 37 °C overnight.

2.1.4 Transformation of DH5α electro-competent cells

About 50 µl of electro competent DH5α cells (New England Biolabs, MA, USA) was added to pre-chilled DNA. The mixture was incubated for 10 min on ice and transferred into 2 mm electroporation cuvettes. The electroporation conditions were set on the Biorad Gene Pulsar Xcell^{TM} Electroporation system as follows: voltage: 2000V, capacitance: 25F and resistance: 200 Ω. The samples were electroporated by pressing pulse and 1 mL of pre-warmed rescue C-media (Thermo Fisher Scientific, MA, USA) was immediately added into the cuvettes. Cells were transferred into 2 mL micro centrifuge tubes, incubated for 1 hr at 37 °C with shaking at 150 rpm. Cells were plated on LB agar plates (Appendix 7.1.5) supplemented with the appropriate antibiotic. The plates were allowed to dry at 37 °C and placed upside down in the incubator at 37 °C overnight.

2.2 Plasmid DNA preparation and purification

2.2.1 Small scale plasmid DNA isolation by alkaline lysis

A single colony containing the plasmid of interest was inoculated in 2 mL of LB (Appendix 7.1.1) media supplemented with the appropriate antibiotic and incubated overnight with shaking at 37 °C. The bacterial cultures were then transferred into sterile 2 mL micro-centrifuge tubes and centrifuged for 30 seconds at maximum speed to pellet the bacteria. The pellet was re-suspended in 180 µl of buffer P1 (Appendix 7.2.1) and 160 of Buffer P2 (Appendix 7.2.2) was added to the cells. To allow cell lysis, the mixture was gently inverted and incubated at room temperature for 5 minutes. Buffer P3 (120 µl, Appendix 7.2.3) was added to neutralise the solution and mixed thoroughly by flicking the tube and incubated on ice for 5 minutes. This was centrifuged for 10 minutes at maximum speed at 4 °C. The supernatant was transferred to a new sterile micro-centrifuge tube and the DNA was precipitated by adding 600 µl of 100 % isopropanol (Merck Chemicals (Pty) Ltd, Darmstadt, Germany). This was inverted to mix, incubated at room temperature for 2 minutes and centrifuged at maximum speed for 20 minutes. The pellet was washed with 150 µl of 70 % ethanol (Merck Chemicals (Pty) Ltd, Darmstadt, Germany), centrifuged at maximum speed for 2 minutes at 4 °C and air-dried for 10 minutes at room temperature. DNA was re-suspended in 50 µl of sterile double deionised water (ddH_2O) and concentration determined using the Nano-drop spectrophotometer at OD_{260}.

2.2.2 Large scale Plasmid DNA isolation

2.2.2.1 Plasmid DNA isolation by QIAGEN Plasmid Maxi Kit

Large scale plasmid DNA preparation and purification was performed using Qiagen Plasmid Maxi Kit (Qiagen, MD, USA) according to manufacturer's instructions. Briefly, a single colony with the plasmid of interest was inoculated in 100 mL of LB (Appendix 7.1.1) media supplemented with the appropriate antibiotic and incubated overnight at 37 °C with shaking at 150 rpm. The bacterial cells were harvested by centrifugation at 6000 g for 15 minutes at 4 °C. The bacterial pellet was re-suspended in 10 mL of chilled buffer P1 (Appendix 7.2.1), 10 mL of buffer P2 (Appendix 7.2.2) was subsequently added and the samples were mixed by inverting the tube then incubated at room temperature for 5 minutes. Ten millilitres of chilled buffer P3 (Appendix 7.2.3)

was added; samples were mixed by inverting the tube and incubated on ice for 15 minutes. The solution was centrifuged at 20 000 g for 30 minutes at 4 °C. During centrifugation step Qiagen-tip 500 was equilibrated by applying 10 mL of QBT buffer and the column was allowed to empty by gravity flow. The supernatant from the centrifuged sample was applied to the Qiagen-tip 500 and allowed to enter the resin by gravity flow. Qiagen-tip 500 was washed twice with 30 mL of QC buffer and placed in a 50 mL Falcon tube. The DNA was eluted with 15 mL of QF buffer and precipitated by adding 10.5 mL of room temperature isopropanol (Merck Chemicals (Pty) Ltd, Darmstadt, Germany). The solution was mixed and centrifuged at 15 000 g for 30 minutes at 4 °C. The supernatant was carefully removed, the pellet was washed with 500 µl of ice cold 70 % ethanol (Merck Chemicals (Pty) Ltd, Darmstadt, Germany) and centrifuged at 15 000 g for 10 minutes at 4 °C. The supernatant was carefully removed, the pellet was air-dried for 10 minutes and the DNA was re-dissolved in 500 µl of sterile dH_2O. The concentration of the plasmid DNA was measured on the Nano-drop Spectrophotometer.

2.2.2.2 Plasmid DNA isolation by Genopure Plasmid Maxi Kit

Plasmid DNA used for the generation of recombinant HDAd in tissue culture was purified using Genopure Plasmid Maxi Kit (Roche Diagnostics, GmbH, Germany), following the manufacturer's instructions with minor modifications. Briefly, a single colony with the plasmid of interest was inoculated in 100 mL of LB (Appendix 7.1.1) media supplemented with the appropriate antibiotic and incubated overnight at 37 °C with shaking at 200-250 rpm. The bacterial cells were centrifuged for 30 minutes at 5000 g and the pellet was re-suspended with 8 mL suspension buffer. Twelve millilitres of lysis buffer was added to the suspension and incubated for 5 minutes at room temperature. This was followed by addition of 12 mL neutralization buffer, gentle mixing by inversion and incubation for 5 minutes on ice. The solution was centrifuged for 1 hour at 12 000 g and the lysate was loaded onto the column equilibrated with 6 mL of equilibration buffer. The column was allowed to empty by gravity and it was washed twice with 16 mL of wash buffer. The column was inserted into a new collection tube and the plasmid was eluted with 15 mL of elution buffer. The eluted plasmid was precipitated with 11 mL of isopropanol (Merck Chemicals (Pty) Ltd, Darmstadt, Germany), incubated for 30 minutes at -20 °C and centrifuged for 30 minutes at 15 000 g. The pellet was washed with 4 mL of chilled 70 % ethanol (Merck Chemicals (Pty) Ltd,

Darmstadt, Germany) and centrifuged for 10 minutes at 15 000 g. The pellet was air-dried for 10 minutes at room temperature and dissolved in 500 µl of sterile distilled water. The concentration of the plasmid DNA was measured on the Nano-drop –Spectrophotometer.

2.3 Manipulation and analysis of DNA

2.3.1 Restriction enzyme (RE) digestion of plasmid DNA

Plasmid DNA isolated using the above mentioned methods was digested with the desired RE for the preparation of cloning vectors, extraction of inserts and screening for positive clones. All the solutions were thawed on ice. Digestion reactions were set up with 1-11 µg of DNA, 1× reaction buffer, 1-11 U enzyme (Thermo Fisher Scientific, MA, USA or New England Biolabs, MA, USA), dH_2O to top up to the required volume and incubated overnight at 37 °C.

2.3.2 Vector dephosphorylation

Plasmid DNA was digested with the RE, which was then inactivated by heating for 20 minutes at 65 °C. To prevent re-circularization of the vector, the DNA was incubated with 1 U/µg of Antarctic Phosphatase (New England Biolabs, MA, USA) for an hour at 37 °C. The reaction was inactivated by heating at 75 °C for 15 minutes on a heating block.

2.3.3 Quantitative Polymerase Chain Reaction (qPCR)

Quantitative PCR (qPCR) was performed by mixing 1 µl of 10 µM forward and reverse primers (**Table 2.2**), 10 µl of 2 × concentrated FastStart Essentila DNA Green Master (Roche Diagnostics, GmbH, Germany), 20-100 ng of DNA and dH_2O was added to top up the final volume to 20 µl in a PCR tube. Reactions with DNA standards were also set-up the same way in parallel. The reactions were put in the Bio-Rad CFX96^{TM} Real Time System (Bio-Rad, CA, USA) machine.

The cycling conditions were set as shown in Table 2.3 and the reactions performed in the 27thermos cycler.

Table 2.2: Primers used in this study

Primer	Sequence	Description	Reference
HDAd F	5'GAAAAAACACACTGGCTTGAAACA'3	Forward primer used for HDAd sequence amplification	(Palmer and Ng, 2003a)
HDAdR	5'TGCCACCTCGTATTTCACCTCTA'3	Reverse primer for HDAd sequence amplification	(Palmer and Ng, 2003a)
HV F	5' TGGGCGTGGTGCCTAAAA'3	Forward primer for helper-virus sequence amplification	(Palmer and Ng, 2003a)
HV R	5' GCCTGCCCCTGGCAAT'3	Reverse primer for helper-virus sequence amplification	(Palmer and Ng, 2003a)

Table 2.3: Cycling conditions for PCR

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	95	5 minutes	1
Denaturation	98	20 seconds	34
Primer annealing	60	30 seconds	
Extension	72	3 minute/kb	
Final extension	72	5 minutes	1
Cooling	4	∞	1

2.3.4 Ligation

For the cloning of RE digested fragments, Fast-Link™ DNA Ligation Kit (Epicentre Biotechnologies, WI, USA) was used following the manufacturer's instructions. Ligation reactions were setup with the insert at a molar excess and various vector to insert ratios (1:3, 1:6, 1:12 and 1:24) were used. The ligation reactions were set up as shown in Table 2.4 in sterile micro-centrifuge tubes. The reactions were incubated at 4°C overnight. The ligation mixture (1 µl) was transformed in DH5α electro competent cells (section 2.1.4, New England Biolabs®Inc, MA, USA).

Table 2.4: Ligation reaction set up for Fast-Link™DNA Ligation

Component	Quantity
10× Fast-Link Ligation Buffer	1.5 µl
10 mM ATP	1.5 µl
Vector DNA	1000 ng
Insert DNA	50 – 500 ng
Sterile Water	Up to 14 µl
Fast-Link DNA Ligase	1 µl

2.3.5 Agarose gel electrophoresis

A 1 % agarose gel was prepared by weighing 1 g of powdered agarose into a conical flask, 100 mL of 1 × tris-acetate-EDTA (TAE, Appendix 7.2.7) electrophoresis running buffer was added and the solution was mixed by swirling. The agarose was melted by microwaving for 3 minutes and cooled to ~ 55 °C. Once the agarose has cooled down, 10 µl of 10 mg/mL ethidium bromide was added. The gel was poured in to the assembled casting tray. The gel was allowed to solidify, the comb and dams were removed from the tray and the gel was placed into the electrophoresis tank. TAE buffer (Appendix 7.2.7) was added to cover the wells. Tracking dye (Thermo Fisher Scientific, MA, USA) was added to DNA to a final concentration of 1 ×. A DNA size molecular weight marker (Thermo Fisher Scientific, MA, USA) was loaded in the first and last wells, whereas the samples were loaded in the middle wells. The current was applied and the gel was run at 90 V until the tracking dye reached the bottom of the gel.

2.3.6 DNA extraction from agarose gel

2.3.6.1 Gel Extraction by QIAquick Gel extraction kit

Plasmid DNA digested with restriction enzyme of interest was run on a 1 % agarose gel and fragments of interest extracted using the QIAquick®Gel Extraction Kit (Qiagen, CA, USA) following the manufacturer's instructions. Briefly, the DNA fragments of interest were excised

from the agarose gel with a clean, sharp scalpel. The gel slices were weighed, three volumes of buffer QG was added (0.1g ~ 100 µl) and incubated at 50 °C for 10 minutes or until the gel slices had completely dissolved. Following addition of isopropanol, the samples were transferred to the QIAquick spin columns in a 2 mL collection tubes and centrifuged for 1 minute at maximum speed. The flow through was discarded and the columns were placed back in the same collection tubes. About 500 µl Buffer QG was added to spin columns and centrifuged for a minute at maximum speed. To wash the DNA sample, 500 µl buffer PE was added and spun for a minute at maximum speed. The spin columns were transferred to a sterile 1.5 mL micro centrifuge tube. Fifty microliters of sterile dH_2O was added to elute the DNA from the columns. This was centrifuged at maximum speed for 1 minute. The concentration of the DNA was measured on the Nano-drop spectrophotometer and 500 -1000 ng was run on a 1 % agarose gel to confirm the correct DNA fragment size.

2.3.6.2 Gel Extraction by phenol Chloroform

The agarose gel was placed under UV light and a sharp clean scalpel was used to excise the DNA fragment of interest. A sterile heated needle was used to pierce a small hole at the bottom of a sterile 0.5 µl micro centrifuge tube and fish tank wool was placed at the bottom of tube. The gel pieces containing DNA fragments of interest were added to this tube, the 0.5 µl tube was placed inside a sterile 2 mL micro centrifuge tube. This was centrifuged at maximum speed until all the buffer had transferred to the 2 mL tube. Phenol and chloroform (100 µl per 300 µl buffer) at a 1:1 ratio were added to the DNA and it was centrifuged at maximum speed for 5 minutes. The upper layer was removed and transferred to a new sterile micro centrifuge tube. Chloroform was added and the mixture was centrifuged for a minute at maximum speed. The upper layer was removed and transferred to a sterile micro centrifuge tube and the volume was adjusted to 300 µl with dH_2O. Sodium acetate to a final concentration of 0.3 M **(**Sigma-Aldrich, MO, USA) and 2.5 × of 100 % ethanol (Merck Chemicals (Pty) Ltd, Darmstadt, Germany) was added. The mixture was incubated for 30 minutes at -20 °C. The solution was centrifuged for 30 minutes at maximum speed and the resultant pellet washed with 500 µl of 70 % ethanol (Merck Chemicals (Pty) Ltd, Darmstadt, Germany). The solution was centrifuged for 5 minutes at maximum speed and the pellet was air-dried and re-suspended in 20 µl of sterile dH_2O.The concentration of the DNA was measured on

the Nano-drop spectrophotometer and 500 - 1000 ng was run on a 1 % agarose gel to confirm the correct DNA fragment size.

2.4 Construction of *lac Z* deficient Adenoviral plasmids expressing anti-HBV sequences from mouse transthyretin (MTTR) promoter

Construction of required plasmids was carried according to standard cloning procedures described above. Table 2.5 shows all the plasmid used and constructed in this study.

For the generation of adenoviral plasmids expressing anti-HBV pri-miRNAs from MTTR promoter, monomeric or trimeric pri-miRNA expression cassettes in pTZ pri-miR-31/5 or pTZ pri-miR-31-5-8-9 previously described by (Mowa et al., 2012) were extracted using AscI restriction enzyme and ligated into the *lac Z* deficient adenoviral genome bearing plasmid (pΔ28E4, kindly donated by Brendan Lee, Baylor College, USA) AscI site.

Table 2.5 Description of plasmids used and constructed in this study

Plasmid name	Description	Selection marker	Reference

pTZMTTR-pri-mi-R-31/5	pTZ57R/T TA cloning vector carrying pri-miR-31/5 MTTR promoter cassette with AscI engineered RE sites	Ampicillin	(Mowa et al., 2014)
pTZMTTR-pri-mi-R-31/5-8-9	pTZ57R/T TA cloning vector carrying pri-miR-31/589 MTTR promoter cassette with AscI engineered RE sites	Ampicillin	(Mowa et al., 2014)
pΔ28E4	*Lac Z* deficient Adenoviral genome bearing plasmid	Kanamycin	Brendan Lee, Baylor College, USA
pΔ28E4CMV*lacZ*	Adenoviral genome bearing plasmid expressing the lac Z reporter gene from the CMV promoter	Kanamycin	Phillip Ng, Baylor College, USA
pHDAd-pri-miRNA-31/5	pΔ28E4expressing anti-HBV pri-miRNA 31/5 from the MTTR promoter	Kanamycin	This study
pHDAd-pri-miRNA-31/589	pΔ28E4expressing anti-HBV pri-miRNA 31/589 from the MTTR promoter	Kanamycin	This study
pCH-9/3091	A target plasmid with a greater genome length HBV sequence	Ampicillin	(Nassal et al., 1990)
pCI-neo-eGFP	pCI-neo mammalian expression vector expressing the enhanced green fluorescence protein (eGFP) from the CMV promoter	Ampicillin	(Passman et al., 2000)

2.5 Tissue culture methods

2.5.1 Cell culturing conditions

Human Embryonic Kidney 293 (HEK293) and Human hepatoma (Huh7) cells were propagated in Gibco® Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific, MA, USA).

HEK293 derived 116 cells were propagated in Eagle's Minimum Essential Medium (EMEM, Thermo Fischer Scientific, MA, USA) or Joklik Eagle's Minimum Essential Medium (JEMEM). DMEM media was supplemented with 10 % (W/V) fetal bovine (FBS, Thermo Fisher Scientific, MA, USA), 100 000 U/mL of penicillin and 100 µg/mL of streptomycin. EMEM media (Appendix 7.3.1) was supplemented with 10 % or 5 % (W/V) Gobco® FBS (Thermo Fisher Scientific, MA, USA), penicillin to 100 000 U/mL, streptomycin to 100 µg/mL, as well as Gibco® L-glutamine (Thermo Fisher Scientific, MA, USA) to 2 mM. JEMEM (Appendix 7.3.3) with 5 % (W/V) faecal calf serum (FBS, Thermo Fisher Scientific, MA, USA), 100 000 U/mL of penicillin, 100 µg/mL of streptomycin and 0.1 mg/mL hygromycin (Sigma-Aldrich, MO, USA) was used. All cell lines were maintained in humidified incubators at 37 °C and 5 % CO_2.

2.5.2 Seeding and growing of cells

To grow up cells from a frozen stock, 1 mL aliquot of cells was defrosted and added to 10 mL of appropriate complete media (with 10 % FBS and 2 mM L-glutamine). This was spun down for 3 minutes at 5000 g to pellet the cells. The media was carefully discarded and the pellet was re-suspended in 2 mL of complete media, transferred into a small flask (25 cm^2) with 5 mL of complete media. The cells were left in the humidified incubator until they were 100 % confluent. To subculture the cells, medium was poured off, 1mL of saline was added to wash the cells. Cells were incubated with 1 mL of saline supplemented with EDTA for 3 minutes at 37 °C in the incubator. For Huh7 cells, saline-EDTA was replaced with 500 µl of 0.5 × Gibco® TrypLE express (Thermo Fisher Scientific, MA, USA). The flask was tapped on the sides to detach the cells, 1mL of complete media (Thermo Fisher Scientific, MA, USA) was added to inactivate triple express and the cells were pipetted up and down to break any clumps. Cells were then transferred to a medium 75 cm^2 flask which had 8 mL of media with 10 % FBS (Thermo Fisher Scientific, MA, USA) and grown in the incubator until the desired confluency was reached. The same procedure was used to sub-culture in to a 175 cm^2 flasks, with 5 mL saline or saline-EDTA, 2 mL TrypLE express, 3 mL complete media to inactivate trypsin and 20 mL final complete media used.

2.5.3 Freezing of cells

To freeze the cells, 100 % confluent cells in a 175 cm^2 flask were washed and detached as described above. Following detaching, 10 mL of complete media was added to the cells and centrifuged for 3 minutes at 5000 g. The supernatant was discarded and the pellet was resuspended with 10 mL of freezing media (Appendix 7.3.4). Aliquots of 1 mL were added to cryovials and stored at -80 °C overnight then moved to liquid nitrogen for long term storage.

2.5.4 Transfection

Cells were seeded at 50 % into 60 mm plates or 24 well-plates a day before transfection as described in section 2.5.2.

2.5.4.1 PEImax transfection

An hour before transfection, the medium from the 60 mm plates with 116 cells was replaced with 5 mL of fresh EMEM media (10 % FBS). The cells were transfected with the *Pme I* digested HDAd plasmid (pHDAd) DNA using the PEImax (Thermo Fischer Scientific, MA, USA) transfection method. Two solutions containing 500 µl of OptI-MEM® (Gibco, Life Technologies, UK) media were made up. The first solution contained 500 µl of Opti-MEM media and 30 µl of PEImax, the second solution contained 500 µl of Opti-MEM and 10 µg in 50 µl *Pme I* linearized pHDAd DNA. The solutions were incubated at room temperature for 10 minutes. The two solutions were then mixed together and were incubated at room temperature for 20 minutes. The solution mixture was added drop wise to 116 cells on the 60 mm plates with 5 mL of EMEM (10 % FBS) and incubated overnight at 37 °C in the incubator at 5 % CO_2.

2.5.4.2 Lipofectamine® 3000 transfection

Transfection of Huh7 cells was carried out using Lipofectamine® 3000 transfection kit (Invitrogen, Life technologies, UK). A day before transfection, Huh7 cells were seeded to 50 %

confluency on a 24 well plate (section 2.5.2) by using DMEM media supplemented with 10 % FBS (Thermo Fischer Scientific, MA, USA) and antibiotics. An hour before transfection, the medium was removed and 500 µl of fresh antibiotic-free DMEM media with 10 % FBS was added. The DNA mix as well as a lipofectamine mix transfection reactions were setup and incubated at room temperature for 5 minutes. The DNA mix comprised of the 100 ng of pCH-9/3091 plasmid, 100 ng of pCI-neo-eGFP plasmid (Table 2.5) and 50 µl of Opti-MEM. For the lipofectamine mix, 0.2 µl of Lipofectamine®3000 was added to 50 µl of Opti-MEM. The two mixes were mixed together and incubated for 15 minutes at room temperature. A total of 100 µl of the transfection solution was added to cells drop wise. The cells were incubated for 5 hours in the incubator at 37 °C.

2.6 Helper virus amplification

To amplify the previously produced helper virus, about 1.5×10^5 HEK293 cells were seeded in a total volume of 15 mL 5 % FBS media in a 150 cm^2 plate. The cells were immediately infected with Helper virus (HV) at an MOI of 0.1 and incubated at 37 °C. The spent media was replaced with fresh media every 48 hours for 10 days, whereby suspended cells were harvested by centrifugation and put back in to the plate. After 10 days, the cells were scraped off in to the spent media and transferred in to 50 mL Falcon tubes. Then 1.5 mL of 40 % sucrose was added, cells were lysed by three freeze-thaw cycles and stored at – 80 °C.

2.6.1 Titration of HV using immunocytochemical staining

Following HV amplification, infectious units (ifus) were determined by immunocytochemical staining using Ultra-Sensitive ABC Peroxidase Mouse IgG staining kit (Thermo Fisher Scientific, MA, USA) according to manufacturer's instructions. Briefly, HEK293 cells were seeded in a 24 well-plate to 50 % confluency in 10 % media (section 2.5.2). The next day the media was removed and cells were washed with 5 % FBS containing media. HV was then diluted (10^0-10^{-3}) in 50 µl of 5 % media and added to the cells. To allow attachment, cells were incubated at 37 ^{0}C for 1 hour rocking the plate every 10 minutes before 450 µl of 5 % FBS DMEM was added to the wells. The

cells were then incubated at 37 °C for 48 hours. The media was removed from the plate, the plate was allowed to dry and the cells were fixed with 300 µl of ice cold methanol and incubated at -20 °C for 10 minutes. The cells were washed 3 × with 1×PBS then blocked with 5 drops of blocking buffer and incubated at room temperature for 20 minutes. The cells were washed once with PBS, permeabilised by adding 200 µl of permeabilisation buffer and incubated for 5 minutes at room temperature. The cells were washed once with PBS, 5 drops of Adenovirus Fiber antibody (BIOCOM biotech, Gauteng, South Africa) diluted 1000× in blocking buffer was added and incubated at room temperature for an hour. The cells were then washed 3× with PBS, 5 drops of biotinylated secondary antibody were added and incubated at room temperature for 30 minutes. PBS was used to wash cells (3×), 5 drops of the ABC reagent were added and incubated at room temperature for 30 minutes. The cells were washed 3× with PBS, 300 µl of the 1× 3,3'-Diaminobenzidine (DAB) substrate (Roche Diagnostics, GmbH, Germany) was added to the cells and incubated at room temperature until the brown stain can be visualised. The cells were viewed and brown stained cells counted under the light microscope.

2.7 HDAds production and amplification

2.7.1 Production of HDAds

Hek293 derived 116 cells were seeded at 50 % into 60 mm plates a day before transfection as described in section 2.5.3. The media was changed an hour before transfection, where 5 mL of spent EMEM was replaced with fresh media. An amount of 10 µg of the pHDAd DNA (pHDAd-pri-miRNA-31/5 or pHDAd-pri-miRNA-31/589 or pΔ28E4 or pΔ28E4*lacZ*CMV) was digested with *Pme I* restriction enzyme in 50 µl was used to transfect 116 cells using PEImax (section 2.5.4.1, Thermo Fischer Scientific, MA, USA) to produce HDAd-pri-miRNA-31/5 or HDAd-pri-miRNA-31/589 or HDAd28E4 or HDAd*lacZ*CMV.

One day after transfection, HV at an MOI of 5 in 1 mL EMEM supplemented with 5 % FBS was added to the cells. To allow attachment, cells were incubated for an hour at 37 °C with rocking every 10 minutes. The media was then topped up to 2.5 mL with EMEM media supplemented with 5% FBS and incubated for 48 hours at 37 °C to allow selective production of HDAds. The cells

were examined for complete cytopathic effect (CPE) and scraped off in to the spent media with a cell scraper. Sucrose was then added to 4 % and the cells were transferred to 2 mL sterile Eppendorf tubes. The cells were subjected to three freeze/thaw cycles in liquid nitrogen and 37 °C water bath. Cell lysates were stored at -80 °C.

2.7.2 Amplification of HDAds

About 70 % 116 cells confluent 60 mm plates were co-infected with HV at MOI 2 and HDAd in the 400 µl of cell lysate from the production step (Section 2.7.1). For adsorption, 600 µl of EMEM media supplemented with 5 % FBS was added to the plate and incubated at 37 °C for an hour, rocking every 10 minutes. An hour post infection, 1.4 mL of EMEM media supplemented with 5 % FBS was added and incubated for 48 hours at 37 °C. The cells were examined for complete cytopathic effect (CPE) and then scraped off in the spent media with a cell scraper. Sucrose was then added to 4 % and the cells were transferred to 2 mL of sterile tubes. The cells were subjected to three freeze/thaw cycles in liquid nitrogen and 37 °C water bath. The cell lysates were stored at -80 °C. This coinfections and harvesting was repeated until maximum HDAd titres were obtained in 60 mm plates. The lowest passage with highest titres was then used to co-infect 70 % confluent 116 cells in a 150 mm dish with helper virus at a MOI of 2. The co-infected cells were harvested after 48 hours and centrifuged at 5000 g for 5 minutes. Supernatant was discarded and the pellet was resuspended in 1mL of 100 mM Tris-HCl (pH 8) and used for large scale production. A control HDAd vector expressing the *lac Z* gene (HDAdlacZCMV) was amplified in parallel for easy quantification of the number of infectious units.

2.7.3 X-gal staining

To assess successful production and monitor HDAd amplification, various dilutions (10^0 -10^{-5}) of the HDAd*lacZ*CMV were made in EMEM media supplemented with 5 % FBS up to a volume of 50 µl. The dilutions were added to 70 % confluent 116 cells in a 24-well plate and incubated for at 37 °C for an hour, rocking every 10 minutes. An hour post infection, 450 µl of EMEM media supplemented with 5 % FBS was added and incubated for 48 hours at 37 °C. After the incubation period, the media was removed from the plate. The plate was allowed to air-dry at room

temperature for 5 minutes, 300 µl of the fixative (1 % formaldehyde and 0.5 % glutaraldehyde) was added to the cells and incubated for 10 minutes at room temperature. The cells were washed twice with 300 µl of 1 × PBS. The staining solution (4 mM K^+ Ferricyanide, 4 mM K^+ Ferrocyanide, 2 mM $MgCl_2$ and 40 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in DMSO was added to the cells and incubated for 2-16 hours at 37 °C. The cells were observed under a microscope and blue stained cells were counted to determine ifus/mL.

2.7.4 Large scale preparation of HDAds

Suspension 116 cells were prepared by transferring 20 mL of spent EMEM media supplemented with 10 % FBS from eight 100 % confluent 175 cm^2 flasks of 116 cells into 3 L spinner flasks. A volume of 5 mL of saline was added to 116 cells in 175cm^2 flask, incubated for 3 minutes at 37 °C. The cells were detached by tapping the flask on the side and transferred to 3 L spinner flasks containing the spent media. The media was topped up to 1 L with fresh JEMEM media (Section 2.5.1) and incubated at 37 °C on a magnetic stirrer at 60 rpm. In two days, 0.5 L of fresh JEMEM media (Section 2.5.1) was added and continued incubating overnight with stirring. A volume of 1 L of fresh JEMEM media (Section 2.5.1) was added and incubated overnight with stirring. To monitor cell growth and health, every time before adding fresh media, 116 cells were counted by transferring 2 mL of cells into a 15 mL Falcon tube and 2 mL of 2 × citric saline was added. The cells were vortexed for 10 seconds, incubated for 10 minutes at 37 °C then vortexed again for 10 seconds. Equal amounts of cells and trypan blue stain were mixed and hemocytometer was used to obtain cell counts. About 100 µl of the cell suspension from the 3 L was added to 400 µl of JEMEM media (Section 2.5.1) in a 24-well plate and incubated at 37 °C.

For co-infections, the cells were harvested from the 3 L culture by centrifuging at 750 g for 5 minutes at room temperature and 0.5 L of spent media was reserved. The cell pellet was resuspended in 100 mL spent media and transferred to a 250 mL spinner flask. The cells were coinfected by adding HV at MOI 2 and 1 mL of the HDAd lysate from the 150 mm plate amplification (Section 2.7.2) and incubated for 2 hours at 37 °C spinning at 60 rpm. The coinfected cells were transferred to a 3 L spinner flask, 0.5 L spent media was added and topped up to 2 L with JEMEM media (Section 2.5.1). The spinner flasks were incubated spinning at 60 rpm for 48

hours at 37 °C. The cells were then harvested by centrifugation at 750 g for 5 minutes at room temperature. The cells were resuspended in 15 mL of 100 mM Tris-HCl (pH 8) supplemented with glycerol to 10 %, transferred to 50 mL Falcon tube and stored at – 80 °C.

2.7.5 HDAd purification

For lysis, 2 mL of 5 % sodium deoxycholate was added to the large scale coinfected cells and incubated for 30 minutes at room temperature with frequent mixing. $MgCl_2$, RNAse A and DNase I were added to 2 mM, 10 µg/mL and 10 µg/mL respectively. The solution was incubated for 1 hour at 37 °C with constant mixing, followed by centrifugation at maximum speed for 10 minutes. The HDAd containing supernatant was removed and the pellet was discarded. Caesium chloride (CsCl) step gradients were prepared in SW 55 ultra-clear ultra-centrifuge tubes (two per virus) by adding 2 mL of 1.35 g/mL CsCl to each tube and overlaying with 3 mL of 1.25 g/mL CsCl. The supernatant was overlaid on top of the two CsCl steps and centrifuged in an ultracentrifuge with an SW 40 rotor at 91 349 g for 1 hour. An 18-guage needle and a 3-cc syringe were used to retrieve the vector from the tubes. The vector was transferred into one SW 55 ultra-clear tube and topped up with 1.35 g/mL CsCl. This was centrifuged overnight at 91 349 g. A 1-cc syringe and 22-guage needle was used to retrieve the virus band and it was transferred into a Slide-A-Lyzer dialysis cassette pre-soaked with dialysis buffer (10 mM Tris-HCl, pH 8) at 4 °C. This was dialyzed at 4 °C with slow stirring and three 500 mL changes of dialysis buffer to remove CsCl. A 22-guage needle with a 1-cc syringe were used to retrieve the virus from the cassette and glycerol was added to 10 %. Aliquots of 50 µl were made and stored at -80 °C.

2.7.6 HDAd quantification and HV contamination determination by qPCR

The quantify CsCl purified HDAd and determine HV contamination, DNA prepared using the QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit (Qiagen, CA, USA) following the blood and body fluid spin protocol according to the manufacturer's instructions was used for qPCR. Briefly, 20 µl of proteinase K was added to 1.5 mL microcentrifuge tube, 50 µl of purified

HDAd was diluted in 150 µl PBS and added to the microcentrifuge tube with proteinase K. A volume of 200 µl of lysis buffer AL was added to the sample and mixed by pulse-vortexing for 15 seconds and incubated at 56 °C for 10 minutes. Absolute ethanol (200 µl) ethanol was added to the sample and mixed by pulse-vortexing for 15 seconds. The mixture was added to QIAamp Spin Column in a 2 mL collection tube and centrifuged at 6000 g for 1 minute. The QIAamp Spin Column was placed in a clean 2 mL collection tube, 500 µl buffer AW1 was added and centrifuged at 6000 g for 1 minute. The QIAamp Spin Column was placed in a clean 2 mL collection tube, 500 µl buffer AW2 was added and centrifuged at 20 000 g for 3 minutes. The QIAamp Spin Column was placed in a new 2 mL collection tube and centrifuged at 6000 g for 1 minute. The QIAamp Spin Column was placed in a clean 1.5 mL microcentrifuge tube, 50 µl dH_2O was added and incubated at room temperature for 1 minute, and then centrifuged at 6000 g for 1 minute. To obtain the Ad titers, 2 µl of the prepared DNA was used for qPCR (Section 2.3.4) using the HV and HDAd primers (Table 2.2) and % HV contamination was calculated.

2.8 Northern blot hybridization

2.8.1 RNA extraction

To determine pri-miRNA expression by recombinant HDAds *in vitro,* Huh 7 cells were seeded and grown to 90 % prior to infection with recombinant HDAds at MOI of 1000. Forty-eight hours post infection, the media was discarded and 1 mL of Trizol (Thermo Fisher Scientific, MA, USA) was added to the cells. The cells were lysed by pipetting up and down and transferred to 2 mL sterile tubes. The lysates were incubated for 5 minutes at room temperature, 200 µl of chloroform was added per mL of Trizol (Thermo Fisher Scientific, MA, USA). The solution was mixed by shaking vigorously for 15 seconds and it was incubated for 3 minutes at room temperature. The mixture was centrifuged at 20 000 g for 15 minutes at 4 °C. The top aqueous phase was transferred to a clean sterile tube and 500 µl of isopropanol was added and mixed. This was incubated at room temperature for 1 minute then centrifuged at 20 000 g for 10 minutes at 4 °C. The supernatant was discarded and the pellet was washed with 1 mL of 75 % ethanol. The pellet was centrifuged at 20 000 g for 5 minutes at 4 °C. The pellet was air dried for 5-10 minutes and resuspended in 50 µl of RNAse free water. The concentration was measured on the Nano-drop spectrophotometer, aliquots of 3 µg were made and stored at -80 °C.

2.8.2 Radioactive labelling of decade RNA marker (ladder)

In a nuclease free tube, 100 ng of the decade RNA marker (1 µl), 6 µl nuclease free water, 1 µl of 10 × kinase reaction buffer, 1 µl of [γ-^{32}P] ATP and 1 µl of T4 polynucleotide kinase were mixed. The solution was incubated for an hour at 37 °C, 8 µl of nuclease free water and 2 µl of cleavage reagent were added and incubated for 5 minutes at room temperature. RNA gel loading buffer II (20 µl, Thermo Fisher Scientific, MA, USA) was added and aliquots of 5 µl were made and stored at – 80 °C.

2.8.3 Radioactive labelling and purification of probes

A volume of 2 µl of 10 µM probe was mixed in a tube with 2 µl of polynucleotide kinase (PNK) buffer A, 1 µl PNK and 1 µl [γ-^{32}P] ATP. The volume was made up to 20 µl with water and the solution was incubated for 45 minutes at 37 °C. The sephadex column was prepared by inserting 1 cm of fish tank wool at the bottom of a 1 mL syringe. Sephadex (0.9 mL, Appendix 7.4.2) was added to the syringe and centrifuged at 5000 g for 2 minute in a 15 mL Falcon tube. The labelled probe was topped up with 30 µl of water and added to the column. The column was centrifuged at 5000 g for 2 minutes and the probe collected in a 15 mL Falcon tube.

2.8.4 Polyacrylamide gel electrophoresis

A 15 % polyacrylamide gel was set the day before running the gel. This was prepared by adding 0.45 g of N-N-methylenebisacrylamide, 8.55 g of acrylamide and 28.8 g of urea to 6 mL of 10 × TBE buffer (pH8, Appendix 6.4.3). About 20 mL of Sabax water was added and the solution was stirred in warm water until it completely dissolved. It was topped up to 60 mL with highly pure (Sabax) water, 300 µl of 1 % ammonium persulfate and 30 µL of N,N,N,N-Tetramethylethyl enediaminne (TEMED) were added. The gel was poured with 1 mL pipette tip and allowed to set. Wet paper towel was used to cover the top of the gel apparatus and it was covered with cling film and left overnight. The next day, the gel was transferred to a running tank and filled with 1 × TBE

(Appendix 7.4.3) buffer and the gel was pre-run for 30 minutes at 150 V before loading. An equal volume of RNA loading dye was added to 30 µg of RNA, these were denatured at 80 °C, whereas the ladder was denatured at 95 °C for 5 minutes. A syringe with a needle on was used to flush each well with running buffer before loading. The samples were loaded with the Hemilton syringe at the bottom of each well and the gel was run at 200 V. The gel was stained with 50 µl of ethidium bromide in 500 mL running buffer for 5 minutes. The gel was put on a plastic and viewed on the gel doc to confirm the quality of RNA.

2.8.5 Blotting

The wells on the gel were trimmed with a scalpel. Six pieces of the thick filter paper and a nylon membrane of the same size as the gel were cut. These were soaked in running buffer and a sandwich was made in a semi-dry blotter by putting three filter papers, the nylon membrane, the gel and three filter papers. Excess liquid was pressed out by rolling a 10 mL plastic pipette. The RNA was transferred to the nylon membrane by running at 0.77 A for 45 minute using a Semi-Dry Electroblotting Unit (Sigma-Aldrich, MO, USA).

2.8.6 UV crosslinking and hybridization

The nylon membrane was removed from the sandwich, put on a filter paper and cross linked for 2 minute using an Ultra-Violet cross linker (UVP Inc., CA, USA). Hybridization buffer (10 mL) was heated to 42 °C in a hybridizing bottle. The membrane was placed in the bottle and pre-hybridized for 20 minutes. The labelled probe was denatured at 95 °C for 5 minutes, added to the bottle and hybridized overnight rotating at 42 °C.

2.8.7 Stringency washes and exposure

The hybridization buffer was removed; the membrane was washed with 50 mL of 5 × SSC and 0.1 % SDS at room temperature for 20 minutes. The membrane was then washed twice with 50 mL of

1 × SSC and 0.1 % SDS at 42 °C for 15 minutes. The membrane was placed in a plastic and put together with imaging plate in a cassette and stored at room temperature in the drawer for one week exposure. The imaging was performed using the FUJIFILM FLA-7000 phosphor-imager (Vacutec, Gauteng, SA).

2.9 Assessment of HBV replication inhibition in cultured cells by Enzyme Linked Immunosorbent Assay (ELISA)

Huh7 cells were transfected with pCH-9/3091 as well as with eGFP plasmids using Lipofectamine® 3000 (section 2.5.4.2). Five hours after transfection, HDAds at various MOIs (500, 1000, 2000 and 4000) in 50 µl of 5 % media was added to the cells. To allow attachment, cells were incubated at 37 ^{0}C for 1 hour rocking the plate every 10 minutes before 450 µl of 5 % FBS DMEM was added. The cells were then incubated at 37 °C. Forty eight hours post infection, knockdown of HBV replication was assessed by measuring HBV surface antigen (HBsAg) which is a marker of HBV gene expression secreted into culture supernatant by ELISA using MONALISA^{TM} HBsAg Ultra Kit (Bio-Rad, CA, USA) following the manufacturer's instructions. Briefly, 100 µl of unknown samples, positive and negative controls were added to the wells of the microplate. A volume of 50 µl of the conjugate solution was added to each well. The plate was covered with adhesive film and incubated for 1 hour 30 minutes at 37 °C. The plate was washed using the automatic microplate washer system and the strips were dried. The development solution was prepared and 100 µl was added to each well. The plate was incubated in the dark for 30 minutes, 100 µl of the stopping solution was added and incubated for 4 minutes at room temperature. The plate was placed inside the microplate reader (Bio-Rad Laboratories, CA, USA) and the optical density was read at 490nm.

2.10 *In vivo* methods

In this study, HBV transgenic mice which express HBV particles were used according to the protocols approved by the University of the Witwatersrand Animal Ethics Committee. All the sampling, handling and injections of animals were done in the Wits central animal service (CAS),

therefore no transportation of animals from CAS was required. Mice which were used were 4-8 weeks old and they were bled by retro-orbital puncture 3 days before injection and used as baseline. The mice (4 - 6 mice/group) were injected with 1×10^{10} HDAd particles via the tail vein; blood was collected via retro-orbital puncture following injections. Weights of the mice were monitored throughout the experiments. The serum samples were used to assess HBV replication inhibition, immune stimulation and toxicity of the HDAds by measuring levels of HBsAg, inflammatory cytokines and alanine liver enzyme levels respectively. For liver transduction and pri-miRNA expression assessment, following injections the mice were sacrificed by carbon dioxide exposure, livers harvested and mice disposed as recommended by CAS staff.

2.10.1 Assessment of liver transduction and transgene expression by recombinant HDAds *in vivo*

As mentioned previously, HDAds expressing pri-miRNA expression cassettes from a liver-specific promoter were used in this study. To assess if these HDAd were able to efficiently deliver these pri-miRNA expression cassettes to the liver, DNA was extracted from the livers of mice injected with 1×10^{10} HDAd particles after one week. DNA was prepared from the livers using QIAamp DNA Mini Kit (Qiagen, CA, USA) following the tissue protocol according to the manufacturer's instructions. Briefly, Proteinase K (20 µl) and 200 µl of buffer ATL were added in to a 2 mL micro-centrifuge tube containing 200 µl of the liver homogenate. This was mixed by vortexing, and incubated at 56 °C for 3 hours. A volume of 200 µl of the lysate was then used to isolate viral DNA according the blood and body fluid spin protocol as outlined in section 2.7.6. The concentration of the DNA was measured on the Nano-drop spectrophotometer and 100 ng was used for qPCR (Section 2.3.3) using HDAd primers (**Table 2.2**).

For pri-miRNA expression and processing *in vivo*, livers were harvested in TRIzol (Thermo Fisher Scientific, MA, USA) from mice at one week post infection with 1×10^{10} HDAd particles. Livers were homogenised, RNA isolated and 30 µg used for Northern blot analysis as described in section 2.8.

2.10.2 Determination of HBV replication inhibition in mice

HBsAg was measured by ELISA in serum samples collected before HDAd injection, at one and two weeks post infection. Serum samples diluted 50 × in 100 µl of saline were used for ELISA using the Monolisa™ HBsAg Ultra Kit (Bio-Rad Laboratories, CA, USA) as per manufacturer's instructions and summerised in section 2.9.

2.10.3 Assessment of inflammatory response and toxicity of recombinant HDAds

For inflammatory response assessments, blood samples were collected and serum harvested at 0, 6 and 24 hours post injection of mice with HDAd. Cytometric bead array (CBA) was performed by using the mouse inflammation kit BD™ (The Scientific Group, South Africa). Briefly, Standards were reconstituted in 2 mL of assay diluent, incubated for 15 minutes at room temperature and mixed by pipetting. Serial dilutions were then performed in the assay diluent. Cytokine Capture beads were mixed by adding 10 µl of each capture bead for each assay tube to be analysed. The standards or serum samples were transferred to tubes containing the capture beads and 50 µl of PE detection reagent was added. Assay tubes were incubated at room temperature in the dark for 2 hours, 1 mL of wash buffer was added and centrifuged at 5000 g for 5 minutes. Each bead pellet was resuspended with 300 µl of wash buffer. Samples were analysed in the LSRFortessa™ flow cytometry instrument and FCAP Array™ software (BD Bioscience, CA, USA) was used to quantify serum levels of inflammatory markers: IL-6, IL-10, MCP-1, IFN-γ, TNF and IL-12p70. For hepatotoxicity determination of the recombinant HDAds, serum samples were collected at 72 hours, one week and two weeks post injection with HDAds and sent to National Health Laboratory Service chemistry laboratory for alanine aminotransferase (ALT) activity assay on an automated photomeric analyser (Roche Diagnostics, Rotkreuz, Switzerland).

2.11 Data analysis

For statistical analysis, data were expressed as the mean ± standard error of the mean (SEM). Statistical difference was determined by using Student's 2-tailed t-test and was considered

significant when $P \leq 0.05$. Calculations were done with GraphPad Prism software (GraphPad Software Inc., CA, USA).

CHAPTER 3

3. RESULTS

3.1 Successful cloning of anti-HBV pri-miRNA expression cassettes into pΔ28E4 backbone

Previously, monomeric and trimeric anti-HBV pri-miRNAs targeting HBx region of HBV were designed (Ely et al., 2009). These were delivered using HDAd co-expressing β-galactosidase as a reporter for easy viral quantification and assessment of transduction efficiency. However, the expression of the reporter gene has been shown to induce an immune response, resulting in quicker vector clearance and decreased HDAd efficacy (Crowther et al., 2014, Mowa et al., 2014). To overcome this, HDAd plasmids devoid of the *lac Z* gene and expressing pri-miRNA sequences from the liver-specific mouse transthyretin receptor (MTTR) promoter were constructed in this study (**Figure 3.1**).

Figure 3.1 Generation of HDAd plasmid constructs with the desired anti-HBV sequences. A) Part of HDAd plasmid with the anti-HBV sequence expressed from a MTTR promoter and a *lac*

Z reporter gene**. B)** Part of HDAd plasmid deficient of *lac Z* and containing pri-miRNA sequence expressed from MTTR promoter, the dotted line represents the deleted region.

To construct HDAd plasmids (pHDAds) containing pri-miRNA sequences under the control of MTTR promoter, anti-HBV pri-miRNA cassettes were cut out from the previously constructed pTZ plasmids [pTZMTTR-pri-mi-R-31/5 and pTZMTTR-pri-mi-R-31/5-8-9, (Mowa et al., 2014)]. These were then ligated in the AscI site of *lac Z* deficient pΔ28E4 plasmid (Donated by Phillip Ng, Baylor College of Education, USA) to form pHDAd-pri-miRNA-31/5 and pHDAd-pri-miRNA-31/589. AscI was used to screen for positive clones (Data not shown). Successful cloning of the anti-HBV expression cassettes into pΔ28E4 was confirmed by restriction enzyme digestion of one positive clone with AscI, NdeI and PmeI restriction enzymes (RE) and **Table 3.1** shows the expected sizes as determined using vector NTI (Thermo Fisher Scientific, MA, USA). **Figure 3.2 A** shows the digestion of constructed pHDAd expressing pri-miRNA-31/5 (pHDAd-pri-miRNA-31/5).

As expected, pHDAd-pri-miRNA-31/5 digested with AscI, NdeI and PmeI resulted in 31 033 bp & 1794 bp or 21 524 bp, 5912 bp & 5391 bp or 29 878 bp & 2949 bp bands respectively. Digestion with NdeI of **Figure 3.2 A** only has 2 bands instead of 3, which is likely to be a result of co-migration of the DNA fragments of 5912 bp and 5391 bp. **Figure 3.2 B** shows the constructed pHDAd expressing pri-miRNA-31/589 (pHDAd-pri-miRNA-31/589). Digestion of pHDAd-pri-miRNA-31/589 with AscI, NdeI and PmeI gave rise to 31 043 bp & 2117 bp or 21 524 bp, 6245 bp & 5391 bp or 30 211 bp & 2949 bp bands respectively. Again, digestion with NdeI of **Figure 3.2 B** only has 2 bands instead of 3, this may be due to the fact the last two bands, 6254 bp and 5391 bp are close to each other and may not have separated from each other. PmeI digestion separated the bacteria from the adenoviral genome, this was important as the PmeI linearized plasmids were later used for HDAd production. As compared to the uncut controls that resulted in one slow migrating band and considering the expected bands for the empty plasmid shown on **Table 3.1**, the fragment sizes obtained following RE digest of the constructs with the various enzymes match the expected sizes obtained from vector NTI software (**Table 3.1**). As a result of their greater length (~ 24 - 35 kb), adenoviral genome-carrying plasmids are very difficult to manipulate for cloning purposes. However, these data show that pri-miRNA expression cassettes were successfully cloned into the pΔ28E4 plasmid, which is 32 827 bp in size.

Table 3.1: Expected fragment sizes for the constructs following restriction enzyme digestion

pHDAd construct	Restriction enzyme	Fragment size (bp)
1. pHDA-pri-miRNA-31/5	AscI	31 033 bp & 1794 bp
	NdeI	21 524 bp, 5912 bp & 5391 bp
	PmeI	29 878 bp & 2949 bp
2. pHDAd-pri-miRNA-31/589	AscI	31 043 bp & 2117 bp
	NdeI	21 524 bp, 6245 bp & 5391 bp
	PmeI	30 211 bp & 2949 bp
3. pΔ28E4	AscI	31 027 bp
	NdeI	21 524bp, 5391 bp & 4112 bp
	PmeI	28 078 bp & 2949 bp

Red indicates the bands that migrate together on the gel

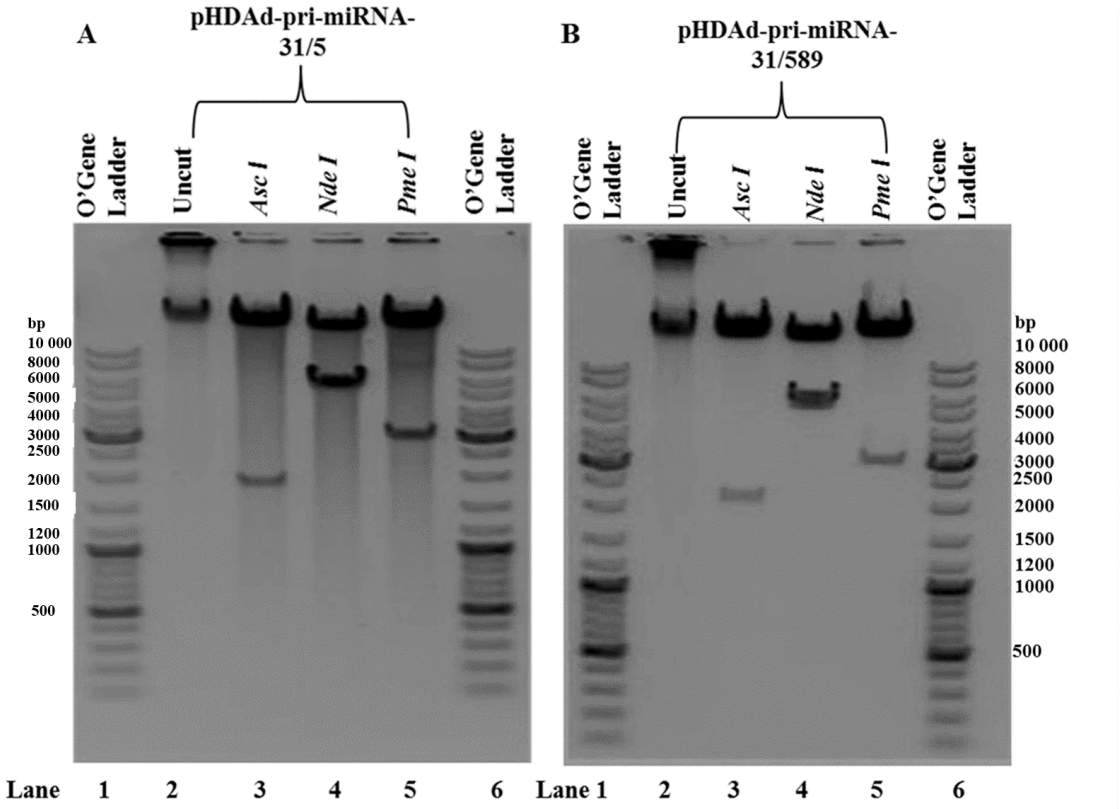

Figure 3.2 Construction of HDAds expressing anti-HBV pri-miRNA expression cassettes from MTTR promoter. Anti-HBV pri-miRNA31/5 (**A**) and pri-miRNA31/5-8-9 (**B**) sequences expressed from MTTR promoter were sub cloned into the HDAd (pΔ28E4) backbone at the AscI restriction enzyme (RE) site. Positive clones were verified by AscI, NdeI and PmeI RE digestion. **A**) Shows the constructed pHDAd expressing pri-miRNA-31/5 (pΔMTTR-pri-miRNA-31/5). Lane 1 and 6 show the O'gene ladder that was used with sizes from 100 bp to 10 000 bp, lane 2 shows uncut pΔMTTR-pri-miRNA-31/5, lane 3 shows pΔMTTR-pri-miRNA-31/5 digested with AscI RE, lane 4 shows pΔMTTR-pri-miRNA-31/5 digested with NdeI RE and lane 5 shows pΔMTTR-pri-miRNA-31/5 digested with PmeI RE. **B**) Shows the constructed pHDAd expressing pri-miRNA-31/589 (pΔMTTR-pri-miRNA-31/589). Lane 1 and 6 show the O'gene ladder that was used with sizes from 100 bp to 10 000 bp, lane 2 shows pΔMTTR-pri-miRNA-31/589 uncut, lane 3 shows pΔMTTR-pri-miRNA-31/589 digested with AscI RE, lane 4 shows pΔMTTR-pri-miRNA-31/589 digested with NdeI RE and lane 5 shows pΔMTTR-pri-miRNA-31/589 digested with PmeI RE.

3.2 HDAd production

Since all the viral coding sequences are removed, HDAds require a helper virus (HV) for their propagation (Palmer and Ng, 2005). Despite the fact that HDAds are devoid of all their viral coding sequences, immune stimulation by the capsid proteins still remains a daunting obstacle that makes these vectors unsafe in clinical application. Several studies have shown that PEGylation of Ads reduce immune stimulation (Kreppel and Kochanek, 2008). Unlike using a non-residue specific amine reactive PEG, introduction of cysteine residues on the adenoviral capsid enable residue specific PEGylation using thiol reactive PEG, and this has been shown to improve transduction efficiency and diminish Ad specific immune stimulation (Prill et al., 2011). This is achieved by using a HV expressing capsid protein with the cysteine modification. Hence in this study, a HV with (AdNG163cys) or without (AdNG163) cysteine modification was used.

3.2.1 Amplification of AdNG163cys and AdNG163 helper-viruses

To obtain enough HV for production of HDAds, previously produced AdNG163cys and AdNG163 HVs were propagated in Hek293T cells. To determine successful amplification, the infectious units (ifus) of the HV were quantified by immunocytochemical staining of infected Hek293T cells. Interestingly, in comparison with AdNG163 (**Figure 3.3 B)**, imaging of stained cells consistently showed brown cells that were clumped together when infected with AdNG163cys **(Figure 3.3 C)** making it difficult to precisely count infected cells (**Figure 3.3**). Enumeration of ifus for AdNG163 showed that 3.85×10^{8} ifus/mL were obtained in a total volume of 20 mL, which was enough for HDAd production. As a result of clumping of AdNG163cys infected cells, qPCR was used to determine total viral particles, which showed that 3.69×10^{9} vps/mL was obtained in a total volume of 20 mL. These were comparable with 4.78×10^{9} vps/mL obtained with AdNG163.

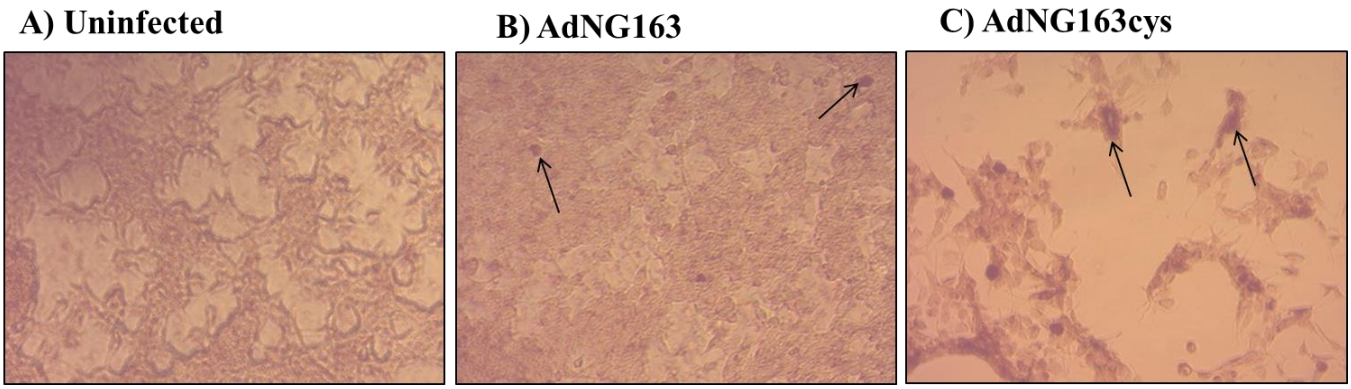

Figure 3.3: Amplification of AdNG163cys and AdNG163 HVs. AdNG163cys and AdNG163 HVs were produced and amplified in Hek293T cells. Immunostaining was done to quantify HV infectious units. The arrows in the images show the brown cells which were observed following staining.

3.2.2 Cysteine modification on the AdNG163cys helper-virus diminish its role in HDAd propagation

Because of the difficulty to determine AdNG163cys ifus, total vps were used to determine the HV quantities required for different MOIs tested for production of cysteine modified HDAd (HDAdcys) or unmodified HDAd. The MOIs that were tested for HDAd production were MOI 5,

10, 20, 50, and 100. HDAd carrying *lac Z* (pΔ28E4CMV*lacZ*) gene was produced in parallel for convenient quantification of ifus produced by X-gal staining. For HDAd production, 116 cells transfected with the pHDAds were infected with AdNG163cys or AdNG163 at various MOIs. Cells were then harvested at 48 hours post infection and lysed. X-gal staining of infected cells showed that AdNG163 resulted in efficient HDAd production at an MOI of 5 with 1.11×10^5 ifu/mL obtained in a total volume of 2.5 mL. In contrast, HDAdcys could only be produced at AdNG163cys MOI of 20. Interestingly, HDAdcys showed similar characteristics with AdNG163cys after X-gal staining of infected cells. Clumped blue cells were observed and again, making it difficult to determine the produced ifus (**Figure 3.4**). The observed clumps of blue cells further suggest that cys modification result in viral aggregation before or during infection. An attempt to amplify HDAdcys at different MOIs of AdNG163cys was unsuccessful (Data not shown).

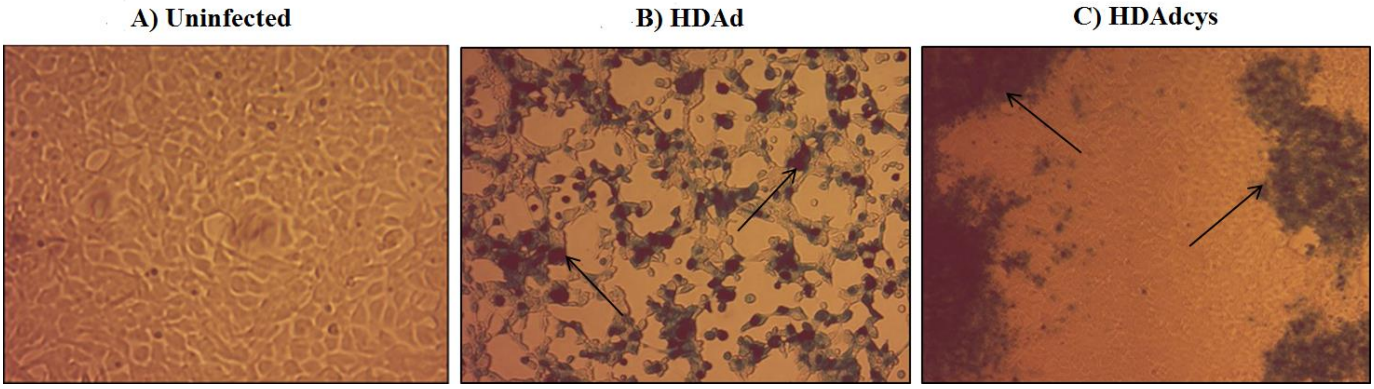

Figure 3.4: X-gal staining of HDAds produced with AdNG163cys or AdNG163 HVs. AdNG163cys and AdNG163 were used for the production of HDAds, **A** shows uninfected cells, **B** shows cells infected with AdNG163 whereas **C** shows cells infected with AdNG163cys respectively. The arrows show the blue cells that were observed following staining.

3.2.3 Effect of cysteine modification on the adenoviral infection efficiency.

Signs of viral aggregation and failure to serve as an efficient HV for production and amplification of HDAds by AdNG163cys may be as a result of capsid structural changes that affect its infection efficiency. Adenoviruses induce morphological changes in the cultured cells upon infection and viral gene expression. This is mainly characterized by rounding of infected cells called cytopathic effect (CPE). This was taken advantage of in order to asses if the cysteine modification of the AdNG163Cys affected the infection efficiency as compared to AdNG163.

To achieve this, cells were infected with AdNG163 or AdNG163cys at an MOI of 2 and CPE assessed by light microscopy after 48 hours. AdNG163 resulted in about 100 % CPE as compared to AdNG163cys, which resulted in no or little CPE (**Figure 3.5**). Hence, suggesting an attenuated transduction and the resultant adenoviral gene expression in cells treated with AdNG163cys.

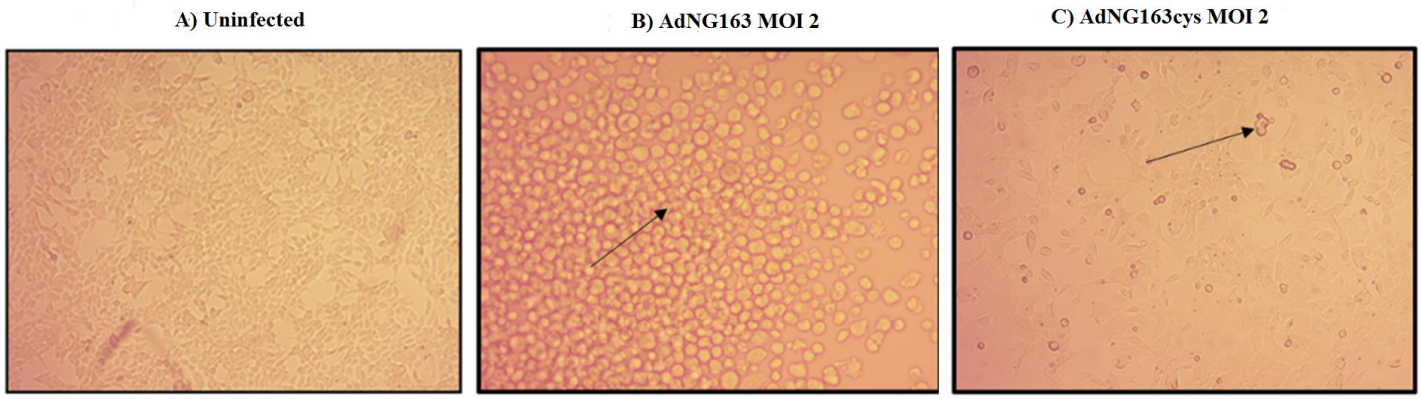

Figure 3.5: Cytophatic effect (CPE) of 116 cells infected with AdNG163 and AdNG163cys. 116 cells were infected with either AdNG163 or AdNG163cys HVs. The arrows indicate CPE which indicated by rounded up cells.

3.3 AdNG163cys has an altered particle structure

The clumping of infected cells suggested that the HV used was aggregating and this might indicate changes in capsid structure. To confirm the structural integrity of the cysteine modified HV, transmission electron microscopy (TEM) was performed. As compared to the HDAd control hexagon structure, the AdNG163cys particles showed obvious differences with no hexagon shape

observed, whereby the particles looked more roundish or collapsed (**Figure 3.6).** These results confirm that modification of the capsids affected the particle structure of the HV.

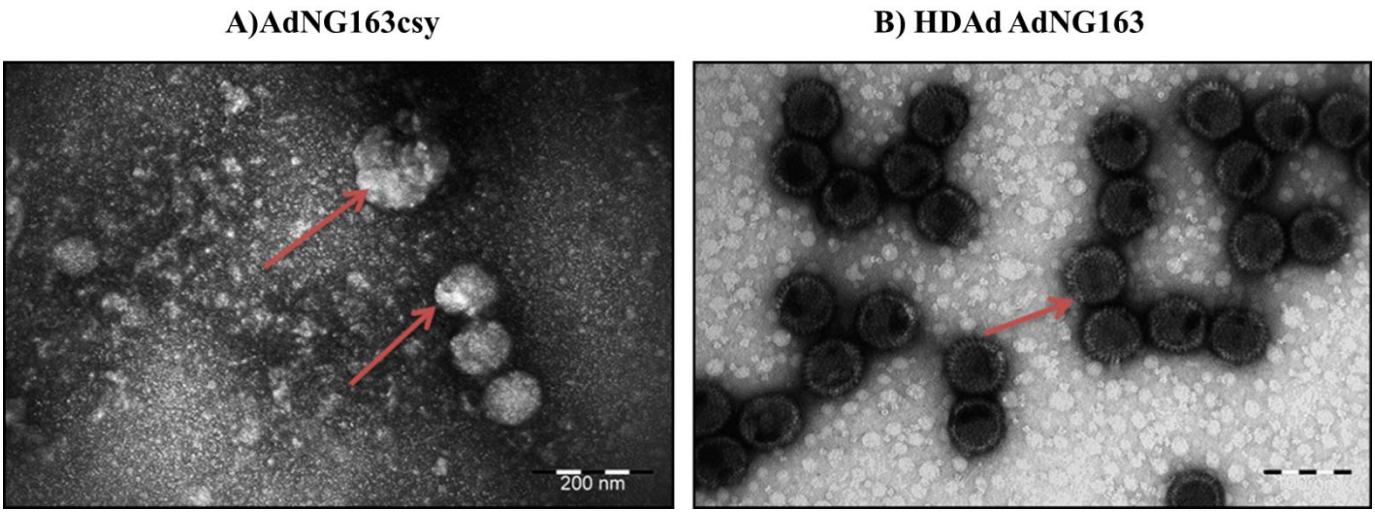

Figure 3.6: Transmission electron microscopy (TEM) of AdNG163cys and HDAd produced from AdNG163 HV. A shows AdNG163cys that was produced whereas **B** shows the HDAd produced by using AdNG163 HV. The arrows are showing the adenoviral particles.

3.4 Successful amplification of HDAds with AdNG163 helper-virus

As a result of AdNG163cys inability to serve as an efficient helper for HDAd production and amplification, *lac Z* deficient HDAds produced with AdNG163 HV at an MOI of 5 were then amplified in parallel with *lac Z* containing HDAd and characterised. Produced HDAds (P0) were amplified by passaging in 116 cells with AdNG163 at MOI 2 until optimal titre were obtained. X-gal staining of the HDAd*lacZ*CMV control (**Figure 3. 7**) showed that ifus/mL obtained were: P1 (4.42×10^5 ifu/mL), P2 (1.86×10^6 ifu/mL), P3 (4.3×10^7 ifu/mL), P4 (3.79×10^9 ifu/mL) and P5 (1.19×10^9 ifu/mL). As expected, there was an amplification with every passage until ~ 1×10^9 ifu/mL were reached. Optimal titres were reached at P5 for a 60 mm plate (**Table 3.2**). Therefore, the lowest passage with higher titre (P4) was amplified in 150 mm plates (P5b) at MOI 2. The cells were lysed and X-gal stain was performed. The ifus/mL were calculated to be 3.3×10^9 ifu/mL (**Table 3.2**) for P5b, which was enough for large scale infections.

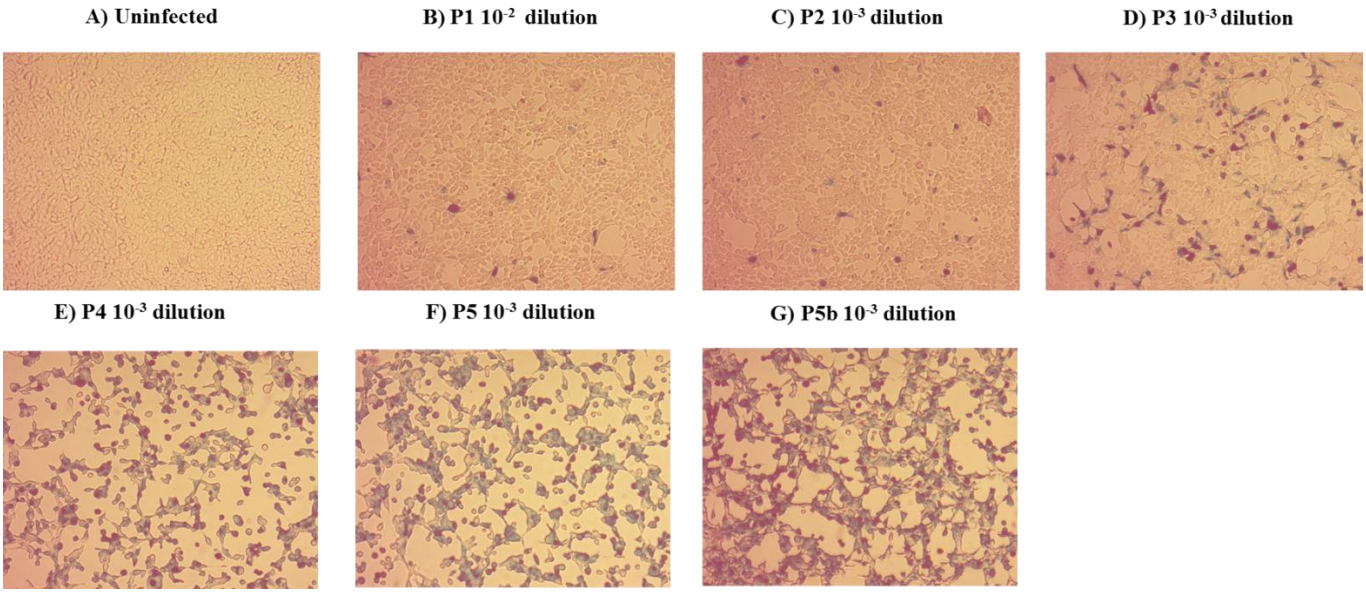

Figure 3.7: Representative images of HDAds amplification with AdNG163. Amplification (P1-P5b) of the HDAds was performed by coinfecting 116 cells with HDAd from previous passage and HV at MOI 2. **A**: uninfected, **B-F:** Passage 1 to 5 in 60 mm plate, **G**: Passage 5b amplified from P4 in a 150 mm plate.

Table 3.2: Ifus/mL for HDAd constructs at various passages

Passage number (P)	Infectious units (ifu/mL)	Fold amplification
P0	1.11×10^5	
P1	4.42×10^5	3.89
P2	1.86×10^6	4.21
P3	4.3×10^7	23.12
P4	3.79×10^9	88.14
P5	1.19×10^9	0.31
P5b	3.3×10^9	2.77

3.5 Large scale production and purification of HDAds

To allow *in vitro* and *in vivo* characterization, HDAds from P5b were amplified in large scale and purified by CsCl gradient. After centrifugation of HDAds in the discontinuous CsCl gradient, as expected, one viral band was observed at the bottom of the tube (**Figure 3.8A**). This was harvested and put through a continuous CsCl gradient centrifugation overnight and one band at the top of the tube was observed (**Figure 3.8B**). This represents a pure HDAd virions and it was harvested and dialysed overnight to remove Cscl. The total viral particles for the various HDAds and HV contamination were determined by q-PCR. HDAdΔ28E4, HDAd-pri-miRNA-31/5 and HDAd-pri-miRNA-31/589 total viral particles obtained were 2.628×10^{13} vps, 9.63×10^{12} vps and 2.328×10^{13} vps respectively (**Table 3.3)**. Because of the low amount of HV used during amplification and the fact that HV genome becomes unpackageable in Cre expressing cells, HV band could not be seen with a naked eye on the gradient, hence this can be harvested and result in HDAd contamination with HV. HDAdΔ28E4 was contaminated by 2 % of HV; HDAd-pri-miRNA-31/5 had HV contamination of 3 % whereas HDAd-pri-miRNA-31/589 had HV contamination of 1.7 %. This HV contamination was negligible and was not a concern for this study. Hence, enough HDAds for *in vitro* and *in vivo* studies were successfully produced and purified.

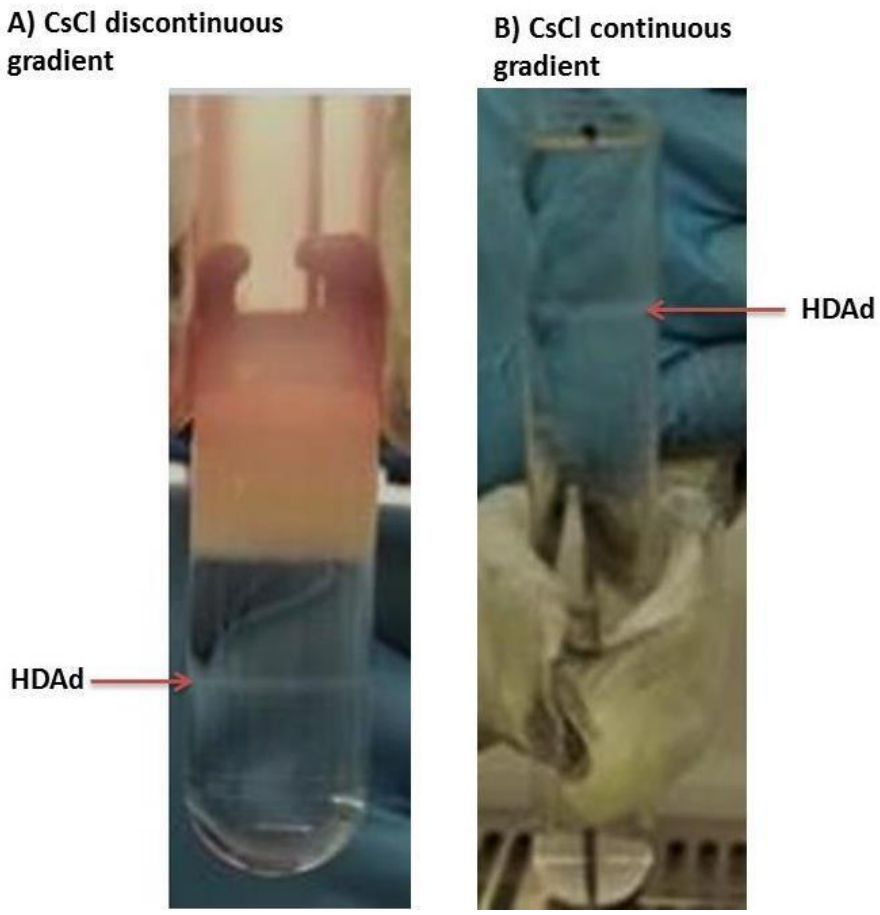

Figure 3.8: A representative CsCl gradient images from large-scale HDAd production and purification. Cells coinfected with HDAds and, AdNG163 HV at MOI 2 were harvested by centrifugation and purified by CsCl gradient centrifugation. A) Discontinuous CsCl gradient after one hour of centrifugation. B) Continuous CsCl gradient after overnight centrifugation. Arrows indicate bands representing the HDAd.

Table 3.3: Purified HDAds, their titres and HV contamination

HDAd	Titres (vps/µl)	Total vps	HV contamination (%)
HDAdΔ28E4	8.76×10^9	2.628×10^{13}	2
HDAd-pri-miRNA-31/5	3.21×10^9	9.63×10^{12}	3
HDAd-pri-miRNA-31/589	7.76×10^9	2.328×10^{13}	1.7

3.6 Anti-HBV pri-miRNA expression and processing in cultured cells

Upon introduction of artificial pri-miRNA expressing cassettes in a host cell, they are expected to enter the host RNAi pathway where they will be expressed by the RNA polymerase II, processed by Drosha and Dicer to produce pre-miRNA and mature miRNA duplexes of about 20 nucleotides (nt) respectively. In this study, *in vitro* anti-HBV pri-miRNA-31/5 and pri-miRNA-31/589 expression from HDAds was assessed by Northern blot using the probes complementary to the expected guides.

As expected, cells infected with HDAd-pri-miRNA 31/5 showed the presence of a single band of ~21 nt when the blot was probed with guide 5 probe only but not guide 8 and 9 probes. HDAD-pri-miRNA 31/589 is expected to produce three different guides. However, only guide 5 could be detected from the RNA isolated from Huh 7 cells infected with HDAD-pri-miRNA 31/589. This could be as a result of differential efficiencies at which the three guides are cleaved from the pre-miRNA, with guide 5 being produced more abundantly than 8 and 9.

As expected, RNA from uninfected cells or cells infected with control empty vector resulted in no band observed. Supporting the above observations, RNA isolated from Huh 7 infected with HDAd co-expressing β-galactosidase and pri-miRNA 31/589 from an MTTR promoter (Mowa et al., 2014) resulted in the detection of both guide 5 and guide 8 at different levels, but not guide 9. Guide 5 probe also detected a higher molecular weight RNA species, which can be from partial processing of precursor-miRNAs (**Figure 3.9**).

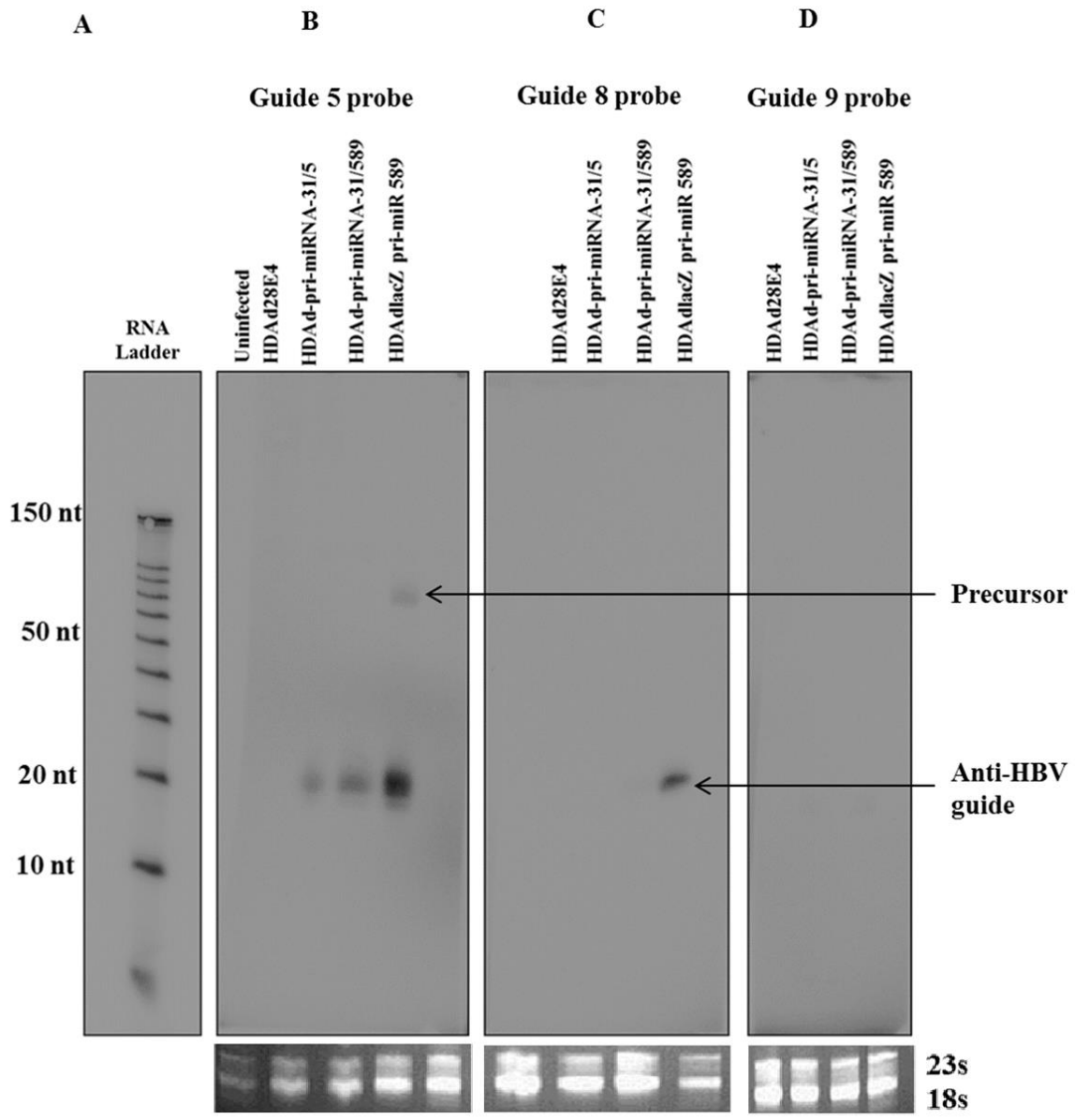

Figure 3.9: Expression and processing of anti-HBV pri-miRNA sequences from HDAds. RNA was extracted from liver-derived Huh7 cells infected with purified HDAds at MOI 1000. The RNA was used for Northern blot hybridization using ^{32}P radioactively labelled probes complementary to the expected guide strands. A decade RNA ladder was used to determine the sizes of RNA. A shows the decade RNA marker that was used with sizes ranging from 10 – 150 nucleotides (nt). B, C and D represent membranes that were probed with guide 5, 8, 9 probes respectively. 18s and 23s rRNAs from ethidium bromide stained gel were used as a loading control.

3.7 Recombinant anti-HBV HDAds significantly inhibit HBV replication in cultured cells

Once the therapeutic pri-miRNAs are expressed and processed to mature miRNA duplexes, they are expected to be recognized by host RISC complex where the guide is selected to bind to the HBV RNA and effect HBV replication inhibition.

To assess if *lac Z* deficient HDAds expressing anti-HBV pri-miRNA sequences can knock down HBV replication *in vitro*, levels of secreted HBsAg (marker of HBV replication) were determined by ELISA using culture supernatants from Huh7 transfected with HBV sequence carrying plasmid pCH9/3091 and infected with HDAds at MOI 500, 1000, 2000 and 4000.

A significant reduction in HBsAg expression was observed in cells infected with HDAd-pri-miRNA-31/5 at MOIs 500, 1000 and 2000 relative to HDAdΔ28E4. Infection of Huh 7 cells with HDAd-pri-miRNA-31/589 resulted in significant and more pronounced HBV gene silencing at MOI 1000 and 2000. Surprisingly MOI 500 did not result in HBV replication inhibition for HDAd-pri-miRNA-31/589. This HBV replication inhibition was dose dependent; however increase of MOI to 2000 or 4000 did not increase anti-HBV effects for both HDAd-pri-miRNA-31/5 and HDAd-pri-miRNA-31/589. This could be as result of high HDAd particles that may affect the health of the cells. ELISA performed after 72 hours post infection, resulted in similar results (**Figure 3.10**).

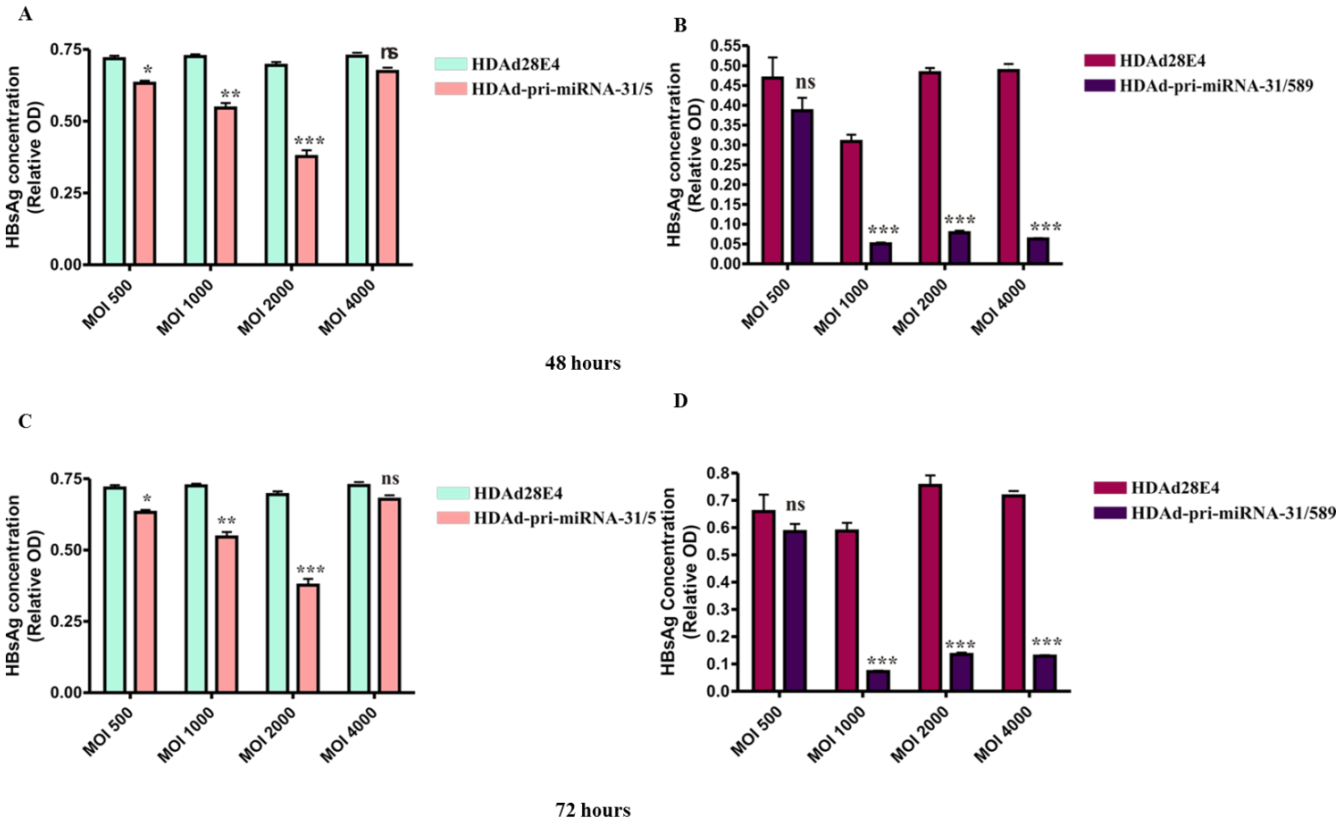

Figure 3.10: Inhibition of HBsAg expression in vitro. Liver-derived Huh7 cells were transfected with pCH-9/3091 and infected with anti-HBV HDAds at MOI 500, 1000, 2000 and 4000. ELISA was performed 48 and 72 hours post infection to measure the levels of HBsAg. A) and B) show HBV gene silencing by HDAd-pri-miRNA-31/5 and HDAd-pri-miRNA-31/589 48 hours post infection, whereas C) and D) show HBV gene silencing by HDAd-pri-miRNA-31/5 and HDAd-pri-miRNA-31/589 72 hours post infection. HDAdΔ28E4 was used as a negative control and used to normalize the data. The graphs were plotted using the normalised mean and normalised standard error of the mean (SEM). The statistical significance was calculated by using a non-paired Student's t-test. P values less than 0.05 (*), 0.01 (**) or 0.0001 (***) were considered statistically significant, whereas ns indicates non-significant.

3.8 Expression of anti-HBV pri-miRNAs and inhibition of HBV replication by HDAds in mice

Long-term *in vivo* transgene expression with reduced immune response stimulation (Suzuki et al., 2010) makes HDAds more appropriate vectors for liver-directed gene therapy. To assess HBV gene silencing *in vivo*, HDAd-pri-miRNA-31/589, which showed the most effective HBV gene silencing *in vitro* and HDAdΔ28E4 as a negative control were used to infect HBV transgenic mice. Mice were grouped into groups of 4-6 mice and injected with 1×10^{10} HDAd particles via the tail vein. Blood samples were collected by retro-orbital puncture at day 0, 1 week and 2 weeks post infection. ELISA was then performed to determine serum HBsAg levels. Surprisingly, the levels of HBsAg in mice infected with HDAd-pri-miRNA-31/589 were not statistically different to those in HDAdΔ28E4 infected mice at both one and two weeks post infection (**Figure 3.11**).

To assess if lack of HBV knockdown was as a result of lack of pri-miRNA expression or processing, mice were infected with HDAd-pri-miRNA-31/589 or HDAdΔ28E4 and RNA was extracted from the livers after 1 week post infection. Northern blotting was then performed with 30 µg RNA of hepatic RNA using ^{32}P radioactively labelled probes complementary to the expected guide 5, 8 and 9. Unlike in the RNA isolated from Huh 7 cells infected with HDAd*lacZ* pri-miR 589, there was no 21 nt guide 5, 8 or 9 detected from RNA isolated from HDAd-pri-miRNA-31/589 infected livers (**Figure 3.12**). This supports the lack of HBV silencing data by ELISA shown on **Figure 3.11**.

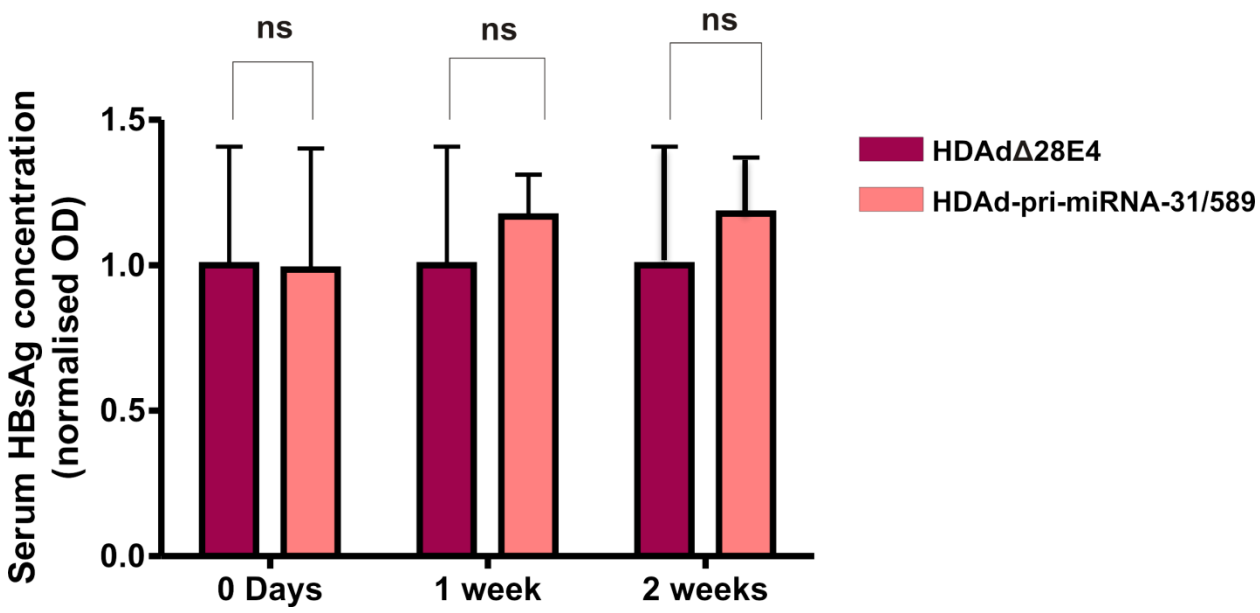

Figure 3.11: Lack of inhibition of HBsAg expression in HBV transgenic mice. HBV transgenic mice were grouped and injected via the tail vein with 1×0^{10} HDAd particles. Blood was collected and the serum was used to analyze HBV replication inhibition by HBsAg ELISA. The graphs were plotted using the normalized mean, error bars represent normalized standard error of the mean (SEM). The statistical significance was calculated by using a non-paired Student's t-test, where ns means non-significant.

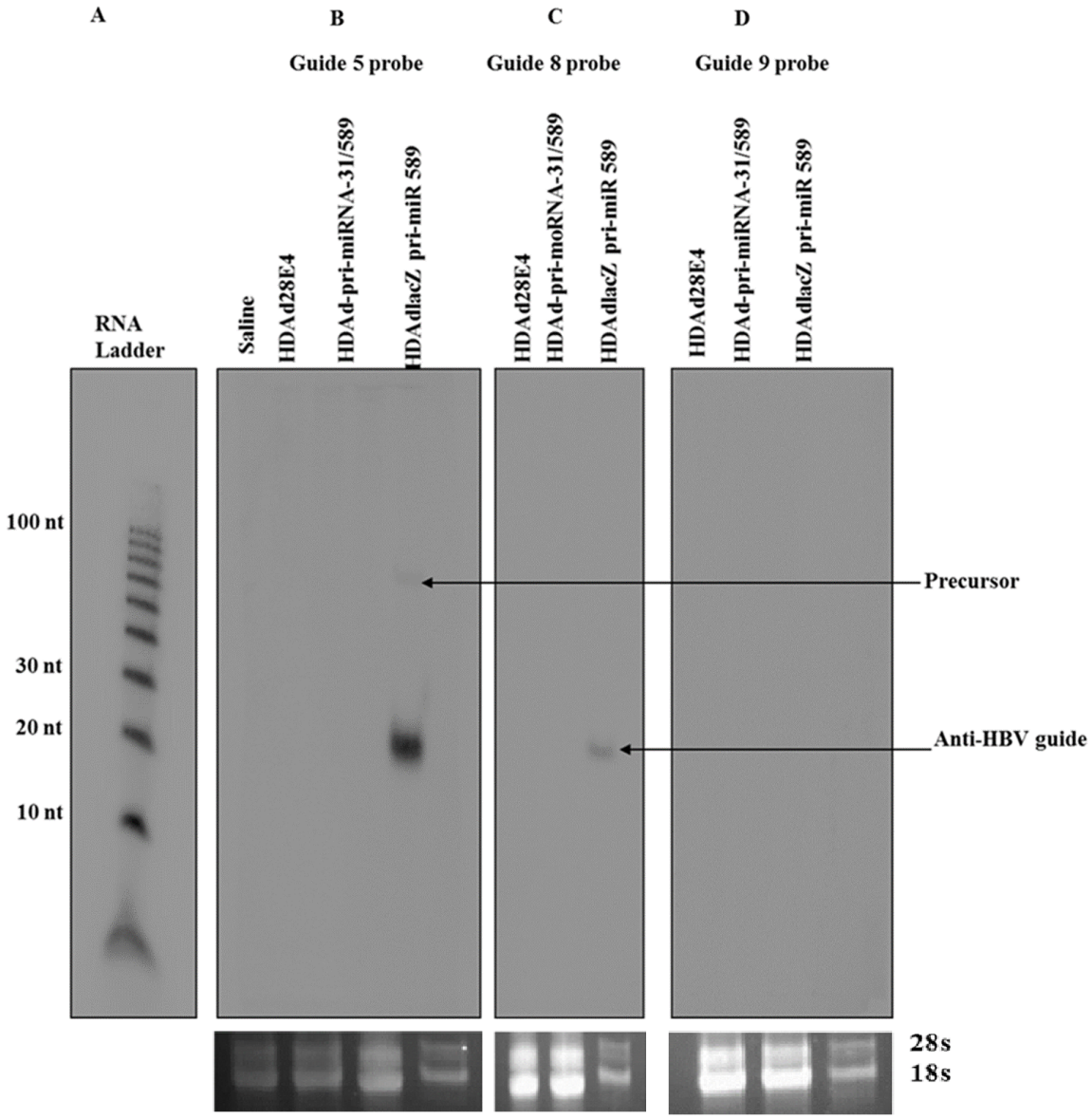

Figure 3.12: Expression and processing of anti-HBV pri-miRNA sequences from HDAds in vivo. HBV transgenic mice were grouped and injected via the tail vein with 1×10^{10} HDAd particles. RNA was extracted from mice livers for Northern blot hybridization using ^{32}P radioactively labelled probes complementary to the expected guide strands. A decade RNA ladder was used to determine the sizes of RNA. **A** shows the decade RNA marker that was used with sizes ranging from 10 – 150 nucleotides (nt). **B**, **C** and **D** represent membranes that were probed with guide 5, 8, 9 probes respectively. 18S and 23S rRNAs from ethidium bromide stained gel were used as a loading control.

There are several possibilities that could result in insufficient pri-miRNA expression and silencing of HBV replication by HDAds: 1) Because total viral particle quantification was used to determine the viral particles required to observe gene knockdown, this observation might have been as a result of insufficient infectious units used to transduce the liver. 2) HDAds might be inducing a strong inflammatory response that may result in clearance of the vector infected cells and toxicity. 3) As a result of time constrains, transgenic mice with moderate HBsAg levels (OD 0.3 to 0.6) could not be obtained when required, hence mice with higher HBsAg titres (OD 08 to 0.9) were used, and in our hands, these do not precisely demonstrate the efficacy of pri-miRNAs used.

3.9 Assessment of HDAds liver transduction, induction of an inflammatory response and liver toxicity

To determine whether HDAds transduced the liver efficiently, DNA was isolated from liver tissue harvested from mice at one week postinfection with 1×10^{10} vps HDAds and used for qPCR using HDAd specific primers. HDAd viral particles of about 4.00×10^{5} VPEs/µg DNA were detected in the livers (**Figure 3.13**). These levels are comparable to levels previously observed and they should result in a significant HBV gene silencing (Mowa et al., 2014).

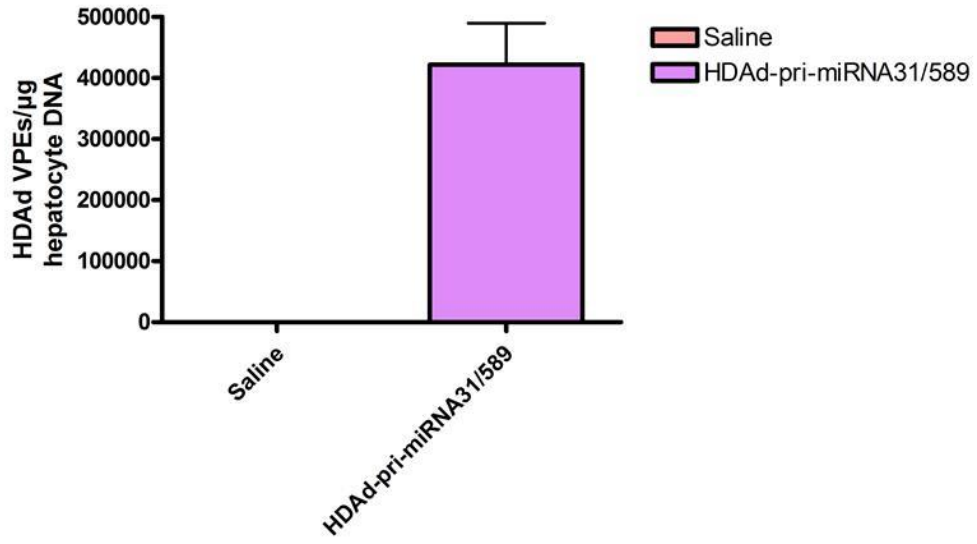

Figure 3.13: HDAd vector copies per µg of hepatocyte DNA. Mice were injected via the tail vein with saline and a single dose of 1 x 10^{10} HDAd particles. Mouse livers were extracted after 1

week and DNA was isolated and subjected to q-PCR to determine HDAd genome copies in the hepatocytes.

To determine if HDAd pri-miRNA 31/589 used induces a strong inflammation of the liver, a cytometric bead array was used to measure levels of circulating inflammatory markers IL-6, IL-10, IL-12p70, MCP-1, IFN-γ and TNF at 0 hours, 6 hours and 24 hours post injection with saline or HDAdΔ28E4 or HDAd-pri-miRNA-31/589 or Poly IC as a positive control. As compared to saline, there was no significant induction of inflammatory marker expression at 0, 6 and 24 hours post infection with either of the HDAds. As expected, poly IC significantly induced expression of TNF, MCP-1, IL-10 and IL-6 6 hours post infection but didn't induce expression of IL-12p70 and IFN-γ. Surprisingly poly IC group had elevated levels of IL-12p70, which must have been from a condition unrelated to the experiment. Even more puzzling, this cytokine was back to normal at 6 hours after injection (**Figure 3.14a**).

To determine whether HDAd pri-miRNA 31/589 induces any liver toxicity, alanine aminotransferase (ALT) levels in serum samples collected at 0 hours, 72 hours, 1 week and 2 weeks post injections was determined by ALT activity assay. Levels of ALT in all the groups remained below the acceptable cut off of 100 U/L at all the time points (**Figure 3.15**); therefore the anti-HBV HDAds used in this study did not induce any obvious liver damage.

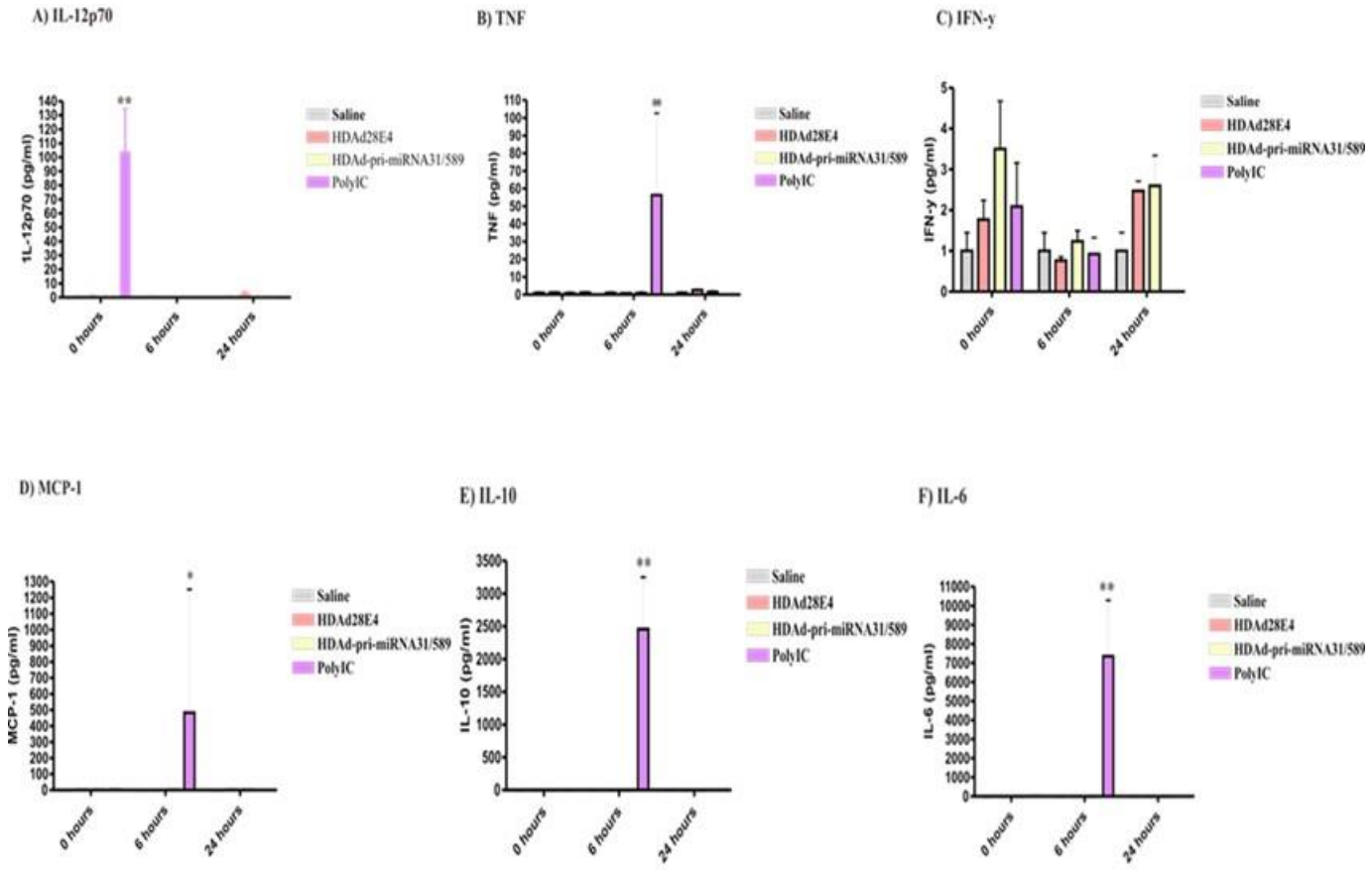

Figure 3.14a: Serum cytokine concentration of mice treated with anti-HBV HDAds. Serum concentrations of cytokines are shown for 0, 6 and 24 hours post injection. The graphs were plotted by using the mean and the standard error of the mean (SEM). The statistical significance was calculated by using a non-paired student T test. P values less than 0.05 (*), 0.01 or (**) were considered statistically significant.

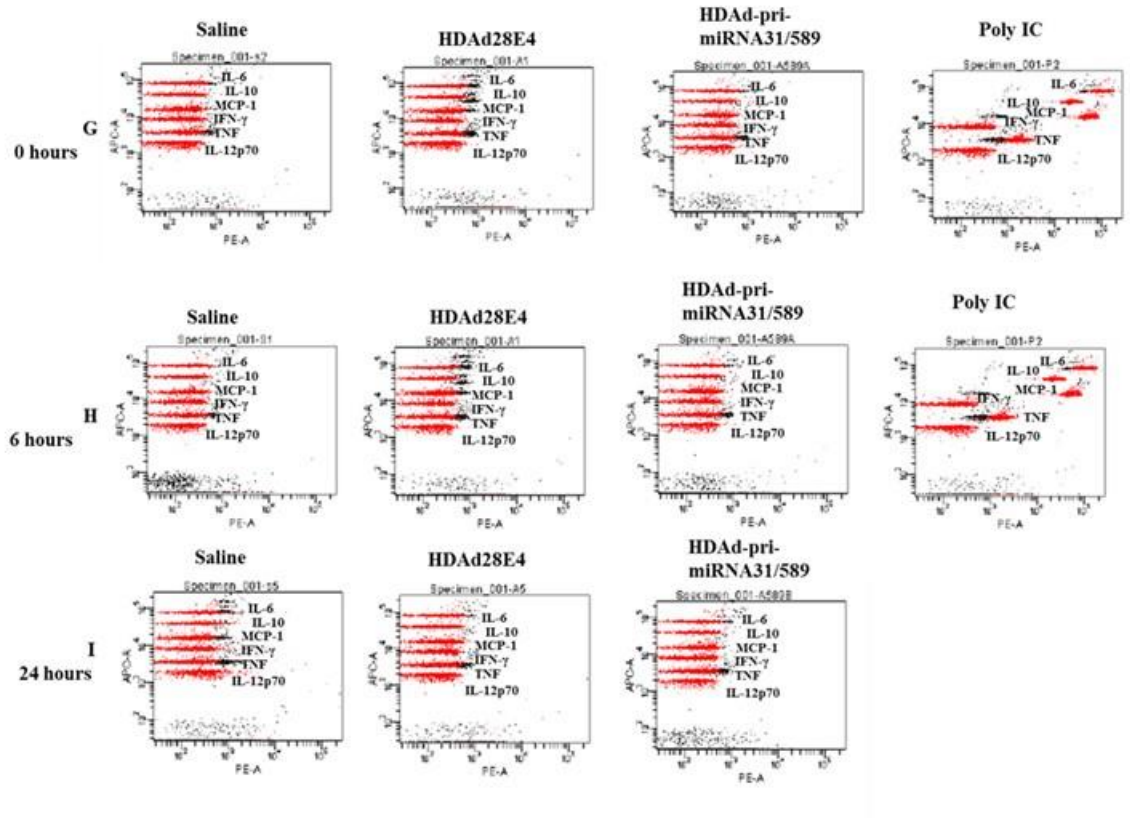

Figure 3.14b: Representative dot plots from CBA assay for IL-10, IL-6, TNF-γ, MCP-1, IFN and IL-12 for 0 hour and 6 hours.

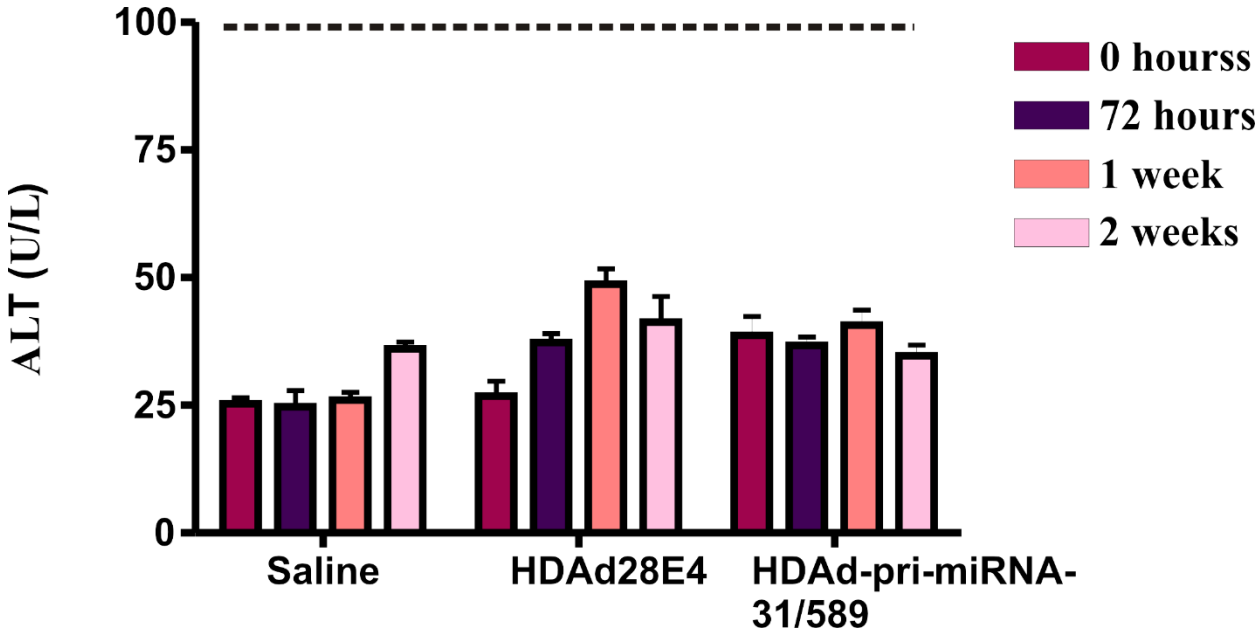

Figure 3.15: Measurement of alanine aminotransferase (ALT) activity. ALT levels in mice serum after 0 hour, 72 hours, 1 week and 2 weeks in mice injected with anti-HBV HDAds were determined. The dotted line represents the baseline or cut-off point for acceptable values that are regarded safe.

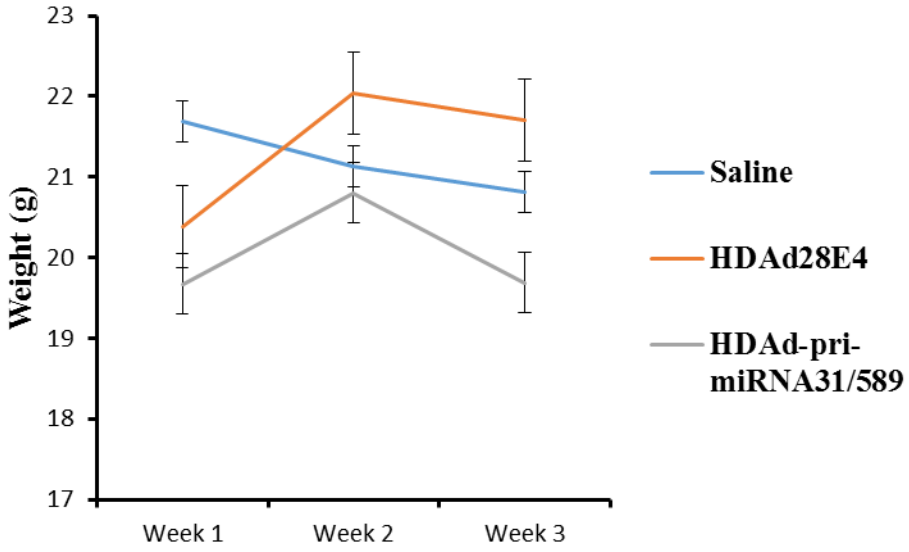

Figure 3.16: Representation of the mice weights following injection. The mice were grouped and injected with either saline (**A**), HDAd28E4 (**B**) or HDAd-pri-miRNA31/589 (**C**). The mice were weighed 1, 2 and 3 weeks post injection.

CHAPTER 4

4. DISCUSSION

Despite the availability of an effective vaccine, chronic infection resulting from HBV is currently incurable. Current treatments are limited by the development of adverse side effects and antiviral drug resistance. The ability to manipulate the RNA interference (RNAi) pathway to regulate gene expression has opened a new outlook into gene therapy. RNAi-based gene silencing has shown therapeutic potential and it is currently being investigated as a potential novel alternative to current HBV treatment. Anti-HBV primary-microRNA (pri-miRNA) sequences, which activate the RNAi pathway have been designed and have successfully been used to inhibit HBV replication *in vitro* and *in vivo* (Ely et al., 2008, Mowa et al., 2014). The major hurdle remains finding safe and efficient delivery vectors for these sequences. With their added advantage of natural liver tropism, Helper-dependent adenoviral vectors (HDAds) have been used with great success for liver-targeted gene therapy. Hence, this study investigated the use of these vectors for delivery of anti-HBV RNAi activators.

4.1 Elimination of the *lac Z* from HDAds

For easy assessment of tissue transduction, reporter genes such as *lac* Z have been co-delivered with therapeutic genes in Adenoviral vectors. However, several studies have suggested that the immune response to β-galactosidase encoded by the gene therapy vectors result in quicker vector clearance and short-term therapeutic effects (Crowther et al., 2014, Sullivan et al., 1997, Morral et al., 1997). A study by Chen and colleagues was one of the earlier studies that investigated the effect of β-galactosidase specific immune response in vector clearance and shortened therapeutic effect (Chen et al., 1997). Interestingly, following the injection of adenoviral vectors expressing β-galactosidase, a complete loss of β-galactosidase expression was observed 42 days after injection

along with vector clearance at 84 days after injection. Hence the elimination of *lac Z* in HDAds is critical to make HDAds more suitable for treatment chronic HBV infection. As a result of larger plasmid size (24-32 kb), cloning into HDAds genome bearing plasmids is tricky. This is mainly attributed to lower ligation and transformation efficiency. To overcome this, instead of conventional restriction manipulations and T4 ligase dependent cloning, a more efficient homologous recombination in *E. coli* is used (Luo et al., 2007). Electroporation is also used as the most efficient way of transforming these plasmids. However, despite these difficulties, T4 ligase mediated ligation and electroporation were successfully used to clone anti-HBV pri-miRNA expression cassettes in to the *lac Z* deficient pD28E4 which is 31.027 kb in size (**Figure 3.2**).

4.2 AdNG163cys fails to serve as an effective helper virus for HDAds production

HDAds are devoid of all their viral coding sequences thus making them less immune stimulatory (Palmer, 2005). However, capsid proteins have been shown to activate innate immunity in non-human primates as well as in mice. Chemical modification of vector particles has been successfully used as a means of improving HDAds transduction as well as reducing capsid specific immune response induction (Prill et al., 2011). Chemical modification using amine involves the covalent attachment of polyethylene glycol (PEG) to amine functional groups of lysine residues on the adenoviral surface. Amine reactive PEGylation has been commonly used to shield epitopes on the Ad capsid, however, this approach of PEGylation has been shown to reduce adenoviral transduction efficiency [Reviewed in (Kim et al., 2012)]. As a result of this limitation, thiol PEGylation was developed; this type of PEGylation involves covalently attaching PEG to cysteine residues on adenoviral surfaces [Reviewed in (Kreppel and Kochanek, 2008)]. Recent studies have showed that thiol reactive PEGylation improve transduction efficiency and results in homogenous particle size (Prill et al., 2011) . Hence, this study investigated the feasibility of using AdNG163cys helper virus with cysteine modification in the HVR-5 for production of HDAds that are amenable to residue specific and thiol reactive PEGylation. To enable production of HDAds using this cysteine modified HV, a previously produced AdNG163cys was first amplified. To determine the amount of infectious units (ifus), the HV was subjected to immunocytochemical staining, which

stains cells infected with the HV. Interestingly, following staining all infected cells were clumped together, making it difficult to count brown stained cells (**Figure 3.3**). Hence, q-PCR was used to determine total viral particles and the values were used to calculate the viral particles required for HDAd production. The HV MOI commonly used for HDAd production is 5 vp/cell, however when AdNG163cys was used, HDAds could only be produced at an MOI of 20. Interestingly, infection of HEK293 cells with cysteine modified and *lac Z* bearing HDAd (pΔ28E4CMV*lacZ*) displayed similar characteristics of clumped blue cells following X-gal staining (**Figure 3.4**). This aggregation of Ads either before or during infection may results from the crosslinking of the vector particles via the sulfhydryl bridges of thiol groups on the cysteine residues. Amplification of this virus was unsuccessful, despite the higher HV MOIs used, suggesting that this modification might diminish transduction efficiency of the HV or the HDAds produced from it. This was confirmed by the fact that infection of cells with AdNG163cys resulted in little or no cytopathic effect as compared to AdNG163 with wild type HVR 5 (**Figure 3.5**).

To investigate wither the modification results in structural changes of the capsid, transmission electron microscopy (TEM) was performed to determine the structural integrity of the cysteine modified HV. As compared to the icosadedral capsid observed with the HDAd previously produced from unmodified HV (**Figure 3.6 B**), the structure of AdNG163cys looked deformed or collapsed (**Figure 3.6 A**). According to Prill and colleagues, these results were unexpected. In their study HV with cysteine modification in the HVR 5 was successfully used to produce and PEGylate Ads using thiol reactive PEG. This improved transduction efficiency of the Ads (Prill et al., 2011). In contradiction and similar to our observations, a study by (Kreppel et al., 2005) generated Ad5-based first generation vectors that had been modified by introduction of cysteine residues within the H1 loop of the Ad fiber protein. These vectors also formed aggregates due to the presence of the cysteine residues. This was however resolved by adding 10 mM of reducing agents such tris (2-carboxyethyl) phosphine) (TCEP) and dithiothreitol (DTT). The addition of these reducing agents did not interfere with the biological functions of the vectors nor their physical integrities. This contradiction raises a necessity to further characterise specific mutations that can be introduced in to the Ads capsid without compromising the integrity of the vector particle and affecting transduction efficiency.

4.3 Successful rescue and amplification of the recombinant HDAds with AdNG163 helper-virus

As a result of the limitations of AdNG163cys HV, unmodified AdNG163 HV was used for the production of *lac* Z deficient HDAds. HDAd plasmids were used to transfect 116 cells, which were later infected with AdNG163 at MOI 2. As a control, HDAds containing the *lac* Z gene (pΔ28E4CMV*lacZ*) were also produced (P0) at MOI 5 and amplified (P1-P5) at MOI 2 using the AdNG163 HV. The amplification of HDAd*lacZ*CMV was monitored by X-gal staining. As expected, about 1.11 $\times 10^5$ ifus/mL were obtained after production. The infectious units per mL obtained with every passage showed an increase until ~ 10^9 ifus/mL were obtained (**Table 3.2**). Maximum titres of 1.19 × 10^9 ifus/mL were obtained at passage 5 in a 60 mm plate and passaging in to 150 mm plates increased the titres to 3.3 × 10^9 ifu/mL which was enough for large scale production. This was not dramatically different with published findings, which showed that the titers of the HDAds should increase until ~ 10^9 ifu/mL are obtained in a 60 mm plate (Palmer and Ng, 2008b).

4.4 Expression of anti-HBV pri-miRNA sequences from liver-specific MTTR promoter

RNA polymerase III (Pol III) such as U6 and H1 have been widely used for the expression of anti-HBV pre-miRNA mimics (shRNAs). However their use has been associated with toxicity as a result of the saturation of the RNAi pathway (Grimm, 2006). Pol II promoters have been shown to be tightly regulated and safer for expression of RNAi activators. Hence, to avoid toxicity, a study by (Ely et al., 2008) compared the expression of shRNAs from Pol II CMV and Pol III U6 promoters. *In vitro* and *in vivo* knockdown of 95-98 % was observed when using U6 promoter to express shRNAs, however expression of shRNAs from CMV promoter reduced HBV knockdown to from 95 % to ~ 60%. These indicate that shRNAs are not compatible with Pol II promoters. Using mimics of pri-miRNAs has more advantages over pre-miRNA mimics as they compatible with safer Pol II promoters and enables a better, regulated tissue specific expression. Use of a liver-specific murine transthyretin (MTTR) promoter to express anti-HBV pri-miRs showed superior

pri-miRNA expression in mice livers than non-tissue specific CMV promoter driven pri-miRNAs (Ely, 2009, Mowa et al., 2014). Hence this study used *lac* Z deficient HDAds to express pri-miRNA sequences from the liver-specific MTTR promoter.

In agreement with published data (Mowa et al., 2012, Mowa et al., 2014, Ely et al., 2008), RNA isolated from Huh7 cells infected with HDAd-pri-miRNA-31/5 showed the presence of a single band of ~21 nt when the blot was probed with guide 5 probe only but not guide 8 and 9 probes (**Figure 3.9**). On the other hand, RNA isolated from Huh7 cells infected with HDAd-pri-miRNA 31/589 is expected to produce three different guides (Ely, 2009, Mowa et al., 2012, Mowa et al., 2014) , surprisingly only guide 5 could be detected in this study. This could be because of differential efficiencies at which the three guides are cleaved from the pre-miRNA, with guide 5 being produced more abundantly than 8 and 9 (Ely et al., 2008).

4.5 Silencing of HBV replication and safety of HDAds expressing anti-HBV pri-miRNA expression cassettes from the MTTR promoter

The anti-HBV pri-miRNA sequences used in this study have been expressed with the CMV or the MTTR promoter before and have been found to significantly inhibit HBV replication *in vitro* and *in vivo* (Ely et al., 2008, Mowa et al., 2012, Mowa et al., 2014). To confirm *in vitro* HBV replication inhibition mediated by these monomeric and trimeric pri-miRNA expression cassettes when *lac Z* deficient HDAds were used, liver-derived Huh7 cells were transfected with HBV genome bearing plasmid and infected with anti-HBV HDAds at MOI of 500, 1000, 2000 and 4000. ELISA was used to determine the levels of secreted HBsAg levels. Relative to the empty vector (HDAdΔ28E4), cells infected with HDAd-pri-miRNA-31/5 at MOIs 500, 1000 and 2000 significantly inhibited HBV replication. This was observed for 48 and 72 hours post infection. Cells infected with HDAd-pri-miRNA-31/589 resulted in a more prominent and significant HBV replication inhibition at MOIs 1000 and 2000. These results are comparable to results obtained by (Mowa et al., 2014), where HBV inhibition was also dose dependent after infecting cells with HDAds expressing pri-miRNA sequences from MTTR promoter. The common MOIs used in both studies are MOI 500 and MOI 1000, in the previous study cells infected with HDAd-pri-miRNA-

31/5 at MOI 500 showed ~85% knockdown and MOI 1000 showed ~77% knockdown. Whereas, in this study cells infected with HDAd-pri-miRNA-31/5 at MOI 500 showed ~10% knockdown and MOI 1000 showed ~25% knockdown. For cells infected with HDAd-pri-miRNA-31/589 in the previous study at MOI 500 showed ~90% knockdown and MOI 1000 showed ~85% knockdown. On the other hand, in this study cells infected with HDAd-pri-miRNA-31/589 at MOI 500 did not inhibit HBV replication, whereas cells infected with MOI 1000 showed ~90% knockdown. These differences may be due to the fact that unlike (Mowa et al., 2014), this study used total viral particles (vps) instead of infectious units (ifus) to calculate number of viral particles required for the different MOIs. This may result in lower ifus used for the experiments. Previous studies have successfully used Ad total viral particles to calculate viral particles required to effect a therapeutic effect (Palmer and Ng, 2004), which suggest that viral preparations from this study may have lower ifus than those used in previous studies.

While the inclusion of reporter genes is not desirable *in vivo*, it serves a valuable tool for titration of vectors. To improve anti-HBV inhibition, ifus could be determined using DNA from HDAd infected cells for q-PCR. This will involve infection of cells with HDAd for 5hrs, total DNA isolation and q-PCR with HDAd specific primers.

To assess the ability of these HDAds to silence HBV replication *in vivo*, HBV transgenic mice were injected with empty HDAdΔ28E4 or HDAd-pri-miRNA-31/589. Serum was collected at 0 days, 1 week and 2 weeks post injection and used for HBsAg ELISA. Surprisingly, HDAd589 did not result in any detectable pri-miRNA expression or significant HBV silencing. In comparison to published data, expression of these anti-HBV pri-miRNAs from the MTTR should result in significant knockdown of HBV replication *in vivo* (Mowa et al., 2014). There are several reasons that may result in these varying results: 1) The nature of the mouse model used in this study can also contribute. 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 born have very high HBsAg levels (OD 0.8-1), these high titter mice were used. Hence, these experiments need to be repeated with medium HBsAg level mice. 2) As stated before, the fact that total viral particles and not ifus were used may result in poor liver transduction. 3) The HDAds used might be inducing a strong immune response that may result in vector clearance and toxicity.

To validate whether HD Ad delivery to hepatocytes was adequate, DNA was extracted from livers of mice injected with anti-HBV HDAds. Q-PCR was performed and interestingly ~4×10^5 copies per µg of hepatocyte DNA for HDAd-pri-miRNA-31/589 were delivered to the hepatocytes. In comparison to 2×10^5 copies per µg of hepatocyte DNA of hepatic HDAd observed by (Mowa et al., 2014), these copy numbers should be enough to trigger HBV knockdown *in vivo*.

To access if HDAds used induce a strong and sustained inflammatory response, CBA was performed to measure the levels of circulating inflammatory markers IL-6, IL-10, IL-12p70, MCP-1, IFN-γ and TNF before, six hours and 24 hours after injection with saline or HDAdΔ28E4 or HDAd-pri-miRNA-31/589 or Poly IC as a positive control. As compared to saline, there was no significant induction of inflammatory marker expression at 0, 6 and 24 hours post infection with either of the HDAds produced in this study. As expected, poly IC which was used as a positive control significantly induced expression of TNF, MCP-1, IL-10 and IL-6 6 hours post infection. These cytokines are said to be involved in important steps of humoral and cell-mediated immunity.

Expression of anti-HBV shRNAs in mice results in hepatotoxicity and fatality in mice (Grimm, 2006). To assess whether HDAds used in this study are toxic, ALT activity was measured for 0 hours, 72 hours, 1 week and 2 weeks post injection with HDAds. In agreement with previous studies (Mowa et al., 2014), levels of ALT in all the groups remained below the acceptable cut off level of 100 U/L at all the time points; therefore, the anti-HBV HDAds used in this study did not induce any obvious liver damage. This was confirmed by normal weight gain observed in all the mice used in this study (**Figure 3.16**).

5. CONCLUSIONS

The aim of this study was to design less immunostimulatory HDAds to prolong anti-HBV effects. *Lac Z* deficient recombinant HDAds expressing pri-miRNA mimics from the MTTR promoter were successfully constructed. The pri-miRNA mimics (pri-miRNA-31/5 and pri-miRNA-31/589) used were able to activate the RNAi pathway, resulting in successful expression and processing into mature miRNA strands *in vitro*. Both pri-miRNA-31/5 and pri-miRNA-31/589 were able to significantly knockdown HBV replication in liver-derived Huh 7 cells in a dose-dependent manner. The quantification of circulating inflammatory and liver toxicity markers reaffirms the safety of HDAds in the development of novel anti-HBV gene therapeutics. However, long term studies are required to determine whether this translates to a sustained HBV replication inhibition *in vivo*. This is the first study to use *lac Z* deficient HDAds to deliver MTTR promoter driven pri-miRNAs against HBV. The findings from this study may contribute to the development of RNAi-based therapeutics with minimised induction of immune response and toxic effects.

6. FUTURE STUDIES

In this study, a helper-virus (HV) with a cysteine modification was used. This HV has been shown by others to function in HDAd production without causing and structural changes to the virions. However, this was not observed in our study. To understand these contradictions, the HV will be taken for sequencing of the Hexon encoding gene to check for additional mutations that may have occurred.

To improve activity of HDAd vectors produced in this study ifus will be determined.

To better asses *in vivo* activity of HDAds generated in this study, mice with HBsAg levels of around 0.4-0.6 will be used.

To assess wither the deletion of *lac Z* from anti-HBV HDAds result in prolonged transgene expression and inhibition of HBV replication long term studies will be carried out in mice.

7. APPENDIX

All general chemicals were obtained from, Merck, Sigma, Quantum Biotech, Qiagen, Thermo Fisher Scientific, Epicentre Biotechnologies

7.1 Bacterial Methods

7.1.1 Luria Bertani (LB) media

Ten grams of tryptone (Quantum Biotech, South Africa), 5 g of yeast extract (Quantum Biotech, South Africa) and 5 g NaCl were dissolved in 1 litre of distilled water (dH_2O) in a 1 litre lab glass bottle and sterilized by autoclaving for 30 min at 121°C and 1 kg/cm^2.

7.1.2 Luria Bertani agar (LA) media

Ten grams of agar (Quantum Biotech, South Africa), 10 g tryptone (Quantum Biotech, South Africa), 5 g of yeast extract (Quantum Biotech, South Africa) and 5 g NaCl were dissolved in 1 litre of distilled water (dH_2O) in a 1 litre lab glass bottle and sterilized by autoclaving for 30 min at 121°C and 1 kg/cm^2.

7.1.3 Ampicillin solution (100 mg/mL)

One gram of ampicillin was added to 5 mL of dH_2O and 5 mL of 100% ethanol. The solution was filter sterilized and stored at -20°C.

7.1.4 Kanamycin solution (50 mg/mL)

An amount of 0.5 g of kanamycin was added to 10 mL dH_2O. The solution was filter sterilized and aliquots were made.

7.1.5 LB Agar plates

Ten grams of tryptone (Quantum Biotech, South Africa), 5 g of yeast extract (Quantum Biotech, South Africa),5 g NaCl and 10 g bacterial agar (Quantum Biotech, South Africa) were dissolved in 1 litre of distilled water (dH_2O) in a 1 litre lab glass bottle and sterilized by autoclaving for 30

min at 121°C and 1 kg/cm^2. Ampicillin (100 mg/mL) or kanamycin (50 mg/mL) was added, and the agar solution was poured in bacterial plates and allowed to solidify at room temperature.

7.1.6 Transformation Buffer

Transformation buffer was prepared by adding 1.47 g of 100 mM $CaCl_2$, 0.30 g of 10 mM PIPES-HCl and 15 mL of 15% glycerol in 80 mL of dH_2O. The pH was adjusted to 7.0 with NaOH, the solution was made up to 100 mL with dH_2O and autoclaved for 30 minutes at 121 °C and 1 kg/cm^2. Transformation buffer was stored at -20°C.

7.1.7 X-gal solution (20 mg/mL)

An amount of 0.2 g of x-gal was added to 10 mL of dimethylformamide, the tube was covered with foil and it was stored at -20°C.

7.2 DNA isolation solutions

7.2.1 Resuspension buffer (Buffer P1)

Tris (hydroxymethyl) aminomethane [Tris base (Sigma-Aldrich, South Africa)] (6.06 g) and 3.72 g $Na_2EDTA.{}_2H2O$ (Sigma-Aldrich, South Africa) were added to 800 mL of distilled water (dH_2O). The pH was adjusted to 8.0 with HCl (Merck Millipore, South Africa) and the volume was topped up to 1 L with dH_2O. The solution was autoclaved for 20 minutes at 121 °C and 1 kg/cm^2, it was allowed to cool and 100 mg of RNase A was added. The buffer was mixed thoroughly and stored at 4 °C.

7.2.2 Lysis buffer (Buffer P2)

Eight grams of 8 sodium hydroxide [(NaOH), Merck Millipore, South Africa)] pellets and 10 g of SDS were added to 500 mL of dH_2O, the volume was adjusted to 1 L with dH_2O and the buffer was stored at room temperature.

7.2.3 Neutralization buffer (Buffer P3)

An amount of 294.5 g of potassium acetate was added to 500 mL dH_2O, about 300 mL of acetic acid was added to adjust the pH to 5.5. The volume was topped up to a final volume of 1 L with dH_2O and it was stored at 4 °C.

7.2.4 QBT buffer

Sodium chloride (NaCl, 43.83 g) and 10.46 g MOPS were dissolved in 800 mL dH2O. The pH was adjusted to 7 with NaOH, 150 mL Isopropanol and 15mL of 10 % Triton X-100 solution (v/v) were added. The final volume was adjusted to 1L with dH_2O and the buffer was stored at room temperature.

7.2.5 QC buffer

NaCl (58.44 g) and 10.46g MOPS were added to 800 mL of dH2O. NaOH was used to adjust the pH to 7, 150 mL Isopropanol was added and the final volume was adjusted to 1 L with dH_2O. The buffer was stored at room temperature.

7.2.6 QF buffer

NaCl (73.05 g) and 6.06 g Tris base were added to 800 mL dH_2O, pH was adjusted to 8.5 with HCl and 150 mL of Isopropanol was added. The final volume was adjusted to 1L with dH_2O and the buffer was stored at room temperature.

7.2.7 50 × Tris-acetate-EDT (TAE) buffer

Tris base (242 g) was added to 57.1mL glacial acetic acid, 100mL 0.5 M EDTA (pH 8) was added and the volume was made up to 1 L with dH_2O. To make 1 L of 1 × TAE buffer, 20 mL of 50 × TAE buffer was added to 980 mL of dH_2O.

7.3 Cell culture methods

7.3.1 Eagle's Minimum Essential Medium (EMEM) media

EMEM media was made by taking 28.23 g of EMEM powder and 6.69 g of sodium hydrogen carbonate and adding to 3 L of sabax water. This was stirred until completely dissolved and the pH was adjusted to 6.8, the media was filter sterilized into autoclaved 1 L bottles and stored in the fridge.

7.3.2 Dulbecco's Modified Eagle's Medium (DMEM) media

DMEM media was purchased from Gibco® (Thermo Fischer Scientific, MA, USA) or made according to the manufacturer's instructions.

7.3.3 Joklik Eagle's Minimum Essential Medium (JEMEM) media

JEMEM media was made by taking 33.6 g of JEMEM powder and 6 g of sodium hydrogen carbonate and adding to 3 L of sabax water. This was stirred until completely dissolved and the pH was adjusted to 6.8, the media was filter sterilized into autoclaved 1 L bottles and stored in the fridge.

7.3.4 Freezing media

Freezing media was made by adding 5.6 mL media (EMEM/DMEM/JEMEM), 3.4 mL FBS and 1 mL DMSO in a 50 mL Falcon tube and filter sterilizing it.

7.4 Northern blot buffers

7.4.1 TE buffer

To make 1 L of TE buffer, 1 M Tris-HCl pH8 was added to 2 mL of 0.5 M EDTA pH 8and the volume was topped up to 1 L with dH_2O.

7.4.2 Sephadex

Sephadex G-25 (5 g) was added to 50 mL TE buffer and left rotating overnight at room temperature. This was centrifuged for 2 minutes at 5000 g twice and resuspended in 50 mL TE buffer.

7.4.3 10 × TBE buffer

Boric acid (27.5 g), and 50 g Tris were added to 400 mL dH_2O. A volume of 20 mL 0.5 M EDTA pH8 was added. The pH was adjusted to 8 and the volume was topped up to 500 mL with dH2O and autocaved for 20 minutes.

7.4.4 20 × SSC

NaCl (3 M) and 3 M of $Na_3C_6H_5O_7.2H_2O$ were added in 800 m dH2O. The pH was adjusted to 7 and topped up to 1 L with dH_2O.

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